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## PHYLOGENETIC CLASSIFICATION OF THE FROG PATHOGEN *AMPHIBIOTHECUM (DERMOSPORIDIUM) PENNERI* BASED ON SMALL RIBOSOMAL SUBUNIT SEQUENCING

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**ABSTRACT:** We determined 1,600 base pairs of DNA sequence in the 18S small ribosomal subunit from two geographically distinct isolates of *Dermosporidium penneri*. Maximum likelihood and parsimony analysis of these sequences place *D. penneri* in the order Dermocystida of the class Mesomycetozoea. The 18S rRNA sequences from these two isolates only differ within a single region of 16 contiguous nucleotides. Based on the distant phylogenetic relationship of these organisms to *Amphibiocystidium ranae* and similarity to *Sphaerothecum destruens* we propose the organism be renamed *Amphibiothecum penneri*.

**Key words:** *Amphibiothecum*, dermocystida, *Dermosporidium*, frog diseases, mesomycetozoea.

### INTRODUCTION

Species of the genus *Dermosporidium* are infrequently studied anuran parasites that were previously characterized based on morphologic observations using light microscopy. *Dermosporidium hylae* was the first species identified and was described from superficial cysts within the dorsal skin of the tree frog *Hyla rubra* in Brazil (Carini, 1940). Several decades later, other organisms of similar morphology, clinical appearance, and specificity for amphibians were described including *Dermosporidium granulosum* from *Rana temporaria* (Broz and Privora, 1952), *Dermosporidium multigranulare* from *Rana esculenta* (Broz and Kulda, 1954), and *Dermosporidium penneri* from the toad *Bufo americanus* (Jay and Pohley, 1981). However, the assignment of the genus *Dermosporidium* was recently challenged based on the lack of supportive data for the morphologic criteria used in the initial descriptions. A single genus “*Amphibiocystidium*” was proposed to encompass microorganisms previously defined in the genera *Dermocycoides*, *Dermosporidium*, and amphibian *Dermocystidium* (Pascolini et al., 2003). Moreover, morphologic and phylogenetic analysis of the small ribosomal subunit of *Amphibiocystidium ranae* re-

vealed the taxon of this amphibian pathogen to be closely related to the Dermocystida infecting fish (Pereira et al., 2005). The sole use of morphologic criteria has led to some confusion regarding the classification of these microorganism(s). Taxonomic genomic characterization of closely related organisms including those in the genus *Dermosporidium* should help to clarify these key relationships. Based on morphologic similarities of microorganisms classified as members of the genus *Dermosporidium* to characteristics of members of the genus *Dermocystidium*, the former was assumed to belong to the class Mesomycetozoea (Mendoza et al., 2002). In our current study, we present molecular evidence of the relationship of *D. penneri* to members of the Order Dermocystida based on phylogenetic analysis of the DNA sequence in the gene encoding the 18S small subunit ribosomal. Based on our findings and the recommendations of Pereira et al. (2005) for changing the nomenclature of amphibian disease organisms in the class Dermocystida, we propose changing the name of *Dermosporidium penneri* to *Amphibiothecum penneri*.

### MATERIALS AND METHODS

We developed oligonucleotide primers unique to the class Mesomycetozoea by se-

quence alignment of the 18S rRNA subunit genes of *Ichthyophonus hoferi* (GenBank accession U25637; Ragan et al., 1996), *Ichthyophonus irregularis* (GenBank accession AF232303; Rand et al., 2000), *Dermocystidium salmonis* (GenBank accession U21337; Jostensen et al., 2002), *Dermocystidium* sp. (GenBank accession U21336; Ragan et al., 1996), *Rhinospordium seeberi* (GenBank accession AF118851; Herr et al., 1999), *Pseudoperkinsus tapetis* (GenBank accession AF192386; Figueras et al., 2000), and *Bufo valliceps* (GenBank accession M59386; Hedges et al., 1990). The primers were designed to amplify as much of the 18S rRNA gene as possible, recognizing members of the order Mesomycetozoea without concurrently amplifying 18S rRNA gene sequence found in toads. The primer sequences are 5'-TAAGCCATGCATGTCAAGTATAA (forward) and 5'-ACTAGGAATTCCTCGTBAAGATS (reverse).

Samples of *A. penneri* microdissected cysts from infected skin of *Bufo americanus* used in this study had been previously collected at New London Submarine Base, Connecticut (designated 18617-002) and Petersburg National Battlefield, Virginia (designated 4825-004) and had been stored frozen in ethanol. Histologically, *A. penneri* elicited a granulatus inflammatory cell response in which spores were extracellular, 10  $\mu$ m in diameter, and contained approximately twenty 1–4  $\mu$ m inclusions and a central nucleus (data not shown). A more comprehensive description of the morphology of *A. penneri* isolates and the pathology elicited by its presence has been published elsewhere (Green and Converse, 2005). DNA was isolated using DNeasy tissue kit (Qiagen Corporation, Valencia, California, USA) according to the manufacturer's recommendations for animal tissues, and sampling emphasized microdissected cysts within infected tissues. Five microliters of the isolated DNA was subjected to amplification in 50  $\mu$ l polymerase chain reactions (PCRs) using Ex-Taq polymerase reagents (Takara Mirus Bio Corp., Madison, Wisconsin, USA) according to manufacturer's recommendations and 0.5  $\mu$ M of each primer. A 9600 gradient Robocycler (Stratagene, La Jolla, California, USA) was set to the following PCR parameters: 95 C hot start for 90 sec, 35 cycles of 95 C (45 sec)/55 C (60 sec)/72 C (140 sec), and a final extension of 72 C for 5 min. Five microliters of each reaction was analyzed by agarose gel electrophoresis. The resulting 1.6 kb amplicons were ligated into TOPO pCR-4 (Invitrogen Corp., Carlsbad, California, USA) and used to transform chemically competent Top 10 *E. coli* (Invitrogen) that were plated onto LB agar containing 100  $\mu$ g/ml ampicillin. Ten colonies from each sam-

ple were selected and grown separately overnight in LB-ampicillin broth. The plasmids were isolated using a Plasmid minikit (Qiagen), eluted with water, and analyzed by *Eco*RI restriction digest (New England Biolabs, Waverly, Massachusetts, USA). Six plasmids containing the amplicon insert from each isolate were submitted for DNA sequencing to Davis Sequencing (Davis, California, USA) in the forward (from the T3 site) and reverse (from the T7 site) directions. The entire procedure of amplification, cloning, and sequence analysis was repeated once with the same sample DNAs and the sequence results combined with those determined initially for unambiguous characterization of the amplified DNA sequence.

Alignment of all forward sequences for each geographic isolate, and separately for all reverse sequences, was performed using Omega 1.1 (Oxford Molecular Ltd., Oxford, UK). Separate consensus sequences were determined for the Connecticut and the Virginia isolates, and the two consensus sequences aligned for comparison. Phylogenetic analysis of the consensus sequences used the same GenBank sequences used for primer development plus *Amphibiocystidium ranae* (GenBank accession AY550245 and AY692319; Pereira et al., 2005), *Dermocystidium percae* (GenBank accession AF533941; Pascolini et al., 2003), *Amoebidium parasiticum* (GenBank accession Y19155; Ustinova et al., 2000), *Anurofeca richardsi* (GenBank accession AF070445; Baker et al., 1999), *Ichthyophonida* gen. sp. LKM51 (GenBank accession AJ130859; Van Hammen et al., 1999), *Psorospermium haeckeli* (GenBank accession U33180; Ragan et al., 1996), *Sphaerofoma arctica* (GenBank accession Y16260; Jostensen et al., 2002), *Sphaerothecum destruens* (GenBank accession AY267346; Arkush et al., 2003), *Capsaspora owczarzaki* (GenBank accession AF436886; Hartel et al., 2002), *Microciona prolifera* (GenBank accession L10825; Wainright et al., 1993), and *Mnemiopsis leidyi* (GenBank accession L10826; Wainright et al., 1993) were used to root the phylograms in the phylogenetic analyses. All GenBank sequences were converted to GCG sequence and truncated at the 5' and 3' ends to coincide with the sequences determined for the *A. penneri* amplicons. All 20 sequences were aligned and analyzed by parsimony analysis and again by maximum likelihood analysis using PHYLIP inference software (Felsenstein, 2004).

The significance of branch points (nodes) for the consensus phylogram generated was determined by bootstrap analysis of 1,000 datasets, each dataset searched for the most parsimonious tree. Bootstrap maximum likelihood analysis of the same 1,000 datasets was performed

(with global rearrangement, the rough option turned off, and with the jumble set and random number seed again set to three). A single phylogram is presented using TREEVIEW (Page, 1996) that represents a consensus of trees from maximum likelihood analysis showing the number of trees in agreement at each node (Fig. 1). *Mnemiopsis leidyi* and *M. prolifera* were used together as an outgroup to root the consensus tree. Phylograms were generated using maximum likelihood analysis with and without imposing a molecular clock (i.e., the tips of all branches were made equidistant from the root). The log likelihood of each maximum likelihood method was analyzed by Chi Square goodness-of-fit testing against the assumption that the small ribosomal subunit gene can function as a molecular clock.

### RESULTS

Amplified 18S rRNA sequences of 18617-002 (Connecticut) and 4825-004 (Virginia) were 1,607 and 1,599 bases in length, respectively, and have been deposited in GenBank (accession numbers AY772000 and AY772001). Alignment of sequences from the two *A. penneri* isolates shows complete sequence agreement with the exception of a single contiguous DNA region. This regional difference spans 16 nucleotides wherein eight nucleotides were deleted in the 18S rRNA gene of the Virginia isolate and two nucleotide substitutions occurred among the remaining eight nucleotides (Fig. 2). The clones from both isolates showed the same variability (A, C, or G) at nucleotide position 1,590 that may have occurred as an artifact resulting from degeneracy in the design of the reverse primer at that position.

The topology of the consensus phylograms generated by bootstrap analyses using parsimony and maximum likelihood methods was the same (data not shown). Using the maximum likelihood method to compare phylograms within the Dermocystida clade, all nodes are well supported (greater than 50% consensus among tree topologies) except at the following nodes: the node distinguishing *Rhinosporidium seeberi* and *Dermocystidium* sp.; distinguishing *A. penneri* from *S. destruens*, the node distinguishing *D. salmonis* from *A.*

*ranae*, and the node distinguishing *C. owczarzaki* from *P. haeckeli*. This analysis showed that *A. penneri* is a member of the order Dermocystida within the class Mesomycetozoea and further suggests that the two *A. penneri* isolates analyzed are distinct from each other. The phylogram generated is identical with others published previously for the class Mesomycetozoea (Wainright et al., 1993; Ragan et al., 1996; Herr et al., 1999; Hertel et al., 2002; Mendoza et al., 2002; Pekkarinen et al., 2003) and similar to the phylogram generated by Pereira et al. (2005). The rosette agent, which is included in many of these phylograms was recently renamed *Sphaerothecum destruens* (Arkus et al., 2003).

Phylograms generated using maximum likelihood methods, with and without a molecular clock assumption, were of the same (unrooted) topology (data not shown). Imposing the molecular clock (natural log likelihood = -8769) when compared with no clock assumption (natural log likelihood = -8679) during analysis of the 18S small ribosomal subunit sequence was not statistically supported ( $\chi^2=180$ , d.f.=18,  $P<0.005$ ). Thus, maximum likelihood tree analysis with molecular clock assumption does not provide a relative estimate of the divergence of species within the class Mesomycetozoea.

### DISCUSSION

The number of parasitic and saprophytic microorganisms assigned to the monophyletic class Mesomycetozoea is continuing to increase (Ragan et al., 1996; Mendoza et al., 2002; Pereira et al., 2005). The 18S rRNA sequence analysis presented in our study supports the categorization of *A. penneri* as an organism closely related to the Dermocystidia parasites of fish, as originally deduced using morphologic characters (Jay and Pohley, 1981). Interestingly, *A. penneri* forms a clade that includes *S. destruens* (since the node separating these is not well supported) presenting a taxonomic quandry given the recent suggestion to use the epithet

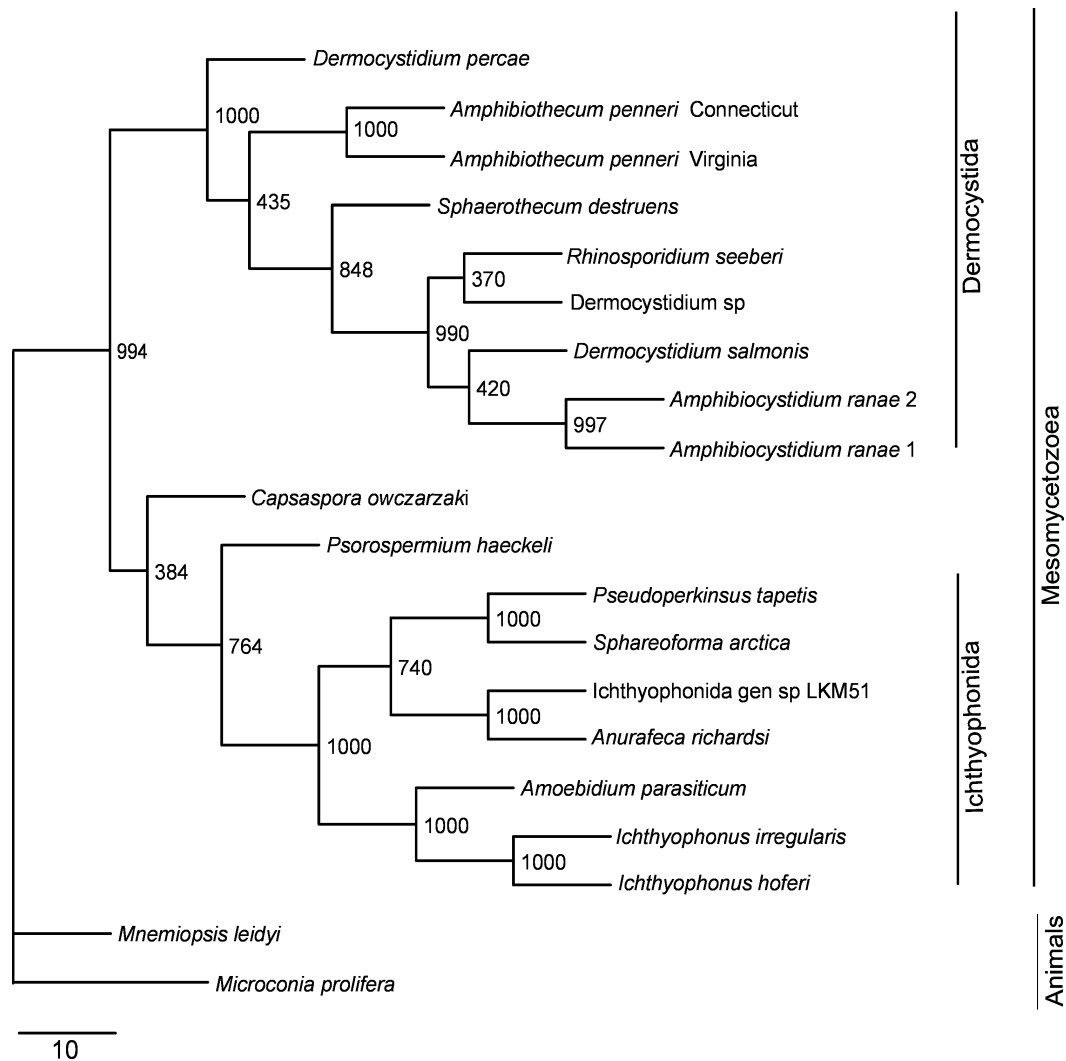


FIGURE 1. This phylogram from maximum likelihood analysis incorporates a molecular clock assumption. The scale shows the branch length equivalent to the occurrence of substitution of 10% of the nucleotide sequence. The relatedness of two microorganisms in this phylogram is the sum of the length of all horizontal branches separating the two species (neglect vertical distances between species). The number to the left at each node is the consensus among 1,000 trees by bootstrap maximum likelihood analysis. This maximum likelihood model allows changes to occur multiple times at any single nucleotide site, thus a branch twice as long as another may not necessarily contain twice as many nucleotides differences. The phylogram shows definitively that *A. penneri* is a member of the order Dermocystida.

150  
A. *penneri* Connecticutt CGACTTCCAT CTTTAGCGGA TGGGGAAGGG  
A. *penneri* Virginia CGACTT.... TTTTACCG...AGGAAGGG

FIGURE 2. The small ribosomal subunit sequences for samples 4825-004 and 18617-002 when aligned demonstrate complete consensus, with the exception one region of 16 nucleotides shown here between positions 146 and 162. Sample 4825-004 (Virginia) lacks eight nucleotides and contains two nucleotide substitutions within this region.



*Dermotheca* for organisms with phylogenetic affinity to *S. destruens* (Pereira et al., 2005). The morphologic character of *A. penneri* cysts is round to oval 4–6 mm in diameter located in divided chambers in the ventral subcutis of the toad *B. americanus* (Jay and Pohley, 1981), unlike the giant elongate-cylindrical intracellular cysts found in liver cells of salmonids characteristic of *S. destruens* (Arkush et al., 2003). The spores of *A. penneri* are 10–12  $\mu\text{m}$  in diameter, whereas the spores of *S. destruens* are 2–6  $\mu\text{m}$ . Thus, the phylogenetic similarity of *A. penneri* and *S. destruens* in the present study is not reflected in host species, or cyst location and morphology. Therefore, we propose assigning a new genus name, *Amphibiothecum*, which reflects both the host species and the phylogenetic similarity to *S. destruens*. The topology of our phylogram is similar to that reported by Pereira et al. (2005), but the node distinguishing *A. ranae* from *D. salmonis* is not well supported in our study whereas the node distinguishing the two *A. ranae* isolates is very well supported. Although our study, as well as studies by others (Pereira et al., 2005), demonstrates that the 18S sequence of *D. percae* is (phylogenetically) more rudimentary when compared with the same region of gene sequence of other members in the class Dermocystida, we have chosen to not address altering the genus designation in this report.

Amphibian population declines and species extinctions have been occurring worldwide over the past 25 yr (Green and Sherman, 2001; Green et al., 2002). In the United States amphibian population declines associated with emerging infectious diseases such as *Batrachochytrium dendrobatidis* (chytrid), iridovirus, and a dermocystidium-like infection (Green et al., 2002; 2003) have adversely affected natural populations. Although infection with *A. penneri* has not been shown to contribute directly to amphibian die-offs (Green et al., 2002), its importance may increase as ecologic stressors change over time. The

18S rRNA sequence determined for *Amphibiothecum penneri* isolates in the present study can be used to develop PCR protocols that are specific for this microorganism; this will allow for rapid, sensitive, and specific identification of this organism from amphibian specimens. These sequences also provide much needed information to better understand the taxonomy of the Mesomycetozoa.

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