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MORPHOLOGICAL, MOLECULAR, AND HISTOPATHOLOGICAL DATA FOR *SEBEKIA MISSISSIPPIENSIS* OVERSTREET, SELF, AND VLIET, 1985 (PENTASTOMIDA: SEBEKIDAE) IN THE AMERICAN ALLIGATOR, *ALLIGATOR MISSISSIPPIENSIS* DAUDIN, AND THE SPOTTED GAR, *LEPISOSTEUS OCULATUS* WINCHELL

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ABSTRACT

Novel molecular data from both mitochondrial (*cytochrome c oxidase subunit 1*) and ribosomal regions (*18S*, *ITS1-5.8S*, *ITS2*, and *28S*) are provided for *Sebekia mississippiensis* Overstreet, Self, & Vliet, 1985, a pentastome infecting the American alligator, *Alligator mississippiensis* Daudin, 1801, and the spotted gar, *Lepisosteus oculatus* Winchell, 1864. Adult and nymphal pentastomes are described from the lungs and liver of the type host, *A. mississippiensis*, collected from Mississippi, while additional nymphs are described from the esophageal lining of *L. oculatus* specimens collected from Louisiana. This sequencing data will facilitate more accurate identification of various life cycle stages of *S. mississippiensis*, enabling future work to resolve many ambiguities in the literature regarding this species. Additionally, histopathological data are provided from both the definitive and intermediate hosts.

Pentastomes are an enigmatic group of crustacean endoparasites most commonly found as adults in the respiratory systems of reptiles, birds, and mammals. Despite significant disease associated with these organisms, they seem to have attracted relatively little attention from parasitologists (Christoffersen and de Assis, 2015). The pentastome *Sebekia mississippiensis* Overstreet, Self, and Vliet, 1985 was described based on adults and nymphs from the American alligator *mississippiensis* Daudin, 1801 by Overstreet et al. (1985). These authors noted a remarkable number of intermediate host species based on nymphs examined from their own work as well as reexamined specimens from previous studies, including the following: bluegill *Lepomis macrochirus* Rafinesque, 1819; gulf killifish *Fundulus grandis* Baird & Girard, 1853; Atlantic croaker *Micropogonias undulatus* (Linnaeus, 1766); green swordtail *Xiphophorus helleri* Heckel, 1848; largemouth bass *Micropterus salmoides* (Lacépède, 1802); a Virginia opossum *Didelphis virginiana* Kerr, 1792; diamond-backed watersnake *Nerodia rhombifer* (Hallowell, 1852); common watersnake *Nerodia sipedon* (Linnaeus, 1758); Mississippi green watersnake *Nerodia cyclopion* (Duméril, Bibron, and Duméril, 1854); broad-banded watersnake *Nerodia fasciata confluens* (Blanchard, 1923); plain-bellied watersnake *Nerodia erythrogaster* (Forster, 1771); red-eared slider *Trachemys scripta elegans* (Wied-Neuwied, 1838); eastern musk turtle *Sternotherus odoratus* (Latreille, in Sonnini & Latreille, 1801); spiny softshell *Apalone spinifera* (LeSueur, 1827); and North American river otter *Lontra canadensis* (Schreber, 1777). In addition to the aforementioned intermediate or paratenic host species, Overstreet et al. (1985) also cited a personal communication indicating the presence of nymphs of an unspecified species in spotted gar *Lepisosteus oculatus* Winchell, 1864, bluegill, and blue catfish *Ictalurus furcatus* (Valenciennes, 1840). They speculated that nymphal pentastomes previously observed in ladyfish *Elops saurus* Linnaeus, 1766 and longnose killifish *Fundulus similis* (Baird and Girard, 1853) were also *S. mississippiensis*, though the authors noted that those nymphs were unavailable for study. This remarkable diversity of intermediate or paratenic host species, some 27 in all, suggests that *S. mississippiensis* exhibits very little host specificity with regard to intermediate or paratenic hosts. Given the brevity of many accounts of *S. mississippiensis*, it is difficult to rule out the possibility that these nymphal accounts may refer to other pentastome species erroneously identified as *S. mississippiensis* or other possibly undescribed species. The paucity of morphological data in many accounts of nymphs of *Sebekia* Sambon

1922, and the absence of prior molecular data on sebekids of the southeastern United States suggest that the diversity of pentastomes may be greatly underestimated for this geographic region, consequently warranting further study.

While Overstreet et al. (1985) provided thorough descriptions of both sexes of adult *S. mississippiensis*, nymphs were only briefly described. Thus, the conspecificity of the nymphs described by Overstreet et al. (1985), as well as nymphs reexamined from other studies by Overstreet et al. (1985), cannot be independently assessed based on the literature alone. Additionally, Overstreet et al. (1985) also assumed *Bdukus ichthyius* Holl, 1928, described by Holl (1928) from yellow bullhead *Ameiurus natalis* (Lesueur, 1819) and pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758), is actually *S. mississippiensis*. Although Holl (1928) did not provide hook measurements, he did provide a line drawing of the nymph showing 64 annuli. This annulus number is consistent with nymphal and adult *S. mississippiensis* individuals as described by Riley et al. (1990), but not the adults reported by Overstreet et al. (1985). Venard and Bangham (1941) reexamined the specimens of Holl (1928) and compared them with 31 additional nymphs collected from the following host species: bowfin *Amia calva* Linnaeus, 1766; eastern mosquitofish *Gambusia holbrooki* Girard, 1859; spotted sunfish *Lepomis punctatus* (Valenciennes, 1831); longear sunfish *Lepomis megalotis* (Rafineqsque, 1820); redear sunfish *Lepomis microlophus* (Günther, 1859); warmouth *Lepomis gulosus* (Cuvier, 1829); and black crappie *Pomoxis nigromaculatus* (Lesueur, 1829). Based on these comparisons, Venard and Bangham (1941) declared Holl's *Bdukus ichthyius* species synonymous with *Sebekia oxycephalum*. While they did provide information about annulus number, Venard and Bangham (1941) unfortunately did not include information about taxonomically informative hook dimensions. As part of a general trend of treating all North American accounts of *S. oxycephalum* as *S. mississippiensis*, Overstreet et al. (1985) suggested these specimens to be *S. mississippiensis* because of their overlapping ranges of annulus number. Curran et al. (2014) concurred with Overstreet et al. (1985) on the synonymy of *B. ichthyius* and *S. mississippiensis* and cited Poore (2012) in support of their claim. However, Poore (2012) cited Junker and Boomker (2006), synonymizing of *B. icthyius* with *S. oxycephalum*, not *S. mississippiensis*, adding further confusion to the history of *Sebekia* species.

Dukes et al. (1971) noted the recovery of nymphs of *S. oxycephalum* from *M. salmoides* and the successful experimental infection of a snapping turtle *Chelydra serpentina* (Linnaeus, 1758). The authors noted the pentastomes recovered from *C. serpentina* were not gravid and still possessed nymphal accessory hooks, and so the turtle's suitability as a definitive host could not be established. Unfortunately, the authors did not provide any data to justify the species identity of the recovered nymphs as *S. oxycephalum*, nor did they make note of any deposited voucher specimens. Overstreet et al. (1985) suggested that these *S. oxycephalum* were in fact *S. mississippiensis* specimens, based on overlapping geography with *A. mississippiensis* previously found to be infected with *S. mississippiensis*.

Deakins (1971) noted the presence of adult *S. oxycephalum* in the lungs and many eggs of *S. oxycephalum* in the interstices of the lungs as well as in fecal samples taken from infected alligators that had apparently died of hemorrhaging of the lungs, ostensibly due to pentastome infection. Hazen et al. (1978) noted the presence of adult *S. oxycephalum* in the lungs and nymphs in the livers of alligators in South Carolina, with considerable hemorrhaging and necrosis noted in both organs. Hazen et al. (1978) further suggested that the lesions associated with *S. oxycephalum* infection may be severe enough to lead to host mortality in coinfections with *Aeromonas hydrophila*. Overstreet et al. (1985) indicated that they confirmed the representative specimens from the studies of Deakins (1971) and Hazen et al. (1978) to be conspecific with *S. mississippiensis*, but neither the original authors nor Overstreet et al. (1985) provided morphometric data to justify this specific diagnosis. Although definitive host range and species support the assessment that these specimens are *S. mississippiensis*, such claims would be more credible if morphological data justifying specific diagnosis were presented. Lastly, Overstreet et al. (1985) and Deardorff and Overstreet (1991) speculated that human infections with *S. mississippiensis* may be possible on the grounds that *S. mississippiensis* nymphs were recovered from other mammalian hosts by Overstreet et al. (1985). The idea of zoonotic infection with *S. mississippiensis* was further expounded on by Overstreet (2013), who noted that humans have been shown to harbor other pentastome species and thus may be susceptible to infection with *S. mississippiensis*. Given that nymphs belonging to the genus *Sebekia* have been reported in a single human case (Mairena et al., 1989), it is plausible that human infection with *S. mississippiensis* may be possible, but this is at present highly speculative, and great care should be taken when implicating a parasite species as a potential zoonotic agent.

Examination of 1 alligator from a eutrophic lake in Florida revealed the presence of 43 pentastomes presumed by the authors to be *S. oxycephalum* (Shotts et al., 1972), and later presumed by Overstreet et al. (1985) to represent *S. mississippiensis*. Significant clinical signs were noted, including the presence of caseous exudate in the lungs and necrotic plugs in the bronchi. Cultures from the lungs indicated the presence of *Aeromonas hydrophila*, *Pseudomonas* sp., and *Enterobacillus* sp. However, the authors noted that it was difficult to determine from this single case the synergistic role, if any, between *S. mississippiensis* and possible secondary bacterial infection in disease pathogenesis (Shotts et al., 1972).

Boyce et al. (1984) described infection with pentastomes identified as *S. oxycephalum* in captively reared juvenile alligator hatchlings from an alligator farm in southern Florida and western mosquitofish *Gambusia affinis* Baird and Girard, 1853 from a pond on the same farm containing adult alligators. Nymphs were recovered from the body cavity, liver, and lungs, with the pentastomes mainly in the pulmonary parenchyma. The authors noted pulmonary hemorrhage, hepatic lipidosis, and collapsed air sacs filled with red blood cells in the affected hatchlings, but no associated inflammatory response was observed. Subsequent to the description by Overstreet et al. (1985) of *S. mississippiensis* as a distinct species from *S. oxycephalum*, the work of Boyce et al. (1984) is referenced as having been with *S. mississippiensis* (Boyce, 1985). However, neither account contains morphometric data justifying species identification. While it is doubtful that these accounts refer to *S. oxycephalum*, the absence of morphometric data consistent with accounts of *S. mississippiensis* makes it difficult to rule out that the reported infections may be of other pentastome species.

Riley et al. (1990), in a reassessment of the genus *Sebekia*, provided the first, and to date only, substantive description of the

nymphs of *S. mississippiensis* based on specimens collected from the lungs of the definitive host. These nymphs were identified as such by the presence of a dorsal accessory piece on the hooks. This description permitted independent morphological comparison of nymphs collected from intermediate hosts for the first time since the original species description by Overstreet et al. (1985).

Boyce and Kazacos (1991) described adverse pathology associated with *S. mississippiensis* infection in a number of experimental paratenic hosts, including the following: mice *Mus musculus* Linnaeus, 1758; golden hamster *Mesocricetus auratus* (Waterhouse, 1839); Florida red-bellied cooter *Pseudemys nelsoni* Carr, 1938; and a pig frog *Lithobates grylio* (Stejneger, 1901). These paratenic hosts were experimentally infected by gavaging them with nymphs identified as *S. mississippiensis* collected from western mosquitofish. Although, no data justifying specific diagnosis were provided, mice and hamsters exhibited marked inflammatory responses to nymphs compared to the cooters and frogs. Additionally, Forrester (1992) cited unpublished data indicating the presence of *S. mississippiensis* in the intestines of river otters collected in various localities in Florida. He speculated that the otters may have become infected through eating infected fish, but he acknowledged the pathological significance remained unknown, and these infections may have been spurious. In reviews of the literature regarding the use of paratenic hosts by pentastomes, Sharpilo (2003) and Sharpilo and Salamatin (2005) summarized many of these accounts of *S. mississippiensis* in reported intermediate, paratenic, and definitive hosts. They indicated that while mosquitofish and alligators serve as intermediate and definitive hosts, respectively, other species from which nymphs of *S. mississippiensis* have been recovered serve as paratenic hosts. More recently, Overstreet and Hawkins (2017) stated that Atlantic croakers, gulf killifish, and bluegill become infected with *S. mississippiensis* after having ingested *G. affinis* infected with *S. mississippiensis* nymphs. However, the citation given in support of this statement (Overstreet, 2013) did not provide direct evidence to support this claim. Instead, it referred to the work of Overstreet et al. (1985), where this was first speculated. While the proposed life cycle is plausible, such claims should remain speculative in the absence of published supporting experimental or molecular data. Scott et al. (1997) examined 55 alligators from southeast Texas and reported *S. mississippiensis* prevalence of 12% and 72% for immature and mature alligators, respectively. Shoeb et al. (2002) examined 10 alligators from Florida and noted the presence of *S. mississippiensis* in the vessels of the liver and lungs of most alligators.

Using *S. mississippiensis* specimens collected from alligators in Florida, Park (2001) provided the first molecular data for *S. mississippiensis* as part of an assessment of length heterogeneity of the external transcribed spacer region. The sequenced regions included partial coverage of the *18S-5′* and *28S-3′* genes as well as the external transcribed spacer region. Unfortunately, the utility of the sequenced regions for molecular characterization has not been adequately established for pentastomes.

Curran et al. (2014), in their original description of the sebekid *Levisunguis subaequalis* Curran, Overstreet, Collins, and Benz 2014, noted having observed nymphs of *S. mississippiensis* in *C. serpentina* and the alligator snapping turtle *Macrochelys temminckii* (Troost in Harlan, 1835), but they did not provide further data for these accounts. Interestingly, Curran et al. (2014) also noted that the nymphs described as *S. oxycephalum* by Venard and Bangham (1941) and later suggested to be *S. mississippiensis* (Overstreet et al., 1985) are in fact consistent with *L. subaequalis*; however, Curran et al. (2014) noted that the data provided by Venard and Bangham (1941) were inadequate for a sufficient comparison to be made.

Overall, *S. mississippiensis* has had a long and convoluted history, both before and since its original description by Overstreet et al. (1985). Although considerable attention has been paid to adverse pathology and the life cycle of *S. mississippiensis* and the definitive and other hosts, relatively little attention has been paid to the systematics of the species. Thus, while much effort has been made to characterize adverse pathology in the definitive and intermediate hosts, failure to justify the species identity of the etiologic agent may conflate the effects of *S. mississippiensis* with those of other pentastome species. In an effort to clarify the long-stated and seldom justified specific diagnoses of *S. mississippiensis*, the present study sought to provide updated morphological and molecular characterization of the species as well as documentation of adverse pathology in the definitive and intermediate hosts. The data provided in the present study are given to help resolve many of the ambiguities in the literature regarding this enigmatic species.

MATERIALS AND METHODS

Specimen collection

During Mississippi's 10-day alligator hunting season in August–September of 2016, 11 alligators, ranging in total body length from 1.37 to 3.58 m, were collected from an alligator processor in Port Gibson, Mississippi, for parasitological examination and transported to the College of Forest Resources at Mississippi State University for necropsy. Organs were separated and transported to the College of Veterinary Medicine at Mississippi State University for further examination. Lungs were opened with scalpels, macerated in physiological saline, and grossly examined for the presence of adult and nymphal pentastomids. Mesenteries and livers were also macerated and grossly examined for the presence of nymphs. Pentastomes were removed with insect pins, with care being taken to avoid puncturing them. Live pentastomes were collected and fixed directly in 70% ethanol for future morphological and molecular characterization. In addition to its suitability as a fixative for molecular characterization, 70% ethanol is generally recommended for fixing pentastomes for morphological characterization, because ethanol-fixed specimens yield the most consistent results (Riley, 1986). One additional alligator was collected in September 2017 and similarly necropsied. While 1 lung was dissected and pentastomes were recovered and stored directly in 70% ethanol, the other lung was stored in 10% neutral-buffered formalin (NBF) for histopathological analysis.

In September 2016, 37 spotted gar, ranging in total body length from 37.5 to 73.5 cm, were collected by boat electrofishing from the Atchafalaya River Basin in Louisiana, for induced spawning at Nicholls State University, Thibodaux, Louisiana. In February and March 2017, 58 additional spotted gar were collected from the Atchafalaya River Basin and Bayou Chevreuil for further study. The fish were not subjected to treatment with anthelmintics or other antiparasitics. At the end of these studies, 37 and 16 whole fish, from 2016 and 2017, respectively, were euthanized by blunt force to the head and transported on ice to the College of

Veterinary Medicine at Mississippi State University for complete parasitological examinations. As part of this examination, the intestinal tract of each fish was examined under an Olympus SZ60 dissecting microscope (Olympus Optical, Tokyo, Japan) for the presence of encysted parasites. Sections of tissue containing pseudocysts were stored in 10% NBF for histological examination. Some sections of heavily parasitized tissue were also incubated overnight at 40 C in a 0.5% pepsin solution acidified with 1% HCl, with the excysted nymphs being recovered the next day. The remaining live nymphs were manually excysted using insect pins and fixed directly in 70% ethanol for morphological and molecular characterization.

Morphological characterization

Several clearing and mounting techniques were employed in an effort to determine the one that produced the most useful specimens. In total, 10 adults and 10 nymphs were mounted and measured.

For 1 adult from *A. mississippiensis* and 3 nymphs from *L. oculatus*, the cephalothoraxes were separated from the hindbodies using scalpel blades. Each cephalothorax was dehydrated through a series of 4 ethanol washes from 70 to 100%, cleared in Hemo-De (Scientific Safety Solvents, Keller, Texas), and ventrally mounted in Canada balsam on glass slides. The hindbodies were likewise dehydrated, cleared, and mounted.

Seven adults and 1 nymph were mounted directly in Hoyer's medium (Hempstead Halide Inc., Galveston, Texas), and measurements were taken as the specimens cleared.

One adult and 1 nymph were stained in Van Cleave's hematoxylin, destained in weak acidic 70% ethanol, transferred to alkaline 70% ethanol, dehydrated as previously described, cleared in Hemo-De, and mounted in Canada balsam on glass slides.

Hologenophores were also made for 1 adult from an alligator and 2 nymphs from gar. The cephalothoraxes were removed using a scalpel blade, dehydrated through a series of 4 ethanol washes, cleared in Hemo-De, and mounted in Canada balsam on a glass slide. The tissues of the hindbodies from the same specimens were dissolved in 180 μL of Buffer ATL and 20 μL of proteinase K overnight (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany). While this lysed the internal structures of the hindbodies, the taxonomically informative annuli were preserved. The unlysed cuticle was recovered, dehydrated as previously described, cleared in Hemo-De, and mounted on the same slide as the cephalothorax. The supernatant was used for DNA extraction following the kit manufacturer's instructions.

Four additional nymphs were also mounted from a total of 3 spotted gar, one of which came from the same host specimen as the hologenophore. After the cuticles were punctured in several places with insect pins, the nymphs were dehydrated as previously described, cleared in Hemo-De, and mounted in Canada balsam on glass slides.

Standard pentastome hook measurements as adapted from Fain (1961) by Barton and Morgan (2016) were taken from all specimens where possible. Additionally, oral cadre lengths and widths were measured where possible as previously reported for adults and nymphs of *Sebekia* (Overstreet et al., 1985; Winch and Riley, 1986; Riley et al., 1990). Annuli, where discernible, were counted for each specimen suspended in ethanol in a concavity slide prior to mounting under cover glass and confirmed after specimens were mounted. Total body length was measured medially from each *S. mississippiensis* individual. Measurements and annuli counts were taken using an Olympus BX53 Microscope (Olympus) with an Olympus DP74 Camera attachment and associated cellSens 1.18 software.

Separate measurements are reported for adult males, adult females, and unsexed nymphs. Additionally, embryonated eggs were measured in utero from females mounted in Hoyer's medium. However, it must be noted that the eggs of other pentastomes have been reported to be larger, once deposited, than those in utero (Riley, 1983). Therefore, measurements reported for eggs in utero may not be consistent with those of eggs recovered via fecal flotation or fecal sedimentation.

Voucher specimens deposited by previous authors yet lacking published morphometric data were borrowed from their respective institutions and subjected to nondestructive morphometric analysis. These included whole mounts of nymphs from alligators as well as whole nymphs in vials from *G. affinis* deposited by Overstreet et al. (1985) as well as whole nymphs and adults in vials from alligators deposited by Tellez et al. (2014). The type specimens for *S. mississippiensis* of Overstreet et al. (1985) were also examined and measured. Data from these specimens are presented with appropriate accession numbers in Suppl. Data Table S1. The following specimens from the present study were deposited in the Smithsonian Institution, National Museum of Natural History (USMN): nymphal hologenophore (USNM 1501649) (Fig. 1B, C), adult hologenophore (USNM 1501648), and nymphal vouchers (USNM 1501650–1501654) (Fig. 1A).

Calculation of intensity, abundance, and prevalence

Intensity, abundance, and prevalence, as defined by Bush et al. (1997) and Rózsa et al. (2000), were calculated for *S. mississippiensis* specimens collected from the alligators, while prevalence was calculated for *S. mississippiensis* specimens collected from spotted gar. All parameters were calculated using Quantitative Parasitology 3.0 (Rózsa et al., 2000) with confidence limits of 95%, to be able to use the implementation Clopper-Pearson method in Quantitative Parasitology 3.0 (Clopper and Pearson, 1934) with 2,000 bootstrap replications. Because large sections of tissue from each infected *L. oculatus* specimen were used for histopathological analysis, abundance and intensity were not calculated for *S. mississippiensis* in the intermediate host.

Molecular characterization

Genomic DNA was extracted from both nymphal and adult pentastomes using a DNeasy Blood and Tissue kit (Qiagen). Ribosomal and mitochondrial genes were amplified from 1 whole adult *S. mississippiensis* using the following primer sets: ERIB1/ERIB10, 1F/5R, Diplo1795F/Diplo2549R, BD1/BD2, LSU5/1500R, Drep2456F/Drep3470R, 28S-5′/28S-3′, LCO1490/HCO2198 (Table I) with Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Fisher, Waltham, Massachusetts). For 2 additional whole adult *S. mississippiensis* specimens, each from a separate alligator, 1 whole nymph from *L. oculatus*, 1 nymphal hologenophore from *L. oculatus*, and 1 hologenophore of an adult *S. mississippiensis* from an alligator, BD1/BD2, LSU5/1500R, and LCO1490/HCO2198 were used to verify conspecificity and assess intraspecific sequence variability for each region. Except for those

Table I. Primers used for DNA amplification.

Primer name	Primer sequence (5′-3′)	Gene target	Reference
ERIB1	ACCTGGTTGATCCTGCCAG	*18S*	Barta et al. (1997)
ERIB10	CTTCCGCAGGTTCACCTACGG	*18S*	Barta et al. (1997)
1F	TACCTGGTTGATCCTGCCAGTAG	*18S*	Carranza et al. (1997)
5R	CTTGGCAAATGCTTTCGC	*18S*	Carranza et al. (1997)
Diplo1795F	CGTCGCTACTACCGATTGAA	*18S* and *ITS*	Rosser et al. (2016)
Diplo2549R	AGTGATCCACCGCTCAGAGT	*18S* and *ITS*	Rosser et al. (2016)
BD1	GTCGTAACAAGGTTTCCGTA	*ITS*	Morgan and Blair (1995)
BD2	TATGCTTAAATTCAGCGGGT	*ITS*	Morgan and Blair (1995)
Drep2456F	CTCGTGTGTCGATGAAGA	*ITS* and *28S*	Alberson et al. (2017)
Drep3470R	CTCCACCCGTTTACCTCTGA	*ITS* and *28S*	Alberson et al. (2017)
28S-5′	TACCCGCTGAACTTAAGCATAT	*28S*	Zehnder and Mariaux (1999)
28S-3′	CTCCTTGGTCCGTGTTTCAAGAC	*28S*	Zehnder and Mariaux (1999)
LSU5	TAGGTCGACCCGCTGAAYTTAAGCA	*28S*	Littlewood et al. (2000)
1500R	GCTATCCTGAGGGAAACTTCG	*28S*	Tkach et al. (2003)
LCO1490	GGTCAACAAATCATAAAGATATTGG	*COI*	Folmer et al. (1994)
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	*COI*	Folmer et al. (1994)

using the primer set LCO1490/HCO2198, all 20-μL PCR reactions were as follows: 10 μL of Phusion Master Mix, 10 μM of each primer, 1 μL of DNA template (~10 ng/μL), and 7 μL of nuclease-free water. For the primer set targeting *cytochrome c oxidase subunit 1* (*COI*), LCO1490/HCO2198, PCR reactions consisted of the following: 20 μL of Phusion Master Mix, 10 μM of each primer, 5 μL of DNA template, and 11 μL of nuclease-free water to a volume of 40 μL. Thermal cycling profiles used with each primer set are listed in Table II.

Intraspecific variability for each gene was determined by aligning sequences from the present study using GUIDANCE2's implementation of the MAFFT algorithm (Katoh and Standley, 2013) on the GUIDANCE2 Web Server (Landan and Graur, 2008; Sela et al., 2015), trimming the resultant alignment by eye in MEGA7, and calculating pairwise distances in MEGA7 (Kumar et al., 2016).

Phylogenetic analysis

Representative *COI* sequences for pentastomes were downloaded from GenBank and aligned with sequences generated in the present study using the MAFFT algorithm as implemented in GUIDANCE2. The resultant alignment was trimmed by eye, and best-fitting substitution models for each codon position were calculated using the Bayesian information criterion in MEGA7 to yield the following models: *COI* position 1 (HKY + G), *COI* position 2 (TN93 + I), and *COI* position 3 (HKY + G). Phylogenetic trees were then constructed using a partitioned data set, with each codon position having the aforementioned models applied, in MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004). Using Markov chain Monte Carlo searches of 2 simultaneous runs of 4 chains with sampling every 100th tree for 1×10^6 generations ensured that the value of the standard deviation of the split frequencies reached <0.01. Additionally, phylogenetic analysis using the maximum likelihood method was performed using IQ-Tree (Nguyen et al., 2015) on the IQ-Tree Web server (Trifinopoulos et al., 2016) with the previously described partitioning scheme. Branch support was tested using ultrafast bootstrap support with 1,000 pseudoreplicates. The resultant trees were formatted in FigTree 1.4.3 (Rambaut, 2016) and Adobe Illustrator CC 2017.1.

Histopathological characterization

Tissues from sampled alligators and gars were fixed in 10% NBF for at least 2 days prior to trimming into cassettes, embedding in paraffin wax, sectioning by microtome into 5-μm ribbons, mounting onto glass slides, and staining with hematoxylin and eosin (standard light microscopy).

TABLE II. Thermal cycling parameters used for DNA amplification.

Primer	Denaturation	Cycling	Extension/elongation
ERIB1/ERIB10 1F/5R	98 C; 10 min	30 cycles of 98 C for 10 sec, 51 C for 30 sec, 72 C for 1 min	72 C; 10 min
BD1/BD2 LSU5/1500R Drep2456F/Drep3470R 28S-5′/28S-3′	98 C; 10 min	45 cycles of 98 C for 10 sec, 57 C for 30 sec, 72 C for 1 min	72 C; 10 min
Diplo1975F/Diplo2549R	98 C; 10 min	35 cycles of 98 C for 10 sec, 58 C for 30 sec, 72 C for 1 min	72 C; 10 min
LCO1490/HCO2198	98 C; 10 min	35 cycles of 98 C for 10 sec, 48 C for 30 sec, 72 C for 1 min	72 C; 10 min

TABLE III. Morphological data for *Sebekia mississippiensis* from the present study, in bold, compared with previously reported data for the species. Abbreviations: BL: body length, BW: body width at cephalothorax, Annuli: number of annuli, AB: hook gape, AC: blade length, BC: base length, DAP: nymphal accessory hook length, FL: fulcrum length, OCL: oral cadre length, OCW: oral cadre width. Measurements are given in micrometers unless otherwise stated. Measurements are rounded to the nearest whole micrometer. Sex of nymphs was not determined. Dashes indicate data not reported in referenced manuscript.

Stage	Sex	BL (mm)	BW	Annuli	AB	AC	BC	DAP	FL	OCL	OCW	Reference
Adult	Male	5.6	0.5	69–70	40	50	30	–	130	170	70	Overstreet et al. (1985)
Adult	Male	5–7.4	–	62–65	–	68–77	–	–	177–205	153–177	–	Riley et al. (1990)
Adult	**Male**	**5.02–7.47**	**–**	**65–68**	**27–38**	**55–75**	**34–45**	**–**	**145–196**	**159–182**	**79–84**	**Present study**
Adult	Female	10	0.6 (max)	70	50	70	40	–	190	210	90	Overstreet et al. (1985)
Adult	Female	–	–	62–69	–	79–91	–	–	200–235	192–220	–	Riley et al. (1990)
Adult	**Female**	**5.69–11.90**	**–**	**70**	**31–40**	**59–75**	**36–50**	**–**	**173–202**	**167–200**	**84–90**	**Present study**
Nymph	–	5.5.–8.0	–	61–67	–	–	–	–	–	–	–	Riley et al. (1990)
Nymph	**–**	**2.93–7.91**	**–**	**62–70**	**27–45**	**65–91**	**42–53**	**74.14–99.47**	**126–216**	**114–181**	**61–83**	**Present study**

RESULTS

Abundance, intensity, and prevalence

Sebekia mississippiensis was found in the lungs of 63.6% (7/11) of sampled alligators. In addition to this typical infection site, pentastomes were also collected from lumen of the gastrointestinal tracts of 27.3% (3/11) and the liver of 9.1% (1/11) of alligators.

In total, *S. mississippiensis* was detected in 7 of 11 alligators examined, yielding a prevalence of 63.6% (95% bias-corrected and accelerated bootstrap confidence limit (BCa), 30.8–89.1%). The number of *S. mississippiensis* collected from each infected alligator ranged from 4 to 37. Mean intensity was 22.3 (95% BCa, 11.1–31.4), while median intensity was 30.0. Mean abundance was 14.2 (95 BCa, 5.6–23.6).

Encysted nymphs were found in 3 of 37 sampled *L. oculatus* specimens in 2016, yielding a prevalence of 8.1% (95% BCa, 1.7–2.19%), and 8 out of 23 *L. oculatus* specimens sampled in 2017, yielding a prevalence of 34.8% (95% BCa, 16.4–57.3%).

Morphological characterization of *Sebekia mississippiensis*

Measurements taken from adult and nymphal *Sebekia mississippiensis* individuals from the present study, compared with those from previous studies, are given in Table III. Ranges were consistent with previously reported values for *S. mississippiensis* adults and nymphs (Overstreet et al., 1985; Riley et al., 1990). Additionally, voucher specimens of nymphs and adults deposited by Overstreet et al. (1985) and Tellez et al. (2014) as *S. mississippiensis* were confirmed to be conspecific with the specimens from the present study. Measurements reported here are rounded to the nearest micrometer and presented in the following format: range (average). Measurements for individual pentastomes from the present study are indicated in Suppl. Table S2.

Adult *Sebekia mississippiensis*

Oral cadre opens anteriorly. Single row of chloride cells occurs on anterior margin of each annulus. Single row of spines occurs on anterior margin of each annulus. Anterior and posterior hooks and fulcra are not markedly different in measurements. Spinous hook shields and fulcra extensions occur on both anterior and posterior hooks (Fig. 2A).

Male *Sebekia mississippiensis*

Based on 5 males, except where otherwise stated: Body 5.02–7.47 (5.02 mm) long, 505–595 (536) wide at greatest cephalothorax width, 969–1141 (1038, n = 3) wide at widest point of abdomen. Annulus number 65–68 (66). Anterior hooks, posterior hooks, and anterior extension of fulcrum spinous. Hook gape (AB) 27–38 (32). Blade length (AC) 55–75 (67). Hook length (AD) 65–90 (73). Base length (BC) 34–45 (41). Fulcrum length (FL) 145–196 (173) long. Oral cadre 159–182 (173) long, 79–84 (82, n = 3) wide. Copulatory spicules ventrally obpyriformis, laterally cowry-shell-shaped with rugose posterior end (Fig. 2B).

Female *Sebekia mississippiensis*

Based on 5 females, except where otherwise stated: Body 5.69–11.90 (9.09 mm) long, 508–539 (524) wide at greatest cephalothorax width, 876–1420 (1223, n = 2) wide at widest point of abdomen. Annulus number 70 (n = 1). Anterior hooks, posterior hooks, and anterior extension of fulcrum spinous. AB 31–40 (34). AC 59–75 (58). AD 75–86 (80). BC 36–50 (42). FL 173–202 (193). Oral cadre 167–200 (186) long, 84–90 (88, n = 3) wide. Eggs 89 (76–93) long, 67 (55–77) wide.

Nymphal *Sebekia mississippiensis*

Based on 11 nymphs, except where otherwise stated: Body 2.93–7.91 (5.10 mm) long, 383–521 (446, n = 10) wide at greatest cephalothorax width, 526–843 (651) wide at widest point of abdomen. Annulus number 62–70 (67, n = 10). Anterior hooks and posterior hooks not spinous. Dorsal accessory piece present on anterior and posterior hooks (Fig. 2D). AB 27–45 (39). AC 65–91 (81). AD 76–94 (87). BC 42–53 (49). Nymphal accessory hook length (DAP) 74–99 (84). FL 126–216 (169). Oral cadre oblong, open anteriorly (Fig. 2E), 114–181 (140, n = 10) long, 61–83 (71, n = 10) wide. Single row of chloride cells in the middle of each annulus. Single row of spines on anterior margin of each annulus (Fig. 2F). Internal organs not discernible.

Molecular characterization and phylogenetic analysis

Intraspecific variability at *ITS1-5.8S–ITS2*, *28S*, and *COI* was 0–0.07%, 0–0.09%, and 0–1.03%, respectively. NCBI accession numbers are listed in Table IV. *COI* intraspecific variability was

Figure 1. Photomicrographs of *Sebekia mississippiensis* nymphs illustrating the proposed technique for producing hologenophores of sebekids. (**A**) Voucher specimen dehydrated in ethanol wash series, cleared in Hemo-De, and mounted in Canada balsam. USNM 1501651. (**B**) Hologenophore cephalothorax. Cephalothorax dehydrated in ethanol wash series, cleared in Hemo-De, and mounted in Canada balsam. USNM 1501649. (**C**) Hologenophore hindbody from the same specimen as in Figure 1B. Internal tissue dissolved with proteinase K, and remaining cuticle dehydrated in ethanol wash series, cleared in Hemo-De, and mounted in Canada balsam. All parts to scale. Color version available online.

consistent with that reported for other pentastomids (Kelehear et al., 2011, 2014).

Results of phylogenetic analyses of *Sebekia mississippiensis COI* sequencing data (Fig. 3) were in agreement with previous phylogenetic placement of *Sebekia* (Barton and Morgan, 2016). *Sebekia mississippiensis* sequences formed a sister clade with other congeners, though very little molecular data are presently available for pentastomes. Therefore, phylogenetic inferences presented here are tentative.

Histopathological characterization

Few *S. mississippiensis* specimens were seen in alligator lung sections, and when present, they were situated away from large airways but near the openings of the foveolae. A gravid female (Fig. 4A) contained many eggs, such that organs were peripherally displaced, and the body wall was highly attenuated. Surrounding tissues were compressed, but lacked inflammation. The pentastomes had thick coats of striated muscle (Fig. 4B) and a fine cuticle. Protein-rich fluid and small numbers of inflammatory cells were often present in the airways. Occasionally, groups of macrophages containing rough brown granular pigment (presumably hemosiderin) were present, indicating previous hemorrhage. Few granulocytes, macrophages, and lymphocytes were present between epithelial cells or in the lamina propria. Inflammation was present throughout the lung, in the form of small, lymphocyte-rich nodular aggregates in bronchi and primary trabeculae. Such inflammation can be expected in an adult wild alligator, and etiologies are many (W. A. Baumgartner, pers. comm.). In 1 area, a large nodule was present (Fig. 4C) that had a core of necrotic debris rimmed by multinucleated giant cells and macrophages (granuloma), all of which was surrounded by myriad lymphocytes many layers thick. The material in the granuloma core was pale pink, hyaline, finely layered, highly infolded, and partially mineralized. Such material is protein rich, poorly digestible, and resembles cuticle. Small numbers of free eggs were present in the air spaces as well (Fig. 4D); no inflammation was seen.

Nymphal *S. mississippiensis* individuals from spotted gar were evident within thin-walled pseudocysts in the muscularis or the serosa (Fig. 5A) of the caudal portion of the esophagus (containing glands). Pseudocyst walls were thin and fibrous, blending into the surrounding musculature. Larvae exhibited characteristic annulations and striated muscle (Fig. 5B), as well as abundant acidophilic glands (Fig. 5C). Each annulus contained many fine sharp spines (Fig. 5C, D). At high power, the pseudocyst wall contained many fibroblasts and small numbers of lymphocytes (Fig. 5D).

DISCUSSION

Unfortunately, many accounts of *S. mississippiensis* do not provide adequate morphological data to justify specific diagnosis. While Overstreet et al. (1985) thoroughly described male and female adults of *S. mississippiensis*, a limited description of nymphal morphology was given. Since this initial description, many publications on this species have relied on host species and geography as the bases for specific diagnoses and have not provided morphometric data consistently, if at all.

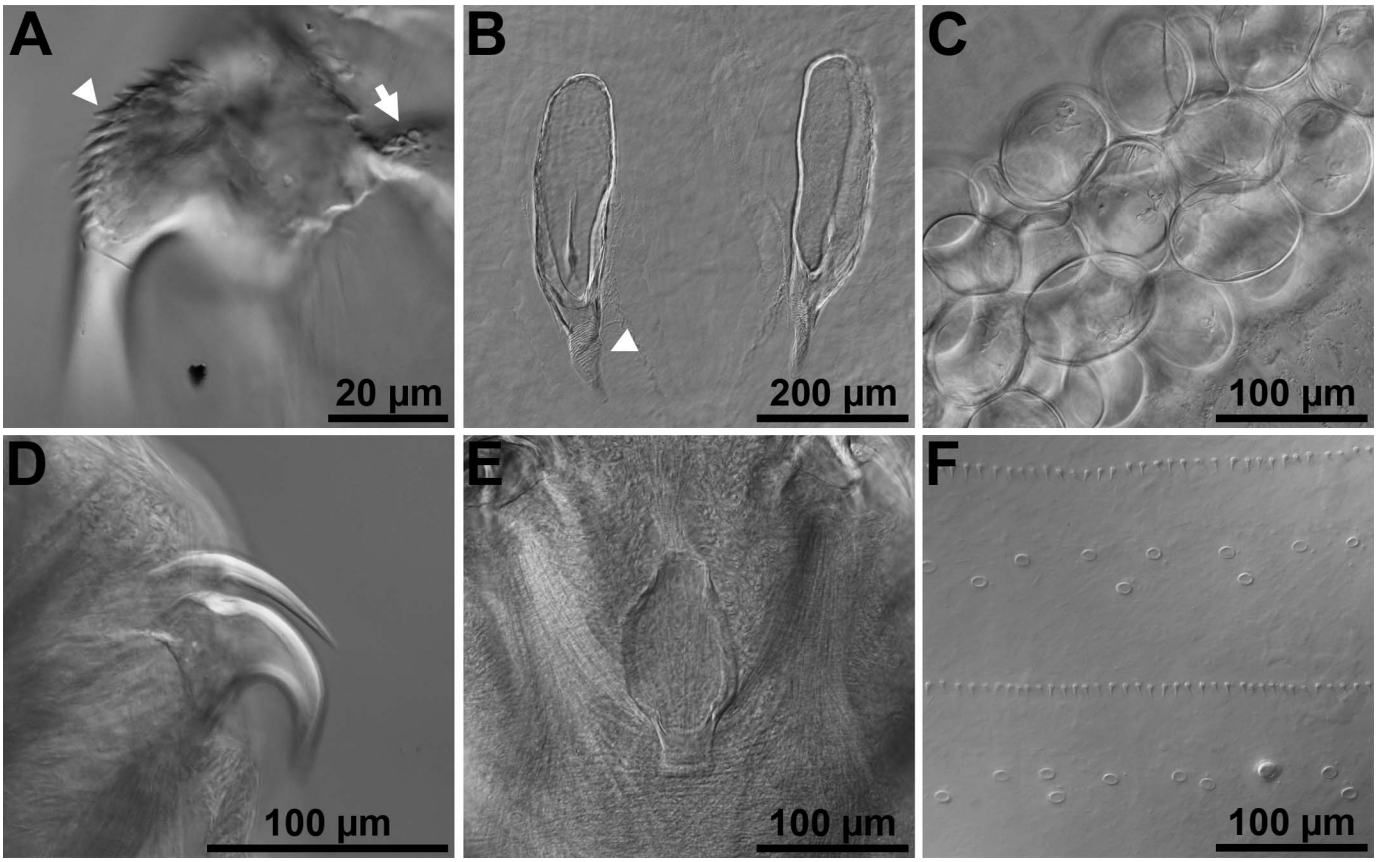

Figure 2. Photomicrographs of qualitative characters of *Sebekia mississippiensis*. (**A**) Spinous hook (arrow head) and shield (arrow) of adult *S. mississippiensis*. (**B**) Copulatory spicules of male *S. mississippiensis* with rugose posterior end (arrowhead). (**C**) Eggs in utero. (**D**) Hook with dorsal accessory piece on *S. mississippiensis* nymph. (**E**) Oral cadre of *S. mississippiensis* nymph. (**F**) Spines and chloride pores of *S. mississippiensis* nymph.

Many accounts of *S. mississippiensis* do not report hook measurements, which are widely used in species identification for porocephalids (Fain, 1961; Riley and Self, 1979, 1981a, 1981b; Ali et al., 1981, 1982, 1984a, 1984b; Self and Rego, 1985). This complicates efforts to compare specimens between studies. Additionally, several studies do not describe parasite morphology directly (Deakins, 1971; Hazen et al., 1978; Boyce et al., 1984, 1987; Boyce, 1985; Tellez et al., 2014), requiring readers to either take the authors at their word regarding species identification or, where available, reexamine voucher specimens. Unfortunately, this undermines the utility of such studies and confounds efforts to better understand the systematics of these enigmatic parasites.

Of additional note, the stigmata described by Venard and Bangham (1941) in the nymphs they examined, analogous to chloride pores according to Riley (1973), were not arranged in uniform rows along the middle of each annulus, as is the case with specimens from Overstreet et al. (1985), Riley et al. (1990), or the present study. However, the taxonomic utility and consistency of this character are not well established. Given that the description provided by Venard and Bangham (1941) did not include hook morphology, it is entirely possible that the examined nymphs represent a different species. Indeed, Curran et al. (2014), in their original description of *L. subaequalis*, noted that the nymphs

TABLE IV. National Center for Biotechnology Information (NCBI) accession numbers for each gene and specimen from the present study of *Sebekia mississippiensis* infecting *Alligator mississippiensis* and *Lepisosteus oculatus*.

		Gene target			
Host ID	**Host species**	***ITS1-5.8S, ITS2***	***28S***	***CO1***	**Type status**
Alligator 1603301	*A. mississippiensis*	MK103087	MK103082	MK248490	–
Alligator 1603302	*A. mississippiensis*	MK103088	MK103083	MK248489	–
Alligator 1603329	*A. mississippiensis*	MK103080		MK248487	–
Alligator 17-2012	*A. mississippiensis*	MK103089	MK103084	MK248488	Hologenophore
Gar G	*L. oculatus*	MK103086	MK103081	MK248491	–
Injected Gar 16	*L. oculatus*	MK103090	MK103085	MK248486	Hologenophore

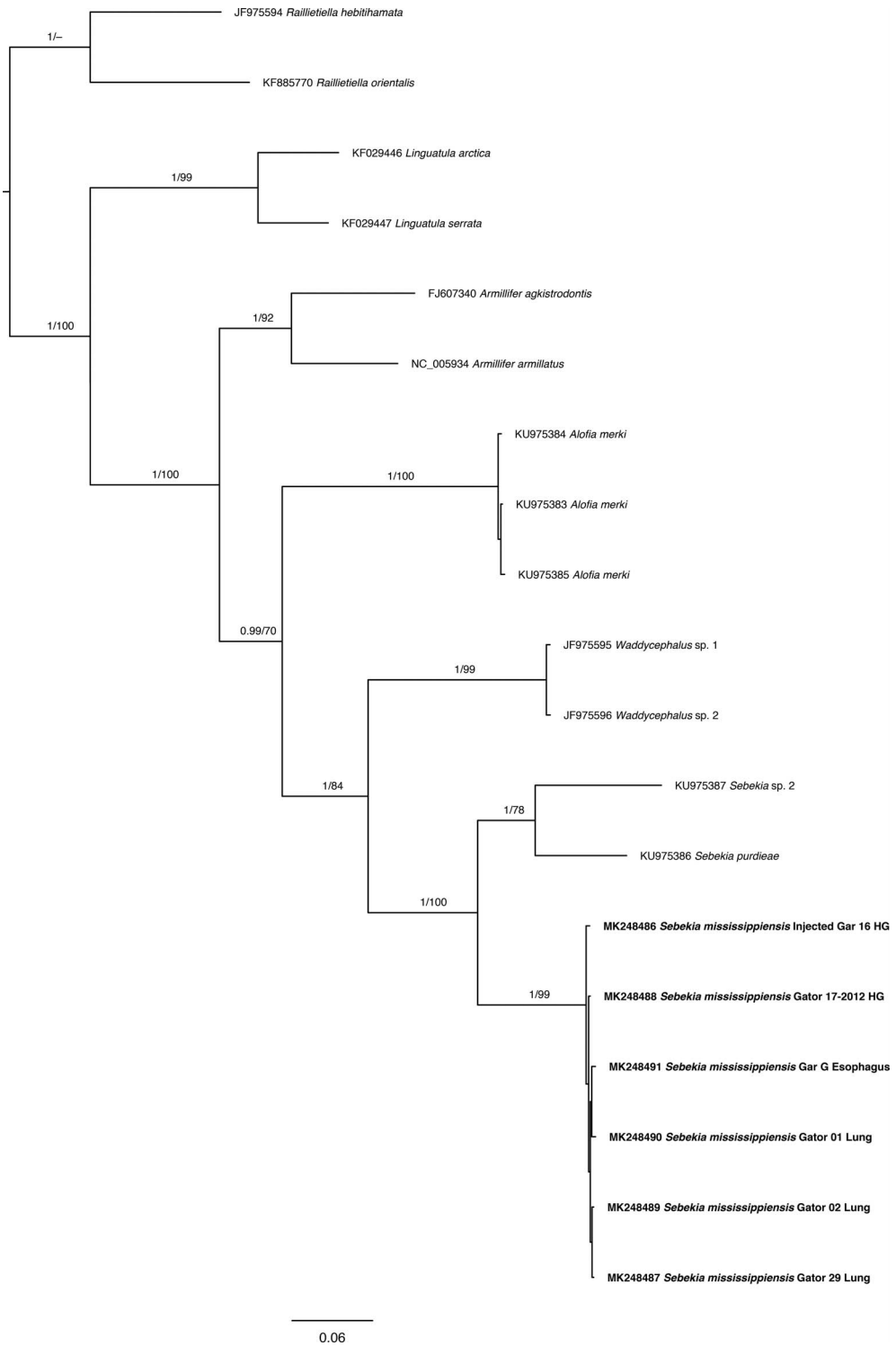

Figure 3. Phylogenetic tree constructed using *cytochrome c oxidase subunit 1* sequencing data from present study and available sequences from GenBank. Numbers indicate Bayesian posterior probabilities/bootstrap values. Scale bar represents number of substitutions per site.

described as *S. oxycephalum* by Venard and Bangham (1941) may actually be *L. subaequalis*.

Overstreet et al. (1985) listed 27 intermediate or paratenic host species for *S. mississippiensis*. Unfortunately, data confirming species identification of the nymphs were not included. The nymphal hook measurements were not included, and the number of annuli was noted to be as low as 61 in some nymphs. Although this annulus number is consistent with the range given by Riley et al. (1990), this character alone is insufficient to establish a specific diagnosis. Given that these 2 characters are generally regarded as

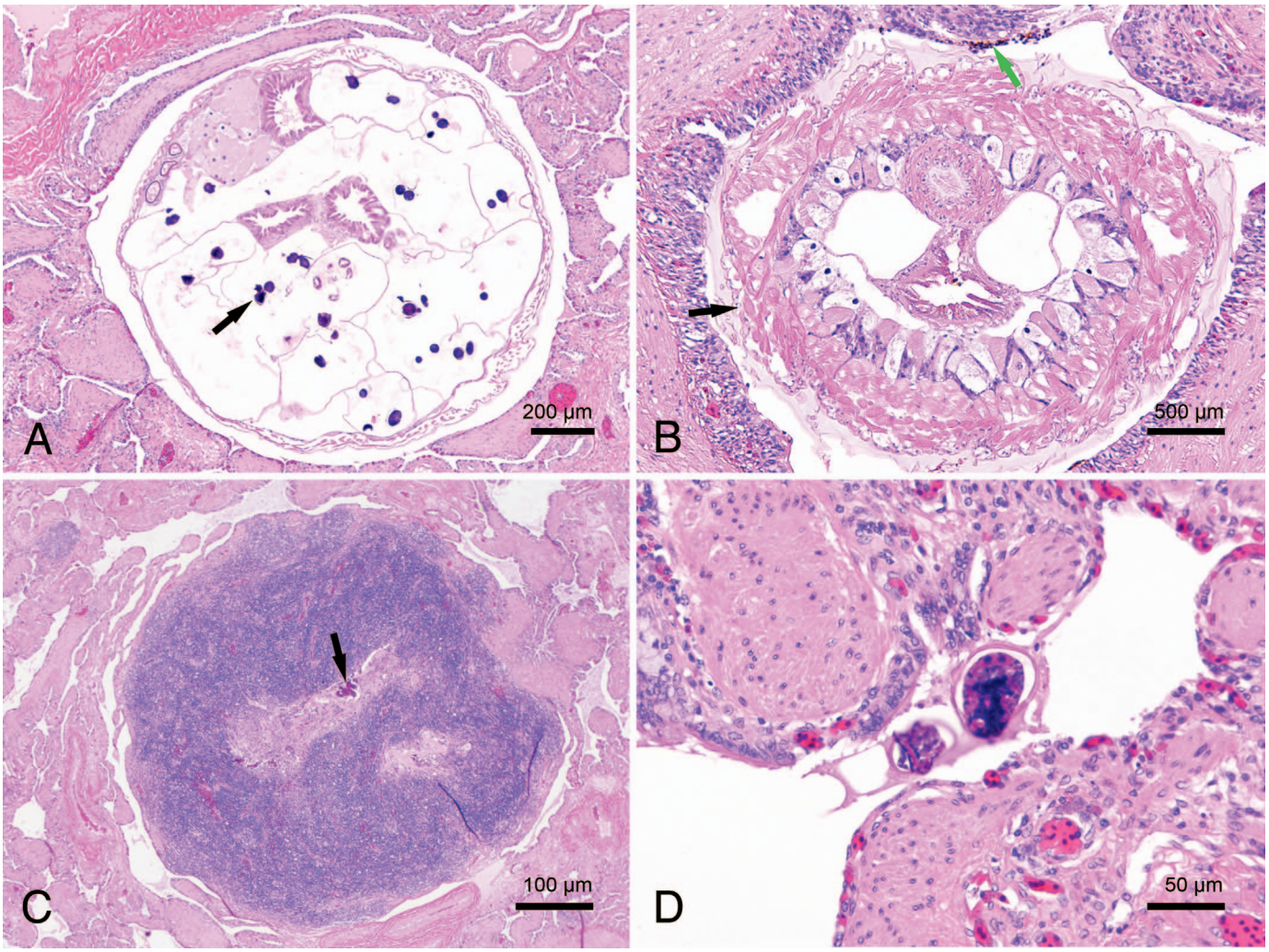

Figure 4. Photomicrographs of *Alligator mississippiensis* lung infected with *Sebekia mississippiensis*. (**A**) Adult female *S. mississippiensis* cross section surrounded by compressed faveolar trabeculae. The ovaries are greatly expanded and contain few eggs (black/dark arrow), due to artifactual loss. The intestine and acidophilic glands (green/light arrow) are displaced by the egg mass. (**B**) Cross section of adult *S. mississippiensis* within the bronchial/trabecular space. Striations in the muscular body wall are evident (black/dark arrow). A thin cuticle with pale amphophilic fluid covers the body. Minimal inflammation is present in the tissues. Macrophages containing coarse brown granular pigment (likely hemosiderin) are present in the protein-rich fluid that covers the epithelial surface (green/light arrow). (**C**) An unusually large inflammatory nodule in the lung. The pale pink center is composed of condensed, hyaline pink, protein-rich, delicate layers that resemble cuticle as well as necrotic tissue, which is sometimes mineralized (purple nidus at black arrow) and is surrounded by multinucleated giant cells and large macrophages. Myriad lymphocytes and macrophages form a thick cortex around the center. (**D**) Two *S. mississippiensis* eggs are present in the upper foveolae. They have thin pale yellow shells that surround primary larva. Stained with hematoxylin and eosin. Color version available online.

being of critical importance for the identification to species among pentastomids and data showing agreement between adults and nymphal stages were not included, such accounts must be regarded with some skepticism. Should further studies employing molecular techniques verify these accounts, this would present a valuable opportunity to examine those morphological characters that are most stable between hosts species and life cycle stages, and thus infer the characters that are most informative for specific diagnosis.

As part of his investigations into the structure of the transcribed spacer region of *S. mississippiensis*, Park (2001) provided the only published molecular data for this species to date. Unfortunately, the regions sequenced by Park (2001) covered partial *18S rRNA*, the extragenic rRNA spacer region, and partial *28S rRNA* sequences. These regions are rarely used for inferring species identities, and their taxonomic utility is not established. Therefore, these regions were not sequenced in the present study.

In characterizing the development of *S. oxycephalum*, Winch and Riley (1986) noted that the nymphs undergo a series of 6 molts during development in the intermediate hosts, with only the latter 3 instars possessing distinguishable annuli and only the final instar possessing the final number of annuli and hook sizes comparable to those of adult specimens. Should the nymphs of *S. mississippiensis* undergo similar development in their intermediate host or hosts, future efforts to identify nymphs to species level would be further confounded by a lack of informative morphometric characters for many instars. Experimental infections of

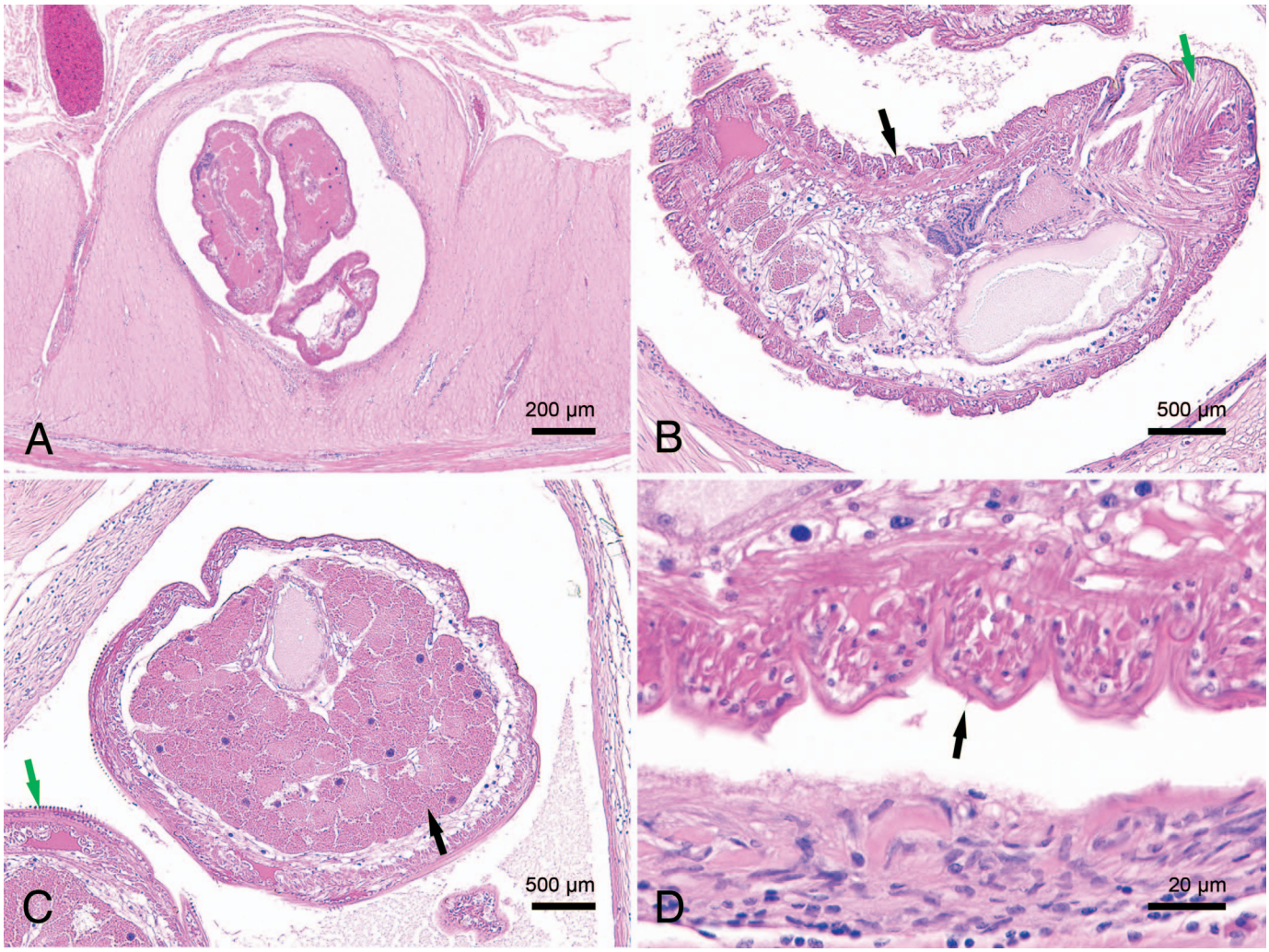

Figure 5. Photomicrographs of *Lepisosteus oculatus* esophagus infected with *Sebekia mississippiensis*. (**A**) Larva encysted within the tunica muscularis. A spacious thin-walled pseudocyst distorts the surrounding myocytes. The submucosa is at top; the serosa is at the bottom. (**B**) An encysted larva. The annulations are readily evident (black arrow), as are the myocyte striations (green arrow). (**C**) Cross section of an encysted larva. Abundant acidophilic glands fill the coelom (black arrow). Tightly spaced, fine spines cover the cuticle (green arrow). The pseudocyst wall is composed of fine collagenous tissue. (**D**) Encysted larva body wall (top) and pseudocyst wall (bottom). Notice the very fine spines (black arrow) on each annulus. The pseudocyst wall is composed of many fibroblasts, with fewer macrophages and lymphocytes. Stained with hematoxylin and eosin. Color version available online.

specific pathogen-free intermediate hosts with *S. mississippiensis*, followed by characterization of each nymphal instar, are needed to better understand if the development of *S. mississippiensis* follows a similar pattern to that of *S. oxycephalum*. Such experimental infections would be difficult because they would require the acquisition of *S. mississippiensis* eggs from adult pentastomes from alligators, feeding those eggs to experimental hosts, and terminally sampling the intermediate hosts at various time points, as was carried out by Winch and Riley (1986) for *S. oxycephalum*. Additionally, experimental intermediate hosts would need to be reared in a controlled setting free of potential pentastome infection, given that there are presently no reliable nonsurgical methods to treat pentastome infections (Tappe and Büttner, 2009). Efforts to treat pentastome infections with antiparasitic drugs have achieved variable success (Paré, 2008). Therefore, experimental hosts cannot otherwise be cleared of existing pentastome infections prior to experimental exposure, thus confounding the results of experimental infections carried out on wild-caught hosts. Therefore, barring the discovery of previously unused morphological characters for distinguishing *Sebekia* spp., particularly earlier instars, comparison of molecular data from nymphs with data collected from more confidently identified adult pentastomes may be the most effective way to identify these larval stages to species.

Although the present definitive host sample size is smaller, prevalence of *S. mississippiensis* from alligators sampled in Mississippi (63.64%) was generally lower than records in Florida and Louisiana (83.3% for Florida, 70.4–92.9% for Louisiana; Tellez et al., 2014), Georgia (50%; Deakins, 1971), Florida (50%; Shotts et al., 1972; 93%; Cherry and Ager, 1982), and Texas (12% and 72% for immature and mature hosts, respectively; Scott et al., 1997). However, it must be noted that the hunted alligators sampled in the present study were mostly male (10/12) and ranged in length from 1.37 to 3.96 m. Therefore, this sample may not be

representative of alligators in Mississippi. The host response of the alligator to *S. mississippiensis* is typical that mentioned in the literature (Jacobson, 2007). Often, infection is rather mild in adult alligators. However, larval migration or heavy pentastome burden may cause significant pulmonary injury, particularly in the young. The large granuloma identified had a core of relatively indigestible, protein-rich, immunogenic material; such material does not typically accumulate in bacterial, fungal, or protistan infections. However, the death of a metazoan parasite, such as an adult specimen of *S. mississippiensis*, may explain it.

The minimal adverse pathology associated with the presence of well-developed *S. mississippiensis* in spotted gar suggests an old host–parasite relationship. Should adverse pathology be observed in other intermediate host species, this may suggest that those species are more recent or aberrant hosts. Conversely, should similar pathology to that presented here be detected in these other hosts species, this would support the view that *S. mississippiensis* has a great number of potential intermediate hosts species. Indeed, histopathological examination of 2 intermediate host species, the western mosquitofish and green swordtails, showed mild and extensive inflammatory responses, respectively, to the presence of *S. mississippiensis* nymphs (Boyce et al., 1987). Assuming that the pentastomes recorded by Boyce et al. (1987) were correctly identified, this suggests that the green swordtail is a more recent intermediate host, whereas the western mosquitofish is a natural host. This is plausible given that the green swordtail is not native to the range of the American alligator, whereas the western mosquitofish is a native species. However, only experimental infections can confirm whether or not these or other species can serve as intermediate or paratenic hosts.

Given that adult pentastomes can generally be more confidently identified than nymphs, sequencing data presented here should be of use for reliably identifying nymphal stages recovered from intermediate or paratenic hosts. Further surveys of fish in the southeastern United States for infections with nymphal pentastomes, coupled with morphological and molecular characterization of recovered nymphs, should shed light on the true diversity of the pentastomes. Given that molecular characterization of larval stages of other parasitic groups (especially platyhelminths) has revealed far greater diversity than was previously anticipated based on morphological data (Poulin, 2010), it is plausible that the same will be revealed for the pentastomes. It is quite possible that many pentastomes previously described as nymphs of *S. mississippiensis* and *S. oxycephalum* may in fact be uncharacterized species. Habitats capable of supporting alligators are typically also ideal for supporting a diversity of other vertebrate species (Newsom et al., 1987). Thus, a habitat capable of supporting the life cycle of *S. mississippiensis* is likely capable of supporting a number of possible pentastome species. Another factor of concern is the fact that the known crocodilian hosts for another pentastome species of the genus *Sebekia*, namely, *Sebekia divestei* Giglioli, in Sambon, 1922 in the American crocodile *Crocodylus acutus* Cuvier, 1807, overlap in range (Neill, 1971). Given the plasticity of species ranges and the fact that these parasites utilize multiple intermediate host species, this suggests the possibility that even definitive host species may be inadequate justification for pentastome species identity, especially in areas of intermediate and/or definitive host range overlap. Pentastomes have been reported from a diverse array of definitive hosts (Paré, 2008). Thus, the possibility should be considered that nymphs recovered in systems large enough to support alligators are not necessarily alligator pentastomes. Should further studies molecularly characterizing pentastome nymphs support historical accounts of *S. mississippiensis*, the following interesting questions would be raised: Why is this particular species so widespread, and why do its nymphs so successfully outcompete species relying on non-crocodilian definitive hosts, such that *S. mississippiensis* nymphs are much more frequently encountered than those of other sebekids, such as *L. subaequalis*? Additionally, this would provide an excellent opportunity to examine the extent of host-derived morphological variation within *S. mississippiensis*, possibly shedding light on the taxonomic utility of morphological characters commonly used in pentastome identification.

Phylogenetic placement of *S. mississippiensis* as a sister clade in the present analysis may suggest that the other molecularly characterized species of *Sebekia*, both from Australia, may in fact represent a distinct clade. However, the dearth of presently available sequencing data from pentastomes precludes accurate phylogenetic inference about the relationships between these parasites.

Regarding the variety of clearing and mounting techniques used in the present study, some discussion is warranted. It has become common practice to mount pentastomes directly in Hoyer's medium under cover glass for morphological characterization (Overstreet et al., 1985; Barton and Morgan, 2016). While this technique provides rapid visibility of taxonomically informative characters and internal structures, these structures quickly clear to the point where all but the hooks are difficult to discern, making comparisons between specimens quite difficult. The specimens are likely to clear further over a long period of time, possibly compromising their utility for future reference (Ash and Orihel, 1987). Indeed, the mounting media from the type material deposited by Overstreet et al. (1985) and examined in the present study appeared to have receded such that many of the specimens are now impossible to measure accurately. The laboratory production of Hoyer's medium also requires chloral hydrate, which is a controlled substance in the United States, and commercially available formulations of the mounting medium are costly. While clearing specimens in lactophenol and mounting in glycerin jelly will avoid these issues, it too results in a semi-permanent mount with special storage requirements (Pritchard and Kruse, 1982). Merely clearing the specimens and examining them in a concavity slide is likely to result in hook measurements that are not easily repeatable due to the difficulty of arranging hooks at precisely the same angle at which they were previously measured. Staining specimens with Van Cleave's hematoxylin and mounting in Canada balsam resulted in easily storable permanent mounts with acceptable visibility of reproductive structures and excellent visibility of digestive systems and chloride pores. However, specimens stained this way had hooks and annuli that were difficult to visualize, even with the aid of differential interference contrast microscopy. In addition to these mounting techniques, it is also common practice to deposit whole pentastomes fixed in 70% ethanol as type material. Unfortunately, examination of taxonomically informative hook morphology is not possible on such material without the application of potentially destructive clearing and mounting techniques. This in fact proved problematic in the present study. Attempts to collect morphometric data from voucher specimens deposited by Tellez et al. (2014) were met with limited success because hooks

were difficult to visualize on most specimens without clearing and mounting. Without being able to confidently detect the presence or absence of a dorsal accessory piece, it is not possible to determine if many of these specimens are adults or nymphs, making direct, independent morphometric comparisons impossible using such specimens. While mounting would have risked mechanical destruction of the specimens, clearing agents have also been suspected of stymying efforts at subsequent DNA extraction from pentastomes (Barton and Morgan, 2016).

Conversely, the clearing and mounting technique that was employed for the hologenophores in the present study provided adequate visibility of both hooks (Fig. 1B) and annuli (Fig. 1C). Separating the cephalothoraces using a scalpel blade, dehydrating in a series of 4 ethanol washes from 70 to 100%, clearing in Hemo-De, and mounting in Canada balsam provided excellent visibility of hooks and the oral cadre. Dissolving the internal tissues of the hindbody with a typical proteinase K-based DNA extraction protocol resulted in a cuticle with distinguishable annuli once dehydrated, cleared, and mounted. Additionally, the supernatant left behind by this technique proved suitable for continued DNA extraction, thus producing an easily stored, permanent voucher specimen with molecular data and readily measurable hook morphology, body length, and number of annuli. We would encourage future researchers to employ this technique for the production of hologenophores of pentastomid species that are small enough to be processed in such a way, in addition to using standard clearing and mounting techniques for morphometric data. This is similar to the technique employed by Literák et al. (2017), who cleared the cephalothoraces in lactophenol while using the hindbodies for molecular analysis. However, the present technique has the added benefit of preserving annulus count for the voucher specimen. It is also worth noting that in addition to adults collected from alligator lungs and nymphs manually excysted from gar tissue, nymphs recovered via pepsin digestion were also suitable for morphological characterization of *S. mississippiensis*, as has previously been reported for other pentastomid taxa (Garedaghi, 2011; Alborzi et al., 2013).

Although the goal of the present work is not to levy criticism against prior researchers, it must be stressed that host records that do not provide adequate morphological or molecular data to justify independent specific diagnoses must be regarded cautiously. Clearly, there is some confusion in the literature regarding specific identification of *S. mississippiensis*, largely owing to a number of accounts of *S. oxycephalum* that are now thought to refer to *S. mississippiensis*. Overstreet et al. (1985) went to great lengths to address these conflicting accounts by examining available specimens from such studies. It would be a shame to undo this work and confound the literature again with further accounts of *S. mississippiensis* that do not adequately justify specific diagnosis. While accounts of *S. mississippiensis* adults from alligators, especially those occurring outside of the reported range of *S. oxycephalum*, are likely accurate, a more cautious approach is warranted regarding the nymphal stages, which lack robust descriptions or molecular data. Adults from seemingly aberrant hosts should be treated likewise. It is hoped that the present work will serve as impetus to provide more robust accounts of pentastomid nymphs accompanied by reliable voucher specimens.

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