J. Parasitol., 92(3), 2006, pp. 611–619
© American Society of Parasitologists 2006

A NEW SPECIES OF *MYXIDIUM* (MYXOSPOREA: MYXIDIIDAE), FROM THE WESTERN CHORUS FROG, *PSEUDACRIS TRISERIATA TRISERIATA*, AND BLANCHARD'S CRICKET FROG, *ACRIS CREPITANS BLANCHARDI* (HYLIDAE), FROM EASTERN NEBRASKA: MORPHOLOGY, PHYLOGENY, AND CRITICAL COMMENTS ON AMPHIBIAN *MYXIDIUM* TAXONOMY

Miloslav Jirků*†, Matthew G. Bolek*‡, Chris M. Whipps§, John Janovy, Jr.‡ Mike L. Kent§, and David Modrý‖

* Department of Parasitology, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences, Palackého 1–3, CZ-612 42 Brno, Czech Republic. *e-mail: miloslav.jirku@seznam.cz* or *mbolek@unlserve.unl.edu*

ABSTRACT: During March 2001–April 2004, 164 adult anurans of 6 species (47 *Rana blairi*, 35 *Rana catesbeiana*, 31 *Hyla chrysoscelis*, 31 *Pseudacris triseriata triseriata*, 11 *Bufo woodhousii*, and 9 *Acris crepitans blanchardi*) from Pawnee Lake, Lancaster County, Nebraska, were surveyed for myxozoan parasites. Of these, 20 of 31 (65%) *P. triseriata triseriata* and 1 of 9 (11%) *A. crepitans blanchardi* were infected with a new species of *Myxidium*. *Myxidium melleni* n. sp. (Myxosporea) is described from the gallbladder of the western chorus frog, *P. triseriata triseriata* (Hylidae). This is the second species of *Myxidium* described from North American amphibians. Mature plasmodia are disc-shaped or elliptical 691 (400–1,375) × 499 (230–1,200) × 23 (16–35) μm, polysporic, producing many disporic pansporoblasts. The mature spores, 12.3 (12.0–13.5) × 7.6 (7.0–9.0) × 6.6 (6.0–8.0) μm, containing a single binucleated sporoplasm, are broadly elliptical, with 2–5 transverse grooves on each valve, and contain 2 equal polar capsules 5.2 (4.8–5.5) × 4.2 (3.8–4.5) μm positioned at opposite ends of the spore. *Myxidium melleni* n. sp. is morphologically consistent with other members of *Myxidium*. However, *M. melleni* n. sp. was phylogenetically distinct from other *Myxidium* species for which DNA sequences are available. Only with improved morphological analyses, accompanied by molecular data, and the deposit of type specimens, can the ambiguous nature of *Myxidium* be resolved. Guidelines for descriptions of new species of *Myxidium* are provided.

There are over 150 described myxozoan species of *Myxidium* Bütschli, 1882. They are distributed worldwide and predominantly infect fish from both marine and freshwater environments. Most *Myxidium* species are coelozoic parasites infecting the gallbladder, urinary bladder, or urinary tubules in the kidneys of fish hosts. However, a few species are known to infect amphibians and reptiles (Lom and Dyková, 1992; Canning et al., 1999; Garner et al., 2005); 4 of these are known to infect the gallbladder of amphibians (Delvinquier et al., 1992). In South America, *Myxidium immersum* was described by Lutz (1889) from 2 anurans: the cane toad, *Bufo marinus*, and the argus frog, *Leptodactylus ocellatus*. Kudo and Sprague (1940) described *Myxidium serotinum* from 2 North American frogs: the northern leopard frog, *Rana pipiens*, and an unidentified true frog, *Rana* sp. Sarkar (1982) described *Myxidium haldari* from an undetermined tree frog (its reported type host, *Hyla arborea*, does not occur in the Indian subcontinent) from West Bengal. Delvinquier et al. (1992) described *Myxidium lesminteri* from the knocking sand frog, *Tomopterna krugerensis* (type host), the natal ghost frog, *Heleophryne natalensis*, and Garman's toad, *Bufo garmani*, from the South African Republic. Additionally, undescribed *Myxidium* spp. have been reported from the Australian tree frog, *Litoria caerulea*, and the LeSueur's frog, *Litoria lesueuri*, from Australia (Hill et al., 1997; Berger et al., 2002). Survey data indicate that *Myxidium* species infecting amphibians may not be host-specific, infecting a wide range of frogs and toads and, in the case of *M. serotinum*, even salamanders (Cordero, 1919; Carini, 1932; Kudo and Sprague, 1940; Kudo, 1943; Clark and Shoemaker, 1973; Delvinquier, 1986; Delvinquier, et al., 1992; McAllister and Trauth, 1995; McAllister and Bursey, 2005).

In North America, descriptions are available for only 3 myxozoan species that infect amphibians: *M. serotinum*, *Sphaerospora ohlmacheri* (Whinery, 1893) from the kidneys of true frogs (Ranidae) and toads (Bufonidae), and *Chloromyxum salamandrae* Upton, McAllister, Trauth, 1995, from the gallbladder of *Eurycea* spp. of plethodontid salamanders (Whinery, 1893; Desser et al., 1986; Upton et al., 1995). Not only are there very few descriptions of myxozoan species from amphibians but also the available information and descriptions of *Myxidium* spp. are somewhat ambiguous. Many relevant articles include descriptions based solely on limited morphological data, such as plasmodia morphology, measurements of few spores, no measurements of polar capsules, and no line drawings. Inadequately described species and undescribed morphotypes confound subsequent identification of species and make distinction and classification of new species problematic. However, comprehensive morphological descriptions accompanied by molecular analyses are likely to alleviate these problems. To date, no molecular studies have been conducted on any myxozoan species from amphibians.

In this article, we describe a new *Myxidium* species (both morphologically and molecularly) from North American anurans, analyze its phylogenetic relationships to other myxozoan taxa as reflected by the small subunit (SSU) ribosomal DNA (rDNA) sequence, and discuss taxonomic problems involving amphibian *Myxidium* spp.

MATERIALS AND METHODS

Sample collection and preparation

Adult anurans were collected at night by hand from Pawnee Lake, Lancaster County, Nebraska (40°51′11″N, 96°53′07″W), during their breeding season from March 2001 to April 2004. These included 47

Received 1 September 2005; revised 4 December 2005; accepted 5 December 2005.

† To whom correspondence should be addressed.
‡ School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, Nebraska, 68588-0118.
§ Center for Fish Disease Research, Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, Oregon 97331-3404.
‖ Institute of Parasitology, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05, České Budějovice, Czech Republic.

TABLE I. Polymerase chain reaction primers used for amplification and sequencing of *Myxidium melleni* n. sp. small subunit ribosomal DNA.

Name	Position*	Sequence	Reference
18E†	1	CTG GTT GAT CCT GCC AGT	Hillis and Dixon, 1991
Myxgen4F	831–850	GTG CCT TGA ATA AAT CAG AG	Diamant et al., 2004
Myxgen3f	1059–1079	GGA CTA ACR AAT GCG AAG GCA	Kent et al., 2000
18J†	1316–1337	GGC TTA ATT TGA CTC AAC ACG G	Hillis and Dixon, 1991
18R	1966–1948	CTA CGG AAA CCT TGT TAC G	Whipps et al., 2003
18K†	1337–1316	CCG TGT TGA GTC AAA TTA AGC C	Hillis and Dixon, 1991
Myxgen2r	1083–1063	CAR ATG CYT TCG CWY TTG TTA	Kent et al., 2000
18I†	385–367	TCT CCG GAA TCG AAC CCT G	Hillis and Dixon, 1991

* Position relative to GenBank DQ003031.
† Primer sequence modified from original description.

Rana blairi, 35 *Rana catesbeiana*, 31 *Hyla chrysoscelis*, 31 *Pseudacris triseriata triseriata*, 11 *Bufo woodhousii*, and 9 *Acris crepitans blanchardi*. Frogs and toads were brought back to the laboratory, pithed, and necropsied within 72 hr of collection. All organs were examined for myxosporeans. Whole gallbladders were removed from dissected frogs, examined fresh using wet-mount preparations or observed under a stereomicroscope to observe plasmodia in situ. Additionally, all fecal contents were examined microscopically at the time of necropsy for the presence of spores. All positive fecal samples containing spores were placed in 2.5% potassium dichromate ($K_2Cr_2O_7$), kept at room temperature in vials, and examined over a period of 6 mo. Permanent slides of plasmodia were made from air-dried bile smears fixed in methanol and stained with Giemsa. A few plasmodia were fixed in 10% neutral buffered formalin (for scanning electron microscopy) and in 95% ethanol (for DNA isolation).

For light microscopy, either fresh preparations of plasmodia were made or samples of fecal sediments were collected from each vial containing dichromate-preserved intestine contents, and observed as wet mounts. Measurements of 30 plasmodia in fresh bile mounts and 30 fresh spores from the fecal content of each species of infected frog were made with the aid of a calibrated ocular micrometer. All spore and plasmodia measurements obtained using light microscopy are reported in micrometers (μm) and indicated by 1 decimal position with a mean followed by the range in parentheses. Light microscopy studies were made using a Wild M20 phase-contrast microscope or Olympus AX 70 microscope equipped with Nomarski interference-contrast optics (NIC).

For scanning electron microscopy (SEM), formalin preserved plasmodia were used. Coverslips were coated with poly-lysine, and air-dried. Spores were removed from plasmodia, and in an aqueous suspension, were allowed to settle on poly-lysine–coated coverslips for 10–20 min. Coverslips with spores were then fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (CB), for 2 hr and washed in CB (3 times for 10 min). Coverslips with spores were then postfixed for 30 min in 4% osmium tetroxide mixed with CB (1:1 ratio). Coverslips were washed with CB (3 times for 10 min), dehydrated in a graded acetone series (30–100%), critical-point dried, mounted on stubs, coated with gold, and examined with a JEOL 6300 (JEOL Ltd., Tokyo, Japan) scanning electron microscope. Measurements obtained using SEM are reported in micrometers (μm) and indicated by 2 decimal positions.

Whole gallbladders containing plasmodia of some frogs were fixed in Bouin's fixative, processed routinely, and embedded in paraffin; sectioned at 12 μm; and affixed to slides, stained with hematoxylin and eosin, mounted in Canada balsam, and examined microscopically. All micrographs were made from material originating from *P. triseriata triseriata*. Description of the new species follows guidelines provided by Lom and Arthur (1989). In the morphological section of this article, we consistently use terms such as valvular, sutural, and polar view and define these terms as follows: valvular view, perpendicular-to-sutural plane; sutural view, perpendicular-to-longitudinal axis of the spore, parallel with the sutural plane; polar view, parallel with the longitudinal axis of the spore. All amphibian common names used are according to Frost (2004).

TABLE II. Prevalence of *Myxidium melleni* n. sp. in 6 anuran species collected from Pawnee Lake, Lancaster County, Nebraska during March 2001–April 2004.

Anuran species	No. infected/ No. examined (%)	95% Confidence interval*
Rana blairi	0/47 (0)	0–8
Rana catesbeiana	0/35 (0)	0–10
Hyla chrysoscelis	0/31 (0)	0–11
Pseudacris triseriata triseriata	20/31 (65)	49–82
Acris crepitans blanchardi	1/9 (11)	0.5–44
Bufo woodhousii	0/11 (0)	0–26

* 95% Confidence intervals were calculated for appropriate sample size based on the tables provided in Rohlf and Sokal (1995).

DNA analysis

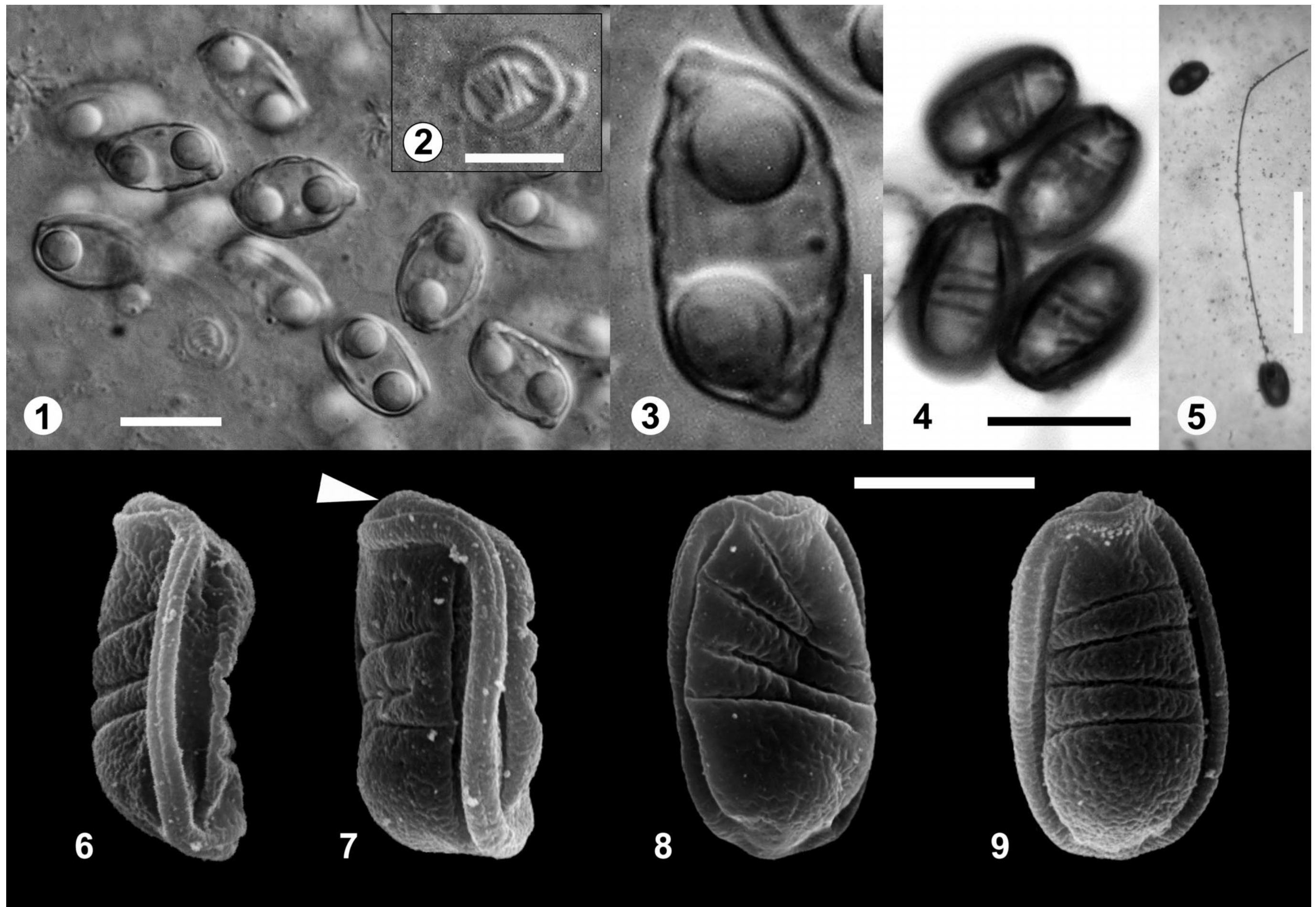
The DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) was used to extract DNA from a plasmodium of *M. melleni* n. sp. Overlapping regions of the SSU rDNA were amplified with polymerase chain reaction (PCR) primers listed in Table I. Amplification products were sequenced in both directions with PCR primers using AP Biotech DYEnamic ET Terminator cycle sequencing chemistry with Thermo Sequenase II (Amersham Biosciences, Piscataway, New Jersey) on an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, California). The resulting sequence fragments were manually aligned, yielding a 1,947 base pair (bp) contiguous sequence of SSU rDNA.

Phylogenetic inference

The SSU rDNA sequence of *M. melleni* n. sp. was aligned to SSU sequences of other species in the suborder Variisporina and sequences from relevant basic logical alignment search tool matches on GenBank. *Tetracapsuloides bryosalmonae* (Gen Bank U70623) was used as an out-group to root the phylogenetic tree. Nucleic acid sequence alignments were conducted in ClustalX (Thompson et al., 1997) using default settings. The resultant sequence alignment was then edited by eye to remove ambiguous regions where we could not confidently identify positions of homology, yielding a 1,400-character alignment. For shorter SSU sequences, unavailable sequence was treated as missing data, and gaps were treated as a fifth character state. Parsimony analyses were conducted in PAUP* Version 4.0b10 (Swofford, 1998), using a heuristic search algorithm with 10 random additions of sequences and tree bisection–reconnection (TBR) branch swapping. Bootstrap values were calculated with 1,000 replicates using a heuristic search algorithm with simple sequence addition and TBR branch swapping.

RESULTS

Of the 6 anuran species examined, myxozoans were only observed in *Pseudacris triseriata triseriata* and *Acris crepitans blanchardi* (Table II). In all infected frogs, plasmodia were found in the gallbladder, and free spores were found in the



FIGURES 1–5. Mature spores of *Myxidium melleni* n. sp. (**1**) Formalin preserved spores, Nomarski interference-contrast (NIC) optics. Note that the distinct transverse grooves are only barely visible. Bar = 10 μm. (**2**) Detail of polar capsule showing 6–7 coils of polar filament, NIC optics. Bar = 5 μm. (**3**) Detail of formalin-preserved spore, in sutural view, polar capsules positioned at opposite poles of the spore and showing opposite orientation along the suture, NIC optics. Bar = 5 μm. (**4**) Giemsa-stained spores. Note that the distinct transverse grooves are clearly visible. Bar = 10 μm. (**5**) Giemsa-stained spores showing extruded polar filament. Bar = 40 μm.

FIGURES 6–9. Scanning electron microscopy (SEM) micrographs of mature spores of *Myxidium melleni* n. sp. (**6**) Sutural view. Note that the sutural ridge is enclosed on either side by a depression on the valve surface. (**7**) Mature spore in sutural view showing polar filament eversion pole. Note the papillary structure (arrowhead) surrounding polar filament eversion pole on the broad end of the shell valve. (**8–9**) Valvular views of mature spores showing variability of spore surface and transverse grooves. All in the same scale. Bar = 5 μm.

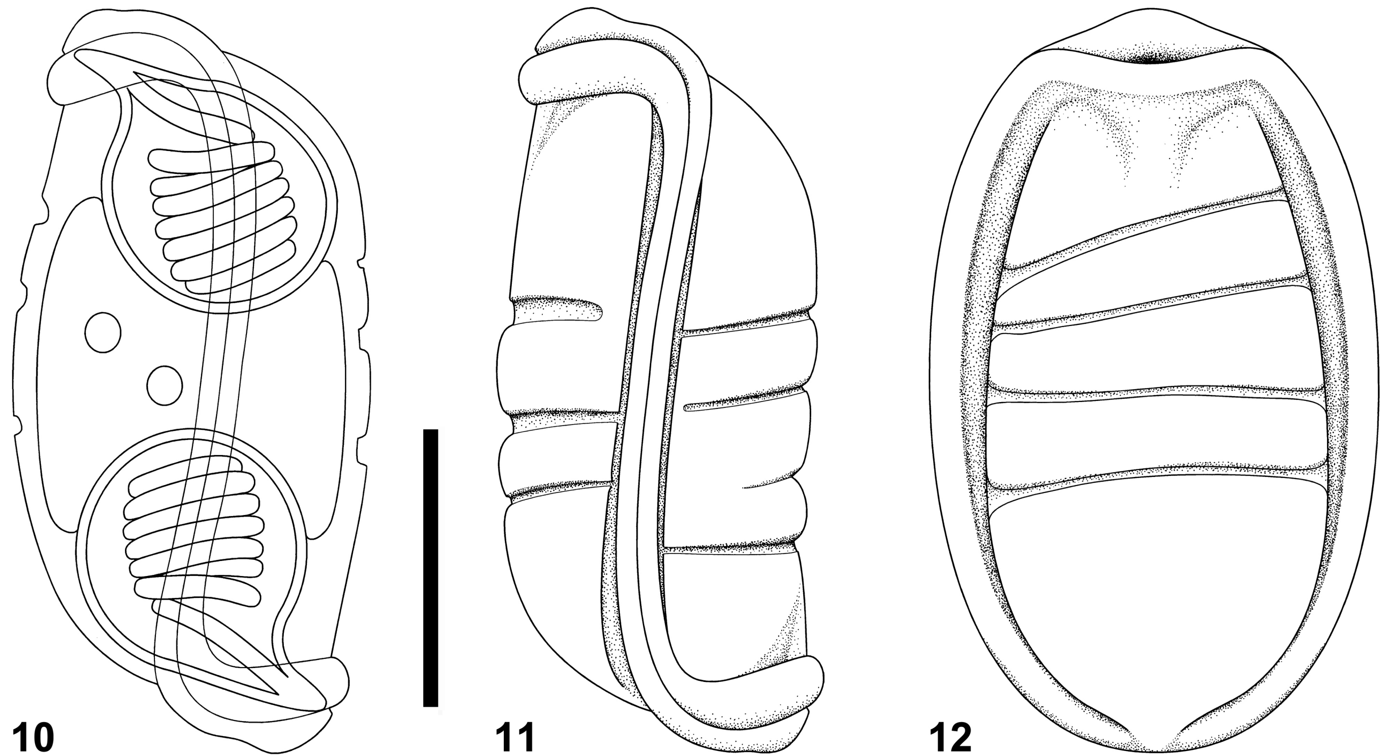
intestine contents. Spore morphology, plasmodium characteristics, and site of infection were consistent with definition of the genus *Myxidium* Bütschli, 1882, and we describe it as a member of this genus.

DESCRIPTION

Myxidium melleni n. sp.
(Figs. 1–9)

Spores: Mature spores, broadly ellipsoidal with slightly flattened ends in valvular view (Figs. 4, 8, 9, 12); elongate and rhomboidal with rounded edges in sutural view (Figs. 3, 6, 7, 10, 11); composed of 2 identical valves. In sutural view, sutural ridge 0.80–0.90 thick, divided by sutural line running down center of ridge (Figs. 6, 7, 10, 11). Sutural ridge turns at angle of approximately 90° near each pole; oriented in opposite directions at opposite poles (Figs. 6, 7, 10, 11). Ridge enclosed on either side by depression varying in depth and width (0.05–1.00), which disappears near poles of spore (Figs. 6–9, 11, 12). Sutural ridge broadens and forms papillary projection around filament eversion pole on broad end of each shell valve (Figs. 6–9, 11, 12). Two equally sized polar capsules: subspherical, sharply attenuated at polar ends, and positioned at opposite poles of spore. In sutural view, axis of each polar capsule diverts (in opposite directions) at an angle of 40–50° from longitudinal axis of spore (Figs. 1, 3, 10). Polar filaments with 6 or 7 coils visible in some formalin-fixed spores (Fig. 2). In methanol-fixed and Giemsa-stained slides, extruded spores observed. Extruded filaments: on average, 78 long (50–103, n = 11) and approximately 0.4 thick. Single extruded filament more commonly observed (Fig. 5) than both extruded filaments. Sporoplasm: binucleate, filling spore cavity between 2 polar capsules (Fig. 10). Valve surface, almost smooth (Fig. 8) or more or less rough/cragged (Figs. 6, 7, 9). In valvular view, each valve with 2–5 distinct transverse grooves, 0.06–0.25 wide, running across whole valve, usually positioned in center of valve, sometimes connected to sutural depression (Figs. 8, 9, 12). Length and direction of each transverse groove often irregular (Figs. 6–9, 11, 12). In valvular view, transverse grooves difficult to discern in fresh and formalin fixed material, but clearly visible in Giemsa-stained spores (Fig. 4) and SEM preparations (Figs. 6–9).

Spore measurements: Pseudacris triseriata triseriata (n = 30), 12.3 long × 7.6 wide (range 12.0–13.5 × 7.0–9.0) × 6.6 thick (range 6.0–8.0), polar capsules 5.2 long × 4.2 wide (range 4.8–5.5 × 3.8–4.5);



FIGURES 10–12. Line drawings of mature spore of *Myxidium melleni* n. sp. (**10**) Internal details of spore. (**11**) Sutural view of spore. (**12**) Valvular view of spore. All in the same scale. Bar = 5 μm.

Acris crepitans blanchardi (n = 30), 11.9 long × 7.2 wide (range 11–12.5 × 6.5–8.0) × 5.9 thick (range 5–7), polar capsules 4.9 long × 4.1 wide (range 4.5–5.5 × 3.8–4.5).

Plasmodia: Parasites float freely in bile, easily seen through gallbladder wall (Fig. 13), stacked on top of each other, occupying significant part of gallbladder volume (Fig. 13). Additionally, larger plasmodia partly wrapped around themselves as well as around neighboring plasmodia. No host tissue damage evident despite heavy infections (Fig. 18). Size and number (2–54) of plasmodia varies among infected frogs. Plasmodia: flattened and disc-shaped or elliptical in shape (Fig. 14, 16, 17), do not exhibit any motility. Plasmodia were 400–1,375 long × 230–1,200 wide (mean 691 × 498, n = 30), 15–36 thick (mean 23.5) from *P. triseriata triseriata*, and 100–720 long × 80–420 wide (mean 326.7 × 247.3, n = 30) from *A. crepitans blanchardi*. Plasmodia not uniformly thick, about 30 in center and gradually decreasing in thickness to 15; 5 at ends. Plasmodia with thin hyaline ectoplasm and granular endoplasm observed in wet mounts and sectioned plasmodia (Figs. 15, 18). Plasmodia polysporic, producing many pansporoblasts distributed in endoplasm (Figs. 17, 18). In Giemsa-stained plasmodia, numerous generative stages observed scattered throughout plasmodia, ranging in number from 10–12 nuclei to fully formed spores (Fig. 19), suggesting active sporogenesis. Pansporoblasts disporic (Figs. 17–19). No relationship between plasmodia size and number of spores produced.

Taxonomic summary

Type host: Pseudacris triseriata triseriata (Wied-Nuweid 1838), western chorus frog (Anura: Hylidae).

Other host: Acris crepitans blanchardi Harper 1947, Blanchard's cricket frog (Anura: Hylidae).

Type locality: Pawnee Lake, Lancaster County, Nebraska (40°51.18′N, 96°53.11′W).

Site of infection: Gallbladder lumen.

Prevalence: Twenty of 31 (65%) *P. triseriata triseriata* were infected during March 2004, and 1 of 9 (11%) *A. crepitans blanchardi* was infected during May of 2002.

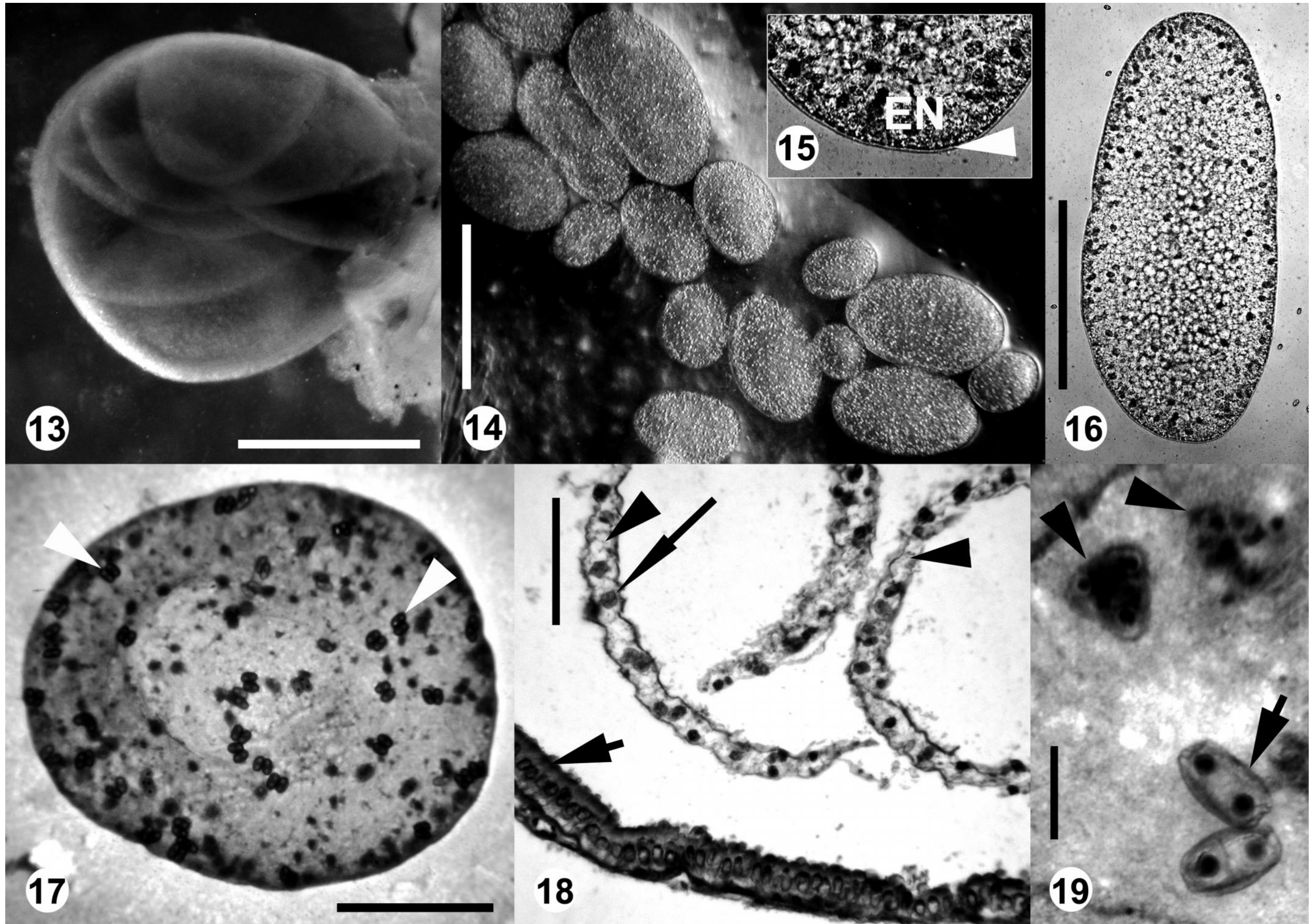
Type specimens: Deposited at the Harold W. Manter Laboratory collection (HWML), University of Nebraska State Museum, Lincoln, Nebraska. Syntype Giemsa-stained slide of plasmodia with spores, HWML 48167; plasmodia preserved in formalin, HWML 48168; gold coated spores, HWML 48169; histological sections of infected gallbladder, HWML 48170; plasmodia preserved in 95% ethanol, HWML 48171; digital images of photomicrographs, HWML 48172.

Type host: P. triseriata triseriata symbiotype and liver sample stored in 95% ethanol for molecular analysis, deposited in the division of herpetology, Natural History Museum and Biodiversity Research Center, University of Kansas, Lawrence, Kansas (KU, 296429).

Etymology: The specific epithet is named in honor of John W. Mellen, who introduced M.G.B. to amphibian parasites during his undergraduate research.

REMARKS

Delvinquier et al. (1992) reviewed the *Myxidium* spp. of amphibians. Of the 4 described species, only *M. lesminteri* from the South African knocking sand frog *Tomopterna krugerensis* overlaps in spore dimensions with *M. melleni* n. sp. However, spores of *M. lesminteri* differ in both suture arrangement (nearly straight or slightly curved vs. distinctly curved near each pole), and spore surface structure (with 2–5 transversal grooves). *Myxidium serotinum*, the only other North American amphibian species of *Myxidium*, differs in having larger spores (16–18 × 9 in *M. serotinum* vs. 12.0–13.5 × 7.0–9.0 in *M. melleni* n. sp.) and in containing 10–13 rough transversal ridges on the spore surface. Similarly, distinct transversal ridges occur on the South American *M. immersum*. *Myxidium haldari*, described from a tree frog in West Bengal, clearly differs in having longitudinal ridges on the spore surface. Finally, there are 19 other named and 11 unnamed species of *Myxidium* spp. reported from strictly freshwater fish and turtles in North America (Mitchell, 1967; Johnson, 1969; Li and Desser, 1985). Of these,



FIGURES 13–19. Plasmodia of *Myxidium melleni* n. sp. (**13**) Gallbladder of *Pseudacris triseriata triseriata* filled with mature plasmodia. Bar = 0.5 mm. (**14**) Wet mount low-power magnification of mature plasmodia showing shape. Bar = 1 mm. (**15**) Peripheral region of plasmodium. Note thin, but well defined, hyaline ectoplasm layer (arrowhead) and granular endoplasm (EN). (**16**) Wet mount of plasmodia. Bar = 300 μm. (**17**) Giemsa-stained plasmodia showing internal distribution of pairs of mature spores (arrowheads). Bar = 100 μm. (**18**) Paraffin section through gallbladder of *Pseudacris triseriata triseriata* containing plasmodia, stained with hematoxylin and eosin. Note that the plasmodia (arrowheads) float freely in gallbladder bile, and no apparent damage is visible to the gallbladder epithelial cells (arrow). Disporic pansporoblasts are visible in endoplasm (long arrow). Bar = 100 μm. (**19**) Higher magnification of Giemsa-stained plasmodium showing numerous generative stages (arrowheads) and fully formed spores (arrow). Bar = 10 μm.

the spore size and shape of *M. melleni* n. sp. most closely resembles *Myxidium macrocapsulare* from the freshwater drum, *Aplodinotus grunniens*, and *Myxidium macrocheili* from the large-scale sucker, *Catostomus macrocheilus*. However, spores of these 2 species have 6–8 and 9–11 striae parallel to the sutural line and are, therefore, morphologically distinct from *M. melleni* n. sp.

Based on these distinct morphological differences, it is unlikely that the form described here is conspecific with previously described *Myxidium* spp. from North American freshwater vertebrates and is described as a new species.

Phylogenetic analysis

In our 1,400-character DNA sequence alignment, 484 characters were constant, 250 were uninformative, and 666 were parsimony informative. Parsimony analysis yielded 3 most-parsimonious trees; from which, we constructed a strict consensus tree (Fig. 20). *Myxidium melleni* n. sp. was a sister taxon to *Sphaeromyxa zaharoni*, within a clade of *Myxidium*, *Chloromyxum*, and *Sphaerospora* species. Although most *Myxidium* species were placed within this clade, the genus was polyphyletic in our analysis. Both *Myxidium lieberkuehni* and *Myxidium giardi* were members of 2 separate clades (Fig. 20).

DISCUSSION

To date, *M. serotinum* Kudo and Sprague, 1940, is the only species of *Myxidium* reported from North American amphibians, originally described from the northern leopard frog, *R. pipiens*, and an unidentified *Rana* sp., from the Midwest (exact locality not given by authors) and Louisiana, respectively. Kudo (1943), in a later study, gave a detailed description of the plasmodia of this species from the gallbladder of the southern toad, *Bufo terrestris*, the bronze frog, *Rana clamitans* (=*R. clamitans clamitans*), and the Florida leopard frog, *Rana sphenocephala utricularia* (=*R. sphenocephala*), all collected in Florida. Unfortunately, his study did not include a morphological analysis

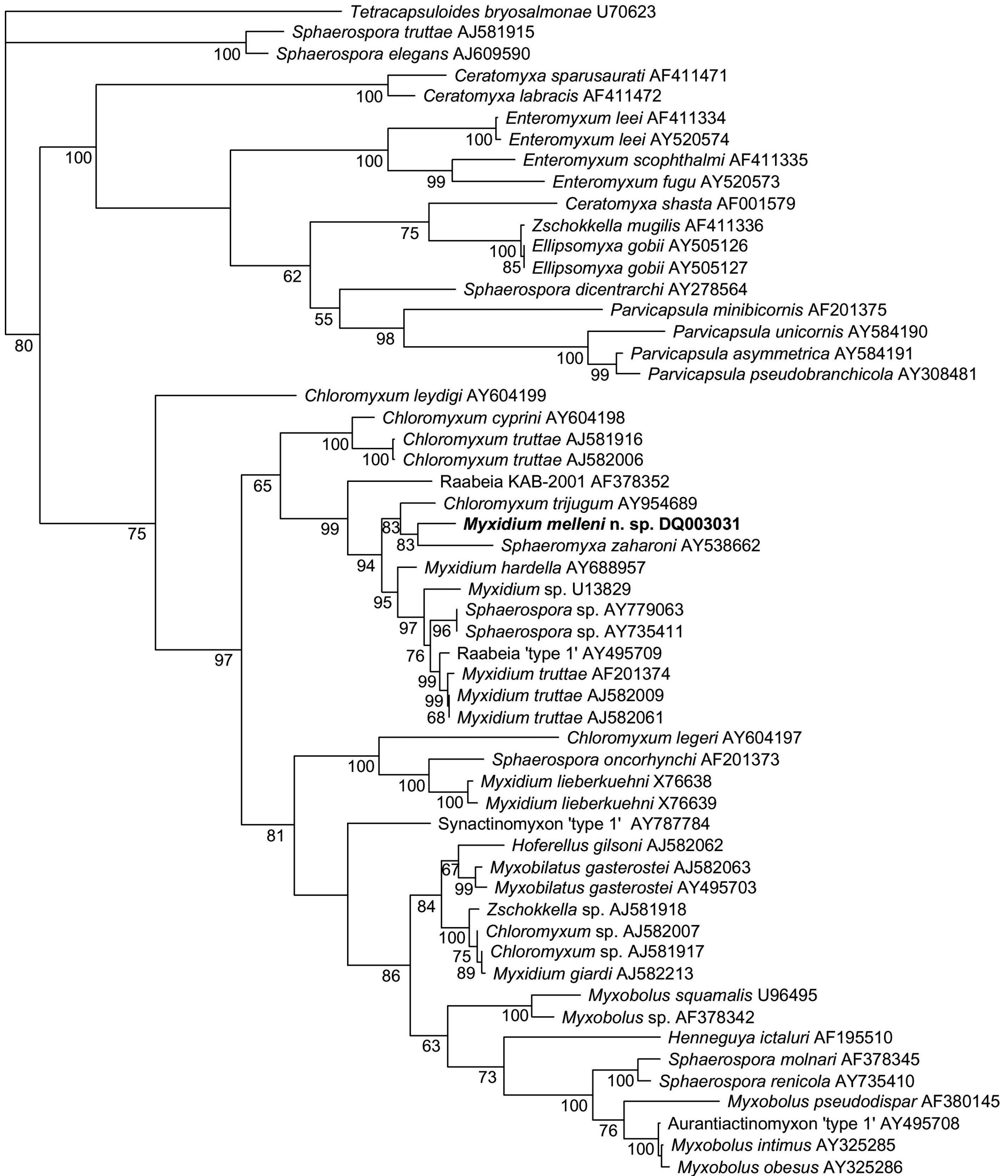


FIGURE 20. Strict consensus tree of 3 equal-length trees obtained by maximum parsimony analysis of Variisporina small subunit ribosomal DNA. Numbers beside species names designate GenBank accession numbers. Bootstrap confidence values are shown at nodes.

of the spores, and it is unclear if his isolates were conspecific with *M. serotinum*. Since the original description by Kudo and Sprague (1940), all *Myxidium*-like myxozoans observed in North American amphibians have been provisionally determined as *M. serotinum* (see McAllister and Trauth, 1995; McAllister and Bursey, 2005). Our study represents the first new description of a *Myxidium* species from American amphibians in over 65 yr.

Other studies on myxozoan parasites of amphibians suggest that they are not host specific (Delvinquier et al., 1992; McAllister and Bursey, 2005). *Myxidium immersum* has been reported from 32 South American and Australian amphibian species in 6 families, and *M. serotinum* has been reported from 20 North American amphibian species, belonging to 5 families (Delvinquier, et al., 1992; McAllister and Bursey, 2005). However, it is unclear whether these parasites truly have broad host specificity or whether they comprise an assemblage of species. Indeed, some authors report various measurements of *M. serotinum* spores differing from those given in the original species description (see McAllister and Trauth, 1995, for review). These differences are repeatedly explained as variations caused by different host species; however, the taxonomic implications of these observed variations are rarely discussed (Clark and Shoemaker, 1973; McAllister and Trauth, 1995; McAllister, et al., 1995; McAllister and Bursey, 2005). Therefore, our discovery of a new *Myxidium* species in *A. crepitans blanchardi*, previously reported as a host for *M. serotinum*, suggests that the diversity of *Myxidium* species in amphibians may be underestimated due to the lack of comprehensive morphological and molecular data and thorough sampling of amphibians throughout different regions. The importance of molecular sequence data is critical because different spore morphotypes (myxosporean/actinosporeans) can represent the same species.

Our study highlights some of the difficulties in evaluation of spore morphology based on light microscopy alone (compare Figs. 1–4 with Figs. 6–9). Our observations suggest that taxonomically important spore surface structures in *Myxidium* are difficult to observe clearly by light microscopy alone and, if possible, should be examined with SEM. Indeed, although superficially similar to *M. serotinum*, spores of *M. melleni* n. sp. possess distinct surface structures, demonstrating the utility of SEM studies for *Myxidium* species descriptions. Only 2 studies by Clark (1982) in an unpublished thesis and McAllister et al. (1995) provide SEM documentation of the North American *M. serotinum*-like spores. Unfortunately, observations by McAllister et al. (1995) were based on material from a single Texas toad, *B. speciosus*, and differences in ultrastructure were discussed marginally. In their study, they confirmed the 10–13 transverse ridges described by Kudo and Sprague (1940). However, their study, based on measurements of 10 spores, indicated that *M. serotinum* spores were 8.0 (8.5–9.5) long and 6.2 (5.8–6.5) wide. These spore dimensions are almost half the size of those in the original description (16–18 × 9 from fresh material; 15 (12–20) × 8.8 (7.5–10) from fixed material) of Kudo and Sprague (1940). These striking morphological differences support our contention that *M. serotinum* consists of an assemblage of species.

In addition to the numerous reports containing inadequate morphological analyses, no topotype material exists for *M. serotinum* (= *M. serotinum* from *R. pipiens* from the "midwest USA" and unidentified *Rana* sp. from Louisiana). Thus, reanalyzing the status of *M. serotinum* is virtually impossible. However, an extensive re-description of *M. serotinum*, according to current standards, including elaborated morphological and, if possible, SEM data from different hosts, together with molecular characterization, could clarify this confusing situation. Furthermore, when new species or new hosts are encountered, the type material must be deposited for future reference. We suggest that, if possible, metal-coated spores along with photosyntypes, alcohol, and formalin-fixed spores, and histological preparations of plasmodia can serve as the type material, fulfilling the requirements of taxonomical rules (International Code of Zoological Nomenclature, 1999). Applying these criteria would clarify the diversity of *Myxidium* spp. parasitizing amphibians.

Morphological analyses alone may be insufficient to distinguish between species and provide evolutionarily accurate taxonomic schemes for the Myxozoa (Andree et al., 1999; Kent et al., 2001; Blaylock et al., 2004; Whipps et al., 2004). Although not absolutely required for a species description, Kent et al. (2001) encouraged researchers to provide rDNA sequences. We endorse such an approach, especially for genera like *Myxidium*, where spore morphology is a poor indication of phylogenetic affinity. Species of *Myxidium* exhibit a variety of morphological characteristics, suggesting a polyphyletic origin of this assemblage of species (Canning et al., 1999), which is supported by several phylogenetic analyses (Kent et al., 2001; Holzer et al., 2004; Garner et al., 2005). Furthermore, distinction between *Myxidium* and *Zschokkella* is problematic, and some species have been assigned to these genera arbitrarily (Lom and Dykova, 1992). Such ambiguities were recognized in *Myxidium leei* and a new species by Palenzuela et al. (2002), and using both morphological and molecular analyses, a new genus *Enteromyxum* was described. The study of Palenzuela et al. (2002) exemplifies the utility of a multidisciplinary approach and suggests that further taxonomic revisions may be warranted to account for the intrageneric heterogeneity of *Myxidium*.

Our phylogenetic analyses were largely consistent with previous analyses of variisporinid SSU rDNA (Kent et al., 2001; Palenzuela et al., 2002; Diamant et al., 2004; Fiala and Dyková, 2004). There were some differences in tree topology when compared when the analyses of Holzer et al. (2004). However, these did not concern the placement of *M. melleni* n. sp. and closely related species. Parsimony analysis of the *M. melleni* n. sp. SSU rDNA sequence placed this species in a clade that comprises other *Myxidium* species and representatives of *Chloromyxum*, *Sphaeromyxa*, and *Sphaerospora*. With the exception of the 2 Raabeia actinosporeans and the 2 *Sphaerospora* sp., all of the representatives (11 of 15) in this clade are parasites of the gallbladder or bile duct. Phylogenetic affinity based on tissue specificity has been suggested for myxozoans (Andree et al., 1999; Holzer et al. 2004). However, like spore morphology, this relationship is not completely clear because there are several other myxozoan species, i.e., *Chloromyxum* spp. in our analysis, that are gallbladder parasites, which fall outside of the clade containing *M. melleni* n. sp. Therefore, tissue specificity is likely a useful character only within a particular clade or phylogenetic grouping of myxozoans, but its utility over a broad range of species, particularly from different genera and families, is less concrete.

Myxidium melleni n. sp. was phylogenetically distinct from

other *Myxidium* species and distant from the type species *Myxidium lieberkuehni*. We can speculate that *Myxidium* species of amphibians may represent a distinct lineage, although this assumption is preliminary at best because only 1 species was included, and rDNA sequences are available for so few other *Myxidium* species. Alternatively, *M. melleni* n. sp. and related species are generally gallbladder parasites, and whether they are piscine or amphibian parasites may have little to do with phylogenetic relationship. Our data are consistent with those of other authors (as mentioned above) in that the clade containing the type species, *M. lieberkuehni*, is phylogenetically distinct from lineages comprising other members of the genus. Thus, future taxonomic division of *Myxidium* into several genera should be considered. However, until more *Myxidium* spp. can be included in molecular phylogenies and characters for each clade of ''*Myxidium*-like'' myxosporeans are defined, we suggest a conservative approach and maintain the current taxonomic scheme.

In our opinion, only careful morphological studies along with molecular and life-cycle studies will resolve the host-specificity and variation problems among amphibian *Myxidium* species. We have identified the following problems that must be addressed in future descriptions and re-descriptions of amphibian myxosporea: (1) absence of the type material, (2) insufficient morphological data obtained solely by light microscopy; and (3) invalid or insufficient host determination and absence of the symbiotype (sensu Frey et al., 1992). In recent years, our state of knowledge of taxonomy and new technologies for species descriptions, together with recent opinions about the taxonomy and curatorial management of other parasite taxa, has increased (Frey et al., 1992; Duszynski and Wilber, 1997). For these reasons, we feel that the following additions for species descriptions of myxosporea should be added to the guidelines provided by Lom and Arthur (1989).

Additional guidelines for descriptions of new species of amphibian Myxosporea

The host: (1) A symbiotype voucher specimens should be deposited in appropriate accredited museum. (2) Because recent phylogenetic and phylogeographical studies of amphibians, including *P. triseriata triseriata*, clearly indicate that there are different lineages within single species of frogs (Austin et al., 2004; Hoffman and Blouin, 2004; Moriarty and Cannatella, 2004), we suggest that DNA sample or ethanol-preserved tissue should be deposited together with the symbiotype to allow future clarification of host taxonomic problems.

The spores and vegetative stages: (1) If possible, SEM data should be incorporated, and to allow further SEM examination of spores, metal-coated spores should be deposited together with other topotype material. (2) To allow future clarification of taxonomic problems, the total DNA sample or ethanol-preserved myxospores should be deposited. (3) Histological preparations and paraffin-embedded tissues should be deposited.

ACKNOWLEDGMENTS

M. G. B. thanks Melissa Bolek for help in collecting frogs; this work was supported by grants from the Initiative for Ecological and Evolutionary Analysis, University of Nebraska–Lincoln; Special Funds, School of Biological Sciences University of Nebraska–Lincoln, and the Center for Great Plains Studies graduate student Grant-in-aid, University of Nebraska–Lincoln. M. J. and D. M. are indebted to Olympus C&S for generous technical support; their work was supported by grants 206/03/1544 and 524/03/H133 from the Grant Agency of the Czech Republic. We also thank 2 anonymous reviewers for improvements on an earlier draft of the manuscript.

LITERATURE CITED

ANDREE, K. B., C. SZÉKELY, K. MOLNÁR, S. J. GRESOVIAC, AND R. P. HEDRICK. 1999. Relationships among members of the genus *Myxobolus* (Myxozoa: Bilvalvidae) based on small subunit ribosomal DNA sequences. Journal of Parasitology **85:** 68–74.

AUSTIN, J. D., S. C. LOUGHEED, AND P. T. BOAG. 2004. Discordant temporal and geographic patterns in maternal lineages of eastern north American frogs, *Rana catesbeiana* (Ranidae) and *Pseudacris crucifer* (Hylidae). Molecular Phylogenetics and Evolution **32:** 799–816.

BERGER, L., A. D. HYATT, V. OLSEN, S. G. HENGSTBERGER, D. BOYLE, G. MARANTELLI, K. HUMPHREYS, AND J. E. LONGCORE. 2002. Production of polyclonal antibodies to *Batrachochytrium dendrobatidis* and their use in an immunoperoxidase test for chytridiomycosis in amphibians. Diseases of Aquatic Organisms **48:** 213–220.

BLAYLOCK, R. B., S. A. BULLARD, AND C. M. WHIPPS. 2004. *Kudoa hypoepicardialis* n. sp. (Myxozoa: Kudoidae) and associated lesions from the heart of seven perciform fishes in the northern Gulf of Mexico. Journal of Parasitology **90:** 584–593.

CANNING, E. U., A. CURRY, C. L. ANDERSON, AND B. OKAMURA. 1999. Ultrastructure of *Myxidium trachinorum* sp. nov. from the gallbladder of the lesser weever fish *Echiichthys vipera*. Parasitology Research **85:** 910–919.

CARINI, A. 1932. *Myxidium lindoyense* n. sp., parasita da vesicula biliar de batrachios do Brasil. Revista de Biologia e Hygiene **3:** 83–84. [In Portuguese].

CLARK, J. G. 1982. Studies on the biology of *Myxidium serotinum* Kudo and Sprague, 1940 (Myxozoa), a parasite of amphibian vertebrates. Ph.D. Thesis. University of Cincinnati, Cincinnati, Ohio, 157 p.

———, AND J. P. SHOEMAKER. 1973. *Eurycea bislineata* (Green), the two-lined salamander, a new host of *Myxidium serotinum* Kudo & Sprague, 1940 (Myxosporida, Myxidiidae). Journal of Protozoology **20:** 365–366.

CORDERO, E. H. 1919. ''*Cystodiscus immersus*'' Mixosporidio de los batracios del Uruguay. Physis **4:** 403–409. [In Spanish].

DELVINQUIER, B. L. J. 1986. *Myxidium immersum* (Protozoa: Myxosporea) of the cane toad, *Bufo marinus*, in Australian Anura, with a synopsis of the genus in amphibians. Australian Journal of Zoology **34:** 843–853.

———, M. B. MARKUS, AND N. I. PASSMORE. 1992. *Myxidium lesminteri* n. sp. (Myxosporea, Myxdiidae) from the gall bladder of three southern African Anura. Systematic Parasitology **23:** 25–30.

DESSER, S. S., J. LOM, AND I. DYKOVÁ. 1986. Developmental stages of *Sphaerospora ohlmacheri* (Whinery, 1893) n. comb. (Myxozoa, Myxosporea) in the renal tubules of bullfrog tadpoles, *Rana catesbeiana*, from Lake of Two Rivers, Algonquin Park, Ontario. Canadian Journal of Zoology **64:** 2213–2217.

DIAMANT, A., C. M. WHIPPS, AND M. L. KENT. 2004. A new species of *Sphaeromyxa* (Myxosporea: Sphaeromyxina: Sphaeromyxidae) in devil firefish, *Pterois miles* (Scorpaenidae), from the northern Red Sea: morphology, ultrastructure, and phylogeny. Journal of Parasitology **90:** 1434–1442.

DUSZYNSKI, D. W., AND P. G. WILBER. 1997. A guideline for the preparation of species descriptions in the Eimeriidae. Journal of Parasitology **83:** 333–336.

FIALA, I., AND I. DYKOVÁ. 2004. The phylogeny of marine and freshwater species of the genus *Chloromyxum* Mingazzini, 1890 (Myxosporea: Bivalvulida) based on small subunit ribosomal RNA gene sequences. Folia Parasitologica **51:** 211–214.

FREY, K. F, T. L. YATES, D. W. DUSZYNSKI, W. L. GANNON, AND S. L. GARDNER. 1992. Designation and curatorial management of type host specimens (symbiotypes) for new parasite species. Journal of Parasitology **78:** 930–932.

FROST, D. R. 2004. Amphibian species of the world: an online reference. Version 3.0 (22 August, 2004). Electronic database accessible at

http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, New York

GARNER, M. M., J. L. BARTHOLOMEW, C. M. WHIPPS, R. W. NORDHAUSEN, AND P. RAITI. 2005. Renal myxozoanosis in Crowned River turtles *Hardella thurjii*: Description of the putative agent, *Myxidium hardella* n. sp. by histopathology, electron microscopy, and DNA sequencing. Veterinary Pathology. Veterinary Pathology **42:** 589–595.

HILL, B. D., P. E. GREEN, AND H. A. LUCKE. 1997. Hepatitis in the green tree frog (*Litoria caerulea*) associated with infection by a species of *Myxidium*. Australian Veterinary Journal **75:** 910–911.

HILLIS, D. M., AND M. T. DIXON. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quarterly Review of Biology **66:** 411–453.

HOFFMAN, E. A., AND M. S. BLOUIN. 2004. Evolutionary history of the northern leopard frog: Reconstruction of phylogeny, phylogeography, and historical changes in population demography from mitochondrial DNA. Evolution **58:** 145–159.

HOLZER, A. S., C. SOMMERVILLE, AND R. WOOTTEN. 2004. Molecular relationships and phylogeny in a community of myxosporeans and actinosporeans based on their 18S rDNA sequences. International Journal for Parasitology **34:** 1099–1111.

INTERNATIONAL CODE OF ZOOLOGICAL NOMENCLATURE. 1999. International code of zoological nomenclature, 4th ed. International Trust for Zoological Nomenclature, The Natural History Museum, London, U.K., 306 p.

JOHNSON, C. A., III. 1969. A redescription of *Myxidium chelonarum* Johnson, 1969 (Cnidospora: Myxidiidae) from various North American turtles. Journal of Protozoology **16:** 700–702.

KENT, M. L., J. KHATTRA, R. P. HEDRICK, AND R. H. DEVLIN. 2000. *Tetracapsula renicola* n. sp. (Myxozoa: Saccosporidae); the PKX myxozoan—The cause of proliferative kidney disease of salmonid fishes. Journal of Parasitology **86:** 103–111.

———, K. B. ANDREE, J. L. BARTHOLOMEW, M. EL-MATBOULI, S. S. DESSER, R. H. DEVLIN, S. W. FEIST, R. P. HEDRICK, R. W. HOFFMANN, J. KHATTRA, S. L. HALLETT, R. J. G. LESTER, M. LONGSHAW, O. PALENZEULA, M. E. SIDDALL, AND C. XIAO. 2001. Recent advances in our knowledge of the Myxozoa. Journal of the Eukaryotic Microbiology **48:** 395–413.

KUDO, R. R. 1943. Further observations on the protozoan, *Myxidium serotinum*, inhabiting the gallbladder of North American Salientia. Journal of Morphology **72:** 263–277.

———, AND V. SPRAGUE. 1940. On *Myxidium immersum* (Lutz) and *M. serotinum* n. sp., two Myxosporidian parasites of Salientia of South and North America. Revista de Medicina Tropical y Parasitología **6:** 65–72.

LI, L., AND S. S. DESSER. 1985. The protozoa parasites of fish from two lakes in Algonquin Park, Ontario. Canadian Journal of Zoology **63:** 1846–1858.

LOM, J., AND J. R. ARTHUR. 1989. A guideline for the preparation of species descriptions in Myxosporea. Journal of Fish Diseases **12:** 151–156.

———, AND I. DYKOVÁ. 1992 Protozoan parasites of fishes. Elsevier, New York, New York, p 315.

LUTZ, A. 1889. Uber ein Myxosporidium aus der Gallenblase brasilianischer Batrachier. Centralblatt für Bakteriologie und Parasitenkunde **5:** 84–88. [In German].

MCALLISTER, C. T., AND C. R. BURSEY. 2005. Endoparasites of the Narrowmouth Toad, *Gastrophryne carolinensis* (Anura: Microhylidae), from Northwestern Louisiana and Northeastern Texas, U.S.A. Comparative Parasitology **72:** 124–128.

———, AND S. E. TRAUTH. 1995. New host records for *Myxidium serotinum* (Protozoa: Myxosporea) from North American amphibians. Journal of Parasitology **81:** 485–488.

———, ———, AND B. L. J. DELVINQUIER. 1995. Ultrastructural observations on *Myxidium serotinum* (Protozoa: Myxosporea) from *Bufo speciosus* (Anura: Bufonidae), in Texas. Journal of the Helminthological Society of Washington **62:** 229–232.

MITCHELL, L. G. 1967. *Myxidium macrocheili* n. sp. (Cnidospora: Myxidiidae) from the largescale sucker *Catostomus macrocheilus* Girard, and a synopsis of the *Myxidium* of North American freshwater vertebrates. Journal of Protozoology **14:** 415–424.

MORIARTY, E. C., AND D. C. CANNATELLA. 2004. Phylogenetic relationships of the North American chorus frogs (*Pseudacris*: Hylidae). Molecular Phylogenetics and Evolution **30:** 409–420.

PALENZUELA, O., M. J. REDONDO, AND P. ALVAREZ-PELLITERO. 2002. Description of *Enteromyxum scophthalmi* gen. nov., sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. Parasitology **124:** 369–379.

ROHLF, F. J., AND R. R. SOKAL. 1995. Statistical tables, 3rd ed. W. H. Freeman and Company, New York, New York, 199 p.

SARKAR, N. K. 1982. Myxosporidian *Myxidium haldari* sp. n. (Myxozoa: Myxidiidae) from Indian tree frog *Hyla arborea*. Acta Protozoologica **21:** 197–199.

SWOFFORD, D. L. 1998. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN, AND D. G. HIGGINS. 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research **24:** 4876–4882.

UPTON, S. J., C. T. MCALLISTER, AND S. E. TRAUTH. 1995. A new species of *Chloromyxum* (Myxozoa, Chloromyxidae) from the gallbladder of *Eurycea* spp. (Caudata, Plethodontidae) in North America. Journal of Wildlife Diseases **31:** 394–396.

WHINERY, J. B. 1893. Some additional notes on a Myxosporidian infection in the common toad. New York Medical Journal **18:** 660–662.

WHIPPS, C. M., R. D. ADLARD, M. S. BRYANT, R. J. G. LESTER, V. FINDLAY, AND M. L. KENT. 2003. First report of three *Kudoa* species from eastern Australia: *Kudoa thyrsites* from Mahi mahi (*Coryphaena hippurus*), *Kudoa amamiensis*, and *Kudoa minithyrsites* n. sp. from sweeper (*Pempheris ypsilychnus*). Journal of Eukaryotic Microbiology **50:** 215–219.

———, G. GROSSEL, R. D. ADLARD, H. YOKOYAMA, M. S. BRYANT, B. L. MUNDAY, AND M. L. KENT. 2004. Phylogeny of the Multivalvulidae (Myxozoa: Myxosporea) based upon comparative rDNA sequence analysis. Journal of Parasitology **90:** 618–622.