Distribution, Abundance, and Genetic Diversity of *Clinostomum* spp. Metacercariae (Trematoda: Digenea) in a Modified Ozark Stream System

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DISTRIBUTION, ABUNDANCE, AND GENETIC DIVERSITY OF *CLINOSTOMUM* SPP. METACERCARIAE (TREMATODA: DIGENEA) IN A MODIFIED OZARK STREAM SYSTEM

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ABSTRACT: Land-use alterations can have profound influences on faunal distributions, including host-parasite relationships. Yellow grub trematodes (*Clinostomum* spp.) have complex life cycles involving 3 hosts: a snail, a fish or amphibian, and a bird. Here, we analyze the distribution, prevalence, intensity, abundance, and genetic diversity of encysting metacercariae of *Clinostomum* spp. in salamanders and fishes throughout an aquatic system that includes a natural Ozark stream and man-made ponds. We found *Clinostomum* sp. infecting permanently aquatic Oklahoma salamanders (*Eurycea tynerensis*; 56% prevalence) and larval grotto salamanders (*Eurycea spelaea*) immediately downstream from a man-made pond. However, *Clinostomum* sp. did not infect any salamanders in the spring that supplies this pond, or in sections farther downstream (~0.5 and 2 km). Metacercariae of *Clinostomum* sp. were present in ~90% of introduced largemouth bass (*Micropterus salmoides*) in the man-made pond adjunct to the stream. Morphological examination and phylogenetic analyses based on the mitochondrial gene *cytochrome oxidase 1* (*Co1*) and the nuclear ribosomal gene *18S* show that fishes and salamanders at this site are primarily infected with *Clinostomum marginatum*. There is a relatively high degree of mitochondrial haplotype diversity in *C. marginatum* at this site but no consistent genetic difference between parasites in largemouth bass from the man-made pond and those in salamanders from the stream. Based on the microgeographic distribution and relationships of metacercariae of *C. marginatum* at this site, we hypothesize that the adjunct man-made pond has created an ecological situation that brings the cercariae of this parasite into contact with novel stream salamander hosts.

Land-use changes can drastically alter the distribution and abundance of wildlife (Sala et al., 2000; Hooper et al., 2005; McKinney, 2006). This, in turn, influences host-parasite relationships, since man-made habitats can initiate novel interactions between members of otherwise different communities (McMichael, 2004; Macdonald and Laurenson, 2006). Anthropogenic landscape modifications, particularly those associated with agriculture, have been shown to influence the prevalence and diversity of digenetic trematode infections in aquatic amphibians (Johnson and Chase, 2004; Koprivnikar et al., 2006; Gray et al., 2007; McKenzie, 2007). However, starkly different effects have been found, depending on the species of trematode, the type of habitat that was disturbed, and the ultimate ecological impacts. For example, increased abundance of trematode *Ribeiroia* sp., which is associated with amphibian limb malformations (Johnson et al., 2002; Johnson and Sutherland, 2003), is thought to be driven by agricultural eutrophication, which promotes the proliferation of the trematodes' intermediate gastropod host (Johnson and Chase, 2004; Johnson et al., 2007). In contrast, sedimentation of cultivated wetlands in the southern Great Plains of North America resulted in reduced prevalence and abundance of metacercariae of *Clinostomum attenuatum* in amphibians, likely due to a decrease in the hydroperiod, which affects the trematode's complex life cycle (Gray et al., 2007).

Clinostomum spp. are cosmopolitan digenetic trematodes that sexually reproduce in the alimentary canal of birds. *Clinostomum* spp. eggs leave the bird from the mouth, or the cloaca in feces (Olsen, 1974; Smyth and Smyth, 1980). The miracidia enter the first intermediate snail host by penetrating an external surface of the body or by being ingested as eggs (Olsen, 1974; Smyth, 1994). The miracidia reproduce asexually in the snail to produce free-swimming cercariae. The cercariae encyst in a second intermediate host (fishes or amphibians) and develop into the metacercariae stage (Olsen, 1974). The encysting metacercariae are the conspicuous "yellow grubs" from which the common name is derived. The life cycle is completed when a piscivorous bird or mammal eats an infected fish or frog (Kanev et al., 2002). *Clinostomum* spp. metacercariae have been documented in a wide diversity of amphibians, but most commonly in species that live in, or breed in, lentic habitats (reviewed in McAllister et al., 2010). While agricultural land-use changes to wetlands have been shown to significantly influence the prevalence and abundance of *Clinostomum* spp. encysting in amphibians (Gray et al., 2007; McKenzie, 2007), novel host interactions resulting from land-use alteration are not well documented. Furthermore, DNA sequence analyses that include *Clinostomum* spp. have been limited to minimal representation in higher-level trematode phylogenies (Olson et al., 2003; Dzikowski et al., 2004; Moszczynska et al., 2009). Consequently, intraspecific and local patterns of genetic diversity have yet to be examined for any species of *Clinostomum.*

Here, we analyze the distribution, prevalence, intensity, abundance, and genetic diversity of *Clinostomum* spp. infecting fishes and stream salamanders in an aquatic system in the Ozarks of Oklahoma. We use the distribution and genetic data to test for an association between a man-made pond that interrupts a native spring-fed stream and the presence of metacercariae of *Clinostomum* spp. in stream-dwelling salamanders downstream from the pond. Additionally, this study documents several novel findings for *Clinostomum* spp., including new host records, infrapopulation genetic variation, and possible mitochondrial heteroplasmy.

MATERIALS AND METHODS

Description of study site

The survey of *Clinostomum* spp. was conducted in Sawmill Hollow in the Nature Conservancy's J.T. Nickel Family Nature and Wildlife Preserve in Cherokee County, Oklahoma (36.0520167°N, 94.811967°W). Sawmill Hollow includes a low-order, gravel-bottomed Ozark stream that originates in the preserve and flows into the Illinois River (Fig. 1). Paedomorphic (permanently aquatic) Oklahoma salamanders (*Eurycea tynerensis*) inhabit the main section of this stream, which is primarily fed by a groundwater

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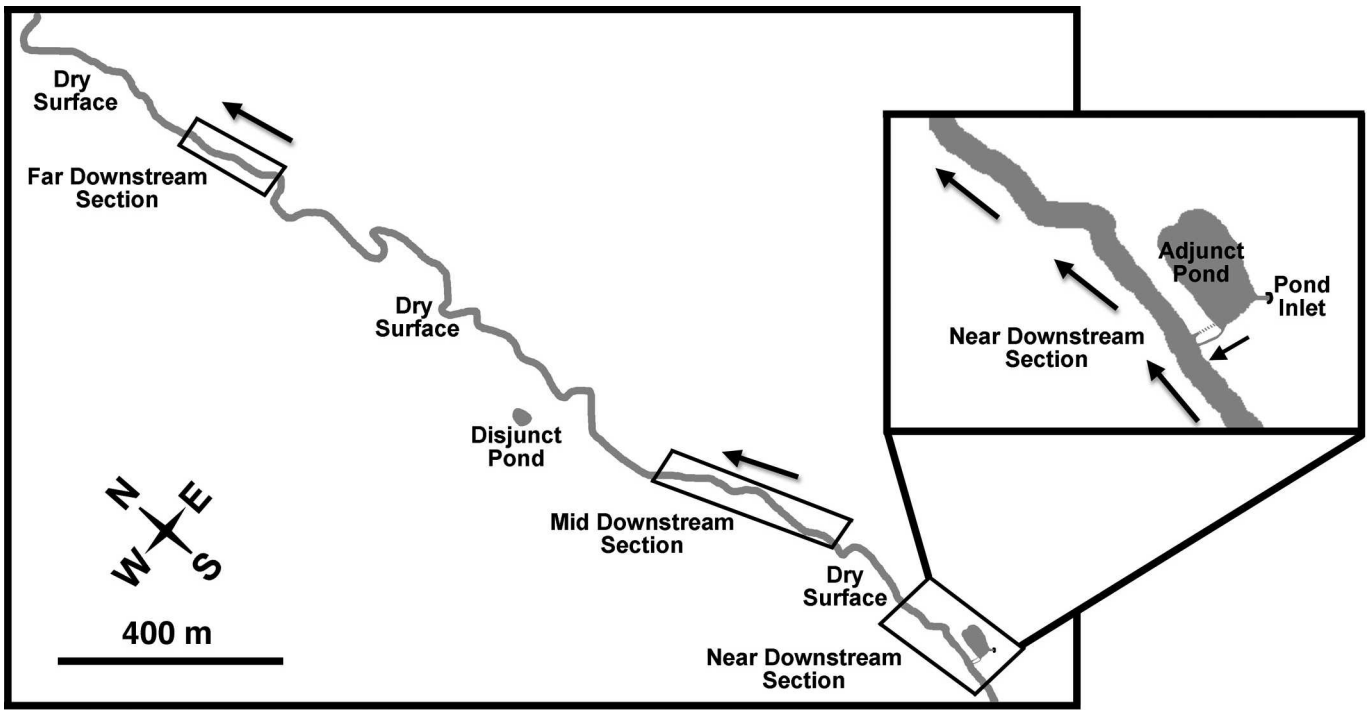

FIGURE 1. Study site in Cherokee County, Oklahoma, where *Clinostomum* spp. metacercariae infection was surveyed. Boxes over the stream indicate the sections where salamanders were surveyed (far, mid, near, and pond inlet), and portions of the stream that were flowing at the surface during our survey. Arrows indicate the direction of stream flow. Intervening sections of the streambed were dry at the surface. Inset map shows the groundwater spring (pond inlet) that feeds into the adjunct pond, which empties into the main-stream channel.

spring (pond inlet) that flows through a man-made pond (adjunct pond) that is ~1,200 m^2. The pond inlet is inhabited by larval grotto salamanders (*Eurycea spelaea*), and it runs from the bottom of a hillside ~3 m across the surface and into the adjunct pond. Largemouth bass (*Micropterus salmoides*) appear to be the only fish inhabiting the adjunct pond. There are 2 shallow outlet streams from the adjunct pond that flow to the main stream. One of the outlet streams flows aboveground about 17 m down a mild gradient before reaching the main stream. The other outlet stream flows about 8.5 m through a pipe then an additional 5.7 m across the surface before reaching the main stream. During our survey period, the main-stream channel was dry ~20 m upstream of the adjunct pond outlets, and in large patches downstream. However, a continuous surface stream is usually present in the winter and spring, and subsurface channels exist in these streams much of the year. During our survey, the water was clearly flowing within the 3 sections of the stream that we surveyed. Sawmill Hollow contains a second small pond that is not directly connected to the stream (disjunct pond). This disjunct pond primarily contains bluegill (*Lepomis macrochirus*) and only has a low density of *M. salmoides*.

Collection methods and analyses

On 18 and 19 November 2009, 2 species of plethodontid salamanders, *E. tynerensis* and *E. spelaea*, were examined for the presence of metacercariae of the digenetic trematode *Clinostomum*. We surveyed 3 sections of stream between 2,030 and 1,860 m downstream (far), 700 and 320 m downstream (mid), and 170 m downstream to 20 m upstream (near) as measured from an outlet of thre small man-made pond feeding into the stream (Fig. 1). These sections included nearly all of the flowing surface water in the study site during our survey. Surveyed salamanders were collected with small dipnets or by hand. Each salamander was visually inspected for presence and number of metacercariae of *Clinostomum* spp. independently by 2 researchers. These data were recorded, and the salamander's coordinates were also determined along the stream. Surveyed salamanders were released after inspection, except for a few infected individuals collected for identification and vouchering. On 3 December 2009, *M. salmoides* individuals were surveyed in the adjunct pond using line and hook, and their mouth, gills, and opercula were examined for the presence and number of metacercariae of *Clinostomum* spp. Largemouth bass in the adjunct pond were resurveyed on 6 March 2010 for a second estimate of prevalence, intensity, and abundance of metacercaria of *Clinostomum* spp. based on a larger sample size. All fish were marked by fin clipping and released after inspection, except for 2 *M. salmoides* individuals from the adjunct pond. We also collected 1 *M. salmoides* and 1 *L. macrochirus* individual from the disjunct pond for genetic analysis of metacercariae.

Parasite methods

We retained 4 *E. tynerensis* (2 males, 2 females, 30–35 mm SVL), 1 *E. spelaea* (female, SVL =37 mm), and 1 *M. salmoides* (18 cm standard length) individuals as vouchers for further processing of metacercariae in the laboratory. Live salamanders were placed in 50-ml plastic conical tubes containing creek water and shipped overnight on ice to one of us (C.T.M.) for examination. Salamanders and fish were killed with a dilute chloretone solution, and encapsulated dermal metacercariae were teased from their capsules or opercula using a dissecting microscope. Metacercariae were flattened and fixed in 70% ethanol, stained with Semichon's acetocarmine, and mounted in Damar. Voucher specimens of *Clinostomum marginatum* metacercariae were deposited in the United States National Parasite Collection (USNPC), Beltsville, Maryland, as follows: USNPC 103062 (from *E. spelaea*), 103065 (from *E. tynerensis*), and 103210 (from *M. salmoides*). Host voucher specimens were deposited in the Arkansas State University Herpetological Museum (ASUMZ), State University, Arkansas, or Henderson State University, Arkadelphia, Arkansas, as follows: *E. spelaea* (ASUMZ 31427), *E. tynerensis* (ASUMZ 31389, 31424–31426; Fig. 2), and *M. salmoides* (HSU 3332).

Analytical methods

Salamanders and fishes were grouped by species and location: *E. tynerensis* samples were classified as near downstream, mid-downstream, or far downstream, based on their proximity to the adjunct pond outlet (Fig. 1). *Eurycea spelaea* samples were classified as pond inlet or near downstream. The 2 surveys of *M. salmoides* in the adjunct pond (December and March) were analyzed separately, but they yielded similar results. Based on the definitions by Bush et al. (1997), we determined: (1) the prevalence of *Clinostomum* spp. (percentage of individuals with any

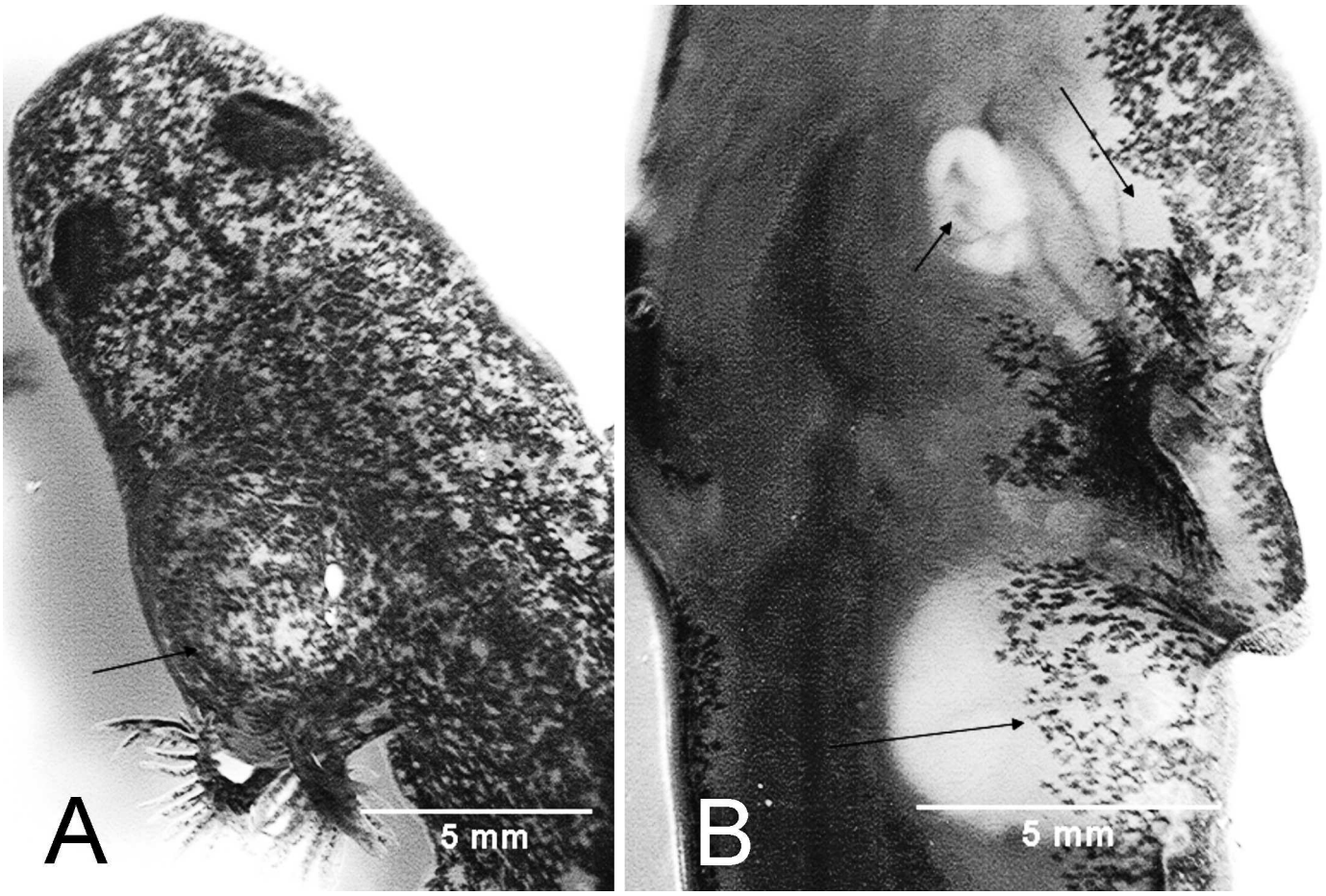

FIGURE 2. Metacercariae of *Clinostomum marginatum*. (**A**) Dorsal view of female *Eurycea tynerensis* (SVL = 33 mm, ASUMZ 31389) from Cherokee County, Oklahoma, showing encysted metacercaria (arrow) on left side in head. (**B**) The same salamander showing 3 metacercariae in gular region and side of body.

metacercariae); (2) the mean intensity of *Clinostomum* spp. infection (number of metacercariae per infected individual); and (3) the mean abundance of *Clinostomum* spp. (number of metacercariae per individual examined). SPSS (Chicago, Illinois) was used to perform 1-way analyses of variance (ANOVA) with Fisher's least significant difference (LSD) tests to determine whether the 3 groups of *E. tynerensis* (near, mid-, and far downstream) differed in their mean abundance of metacercariae of *Clinostomum* spp. This was to test whether metacercariae of *Clinostomum* spp. were more abundant in the section of stream immediately downstream from the pond (near-downstream section) compared to sections farther downstream (mid- and far-downstream sections). The pond inlet only contained *E. spelaea*, so we were unable to directly include this section in the statistical analysis, although the lack of infection by *Clinostomum* spp. in salamanders from the inlet is consistent with our interpretation of the results.

Molecular methods and genetic analyses

Twenty-two metacercariae of *Clinostomum* spp. were dissected from *E. tynerensis*, *M. salmoides*, and *L. macrochirus* collected from the study area. Nine of the metacercariae were from 2 *E. tynerensis* individuals from the near-downstream section, 9 of the metacercariae were from 2 *M. salmoides* individuals from the adjunct pond, 2 of the metacercariae were from 1 *M. salmoides* individual from the disjunct pond, and 2 of the metacercariae were from 1 *L. macrochirus* individual also from the disjunct pond. Qiagen (Hilden, Germany) DNeasy extraction columns were used to isolate DNA from whole frozen, or ethanol-preserved, metacercariae. The mitochondrial gene *cytochrome oxidase 1* (*Co1*; 557 bp) was amplified via polymerase chain reaction (PCR) using the newly designed primers Trem_Co1F 5′-TTTCGTTGGATCATAAGCG-3′ and Trem_Co1R 5′-GCAGCACTAAATTTACGATCAAA-3′. The primers were designed from 3 trematode sequences from GenBank: *Clinostomum* sp. (FJ477191) for which the primers are perfect matches, and *Schistosoma haematobium* (DQ157222) and *Fasciola hepatica* (FJ477191), which also have high sequence similarity. Approximately 430 bp of the nuclear ribosomal gene *18S* were also amplified by PCR with other newly designed primers Clino_18Sa_F 5′-CCGCAAGGGAATGGGTGGATTTAT-3′ and Clino_18Sa_R 5′-TCAAAGTAAAGATGCCGTCCGCTC-3′. The *18S* primers were designed using an alignment of all *Clinostomum* sequences from GenBank (*Clinostomum complanatum* FJ609420 and AY245701, *Clinostomum giganticum* FJ970654, *Clinostomum marginatum* AY245760, *Clinostomum piscidium* FJ970655, *Clinostomum phalacrocoracis* FJ609422 and FJ609423, *Clinostomum* sp. Australia AY222094, *Clinostomum* sp. "cutaneum" GQ339114 and FJ609421, and *Clinostomum* sp. U.S.A. AY222095), and they were a perfect match to all sequences.

PCR products underwent electrophoresis on 1% agarose gels, and strong PCR products of the expected molecular weight were cleaned with ExosapIT (USB Corp., Cleveland, Ohio). Big Dye v. 3.1 (Applied Biosystems Inc., Foster City, California) was used for cycle sequencing reactions, which were cleaned with Sephadex (Sigma, St. Louis, Missouri) and sequenced on a 3130×l capillary sequencer at the University of Tulsa (Tulsa, Oklahoma). Sequences were edited and aligned in Sequencher™ v. 4.8 (Gene Codes Corp., Ann Arbor, Michigan). The "trematode mitochondrial code" in Sequencher was used to translate the *Co1* sequences to check for the absence of stop codons in each sequence. Sequences were deposited on GenBank (HQ439560 to HQ439586). The *Co1* alignment was completely unambiguous, and there were no stop codons in any of the *Co1* sequences. The *18S* alignment was mostly unambiguous except for a short variable region (6 to 7 bp in length) that was removed prior to phylogenetic analyses.

Alignments of each gene were analyzed with Bayesian phylogenetic methods and the *Co1* alignment was also analyzed using a haplotype network. MrModeltest v. 2.2 (Nylander, 2004) was used to determine the most appropriate model of nucleotide substitution for each gene. The models chosen were HKY for *Co1* and HKY + I for *18S*. For both *Co1* and *18S*, Bayesian phylogenetic analyses performed in MrBayes v. 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) were run with 4 chains (3 hot and 1 cold) and uniform priors for 20 million generations (with a tree saved at every 1,000 generations). We discarded the first 5 million generations (5,000 trees) as burn-in, and the topology and branch lengths were summed across 15,001 post–burn-in trees. A 50% majority-rule consensus of the post–burn-in trees was computed using PAUP* (Swofford, 2001) to determine Bayesian posterior probabilities (BAPP) for relationships. The 2 simultaneous independent runs performed in MrBayes 3.1 converged on the same topology and posterior probabilities. The *Co1* phylogeny was rooted with the most divergent *Co1* sequence for our sample. The *18S* phylogeny was rooted with *Shistosomatium douthitti* (GenBank AY157221).

PAUP* (Swofford, 2001) was also used to calculate uncorrected pairwise genetic divergence for *Co1*. Redundant *Co1* haplotypes were consolidated using Collapse, v. 1.1 (available at http://darwin.uvigo.es/), and a minimum spanning network based on a pairwise matrix was calculated using Arlequin v. 3.0 (Excoffier et al., 2005). Arlequin was also used to calculate standard diversity indices (''gene diversity'') and molecular indices (mean number of pairwise differences [π] and nucleotide diversity) for *Co1* of metacercariae from: (1) all hosts at our site; (2) all salamanders; (3) all *M. salmoides* from the adjunct pond; and (4) all fishes from the disjunct pond. The pairwise F_{st} values with 16,000 permutations were used to evaluate genetic distances between host and/or location groups, and to test the following hypotheses: *Clinostomum* spp. infecting *M. salmoides* from the adjunct pond and *E. tynerensis* from the near-downstream section are genetically different (significant F_{st}, $P \leq 0.05$), or are part of the same population (non-significant F_{st}). This test is particularly important given the host specificity of many trematodes, and the current lack of genetic data or tests of cryptic diversity in *Clinostomum* spp. All *18S* sequences collected from our study site were identical and, therefore, were not useful for population-level analyses, but they were used for species-level corroboration of some of our most mitochondrially divergent metacercariae.

RESULTS

Distribution, prevalence, intensity, and abundance

We examined a total of 74 *E. tynerensis* individuals from Sawmill Hollow. The 3 groups (far, mid-, and near-downstream) yielded 25, 31, and 18 salamanders, respectively. Salamanders from the far- and mid-downstream sections did not show any signs of infection by *Clinostomum* spp. Fifty-six percent of the *E. tynerensis* individuals found in the near-downstream section were infected with *Clinostomum* sp. (Fig. 3), with a mean intensity of 2.2 (±1.9; average ± standard deviation) metacercariae per infected individual and a mean abundance of 1.2 (±1.8) metacercariae per individual examined (Fig. 3). ANOVAs with Fisher's LSD for abundance of *Clinostomum* sp. on *E. tynerensis* showed significant variation among groups ($F_{(2,\ 71)} = 13.107$, $P < 0.0001$). The *Clinostomum* sp. mean abundance was significantly higher in the near-downstream section than the mid- ($P < 0.0001$) and far-downstream ($P < 0.0001$) sections. There were no significant differences between the mid- and far-downstream sections ($P = 1.00$), because *Clinostomum* spp. were absent in both. Fewer *E. spelaea* individuals were found during our search (n = 12); the near-downstream section and pond inlet yielded 2 and 10 salamanders respectively, with 1 infected individual in the near-downstream section. Due to the low numbers of *E. spelaea* collected in the near-downstream section, we were unable to statistically compare the mean abundance of metacercariae in *E. spelaea* from the pond inlet and near-downstream section.

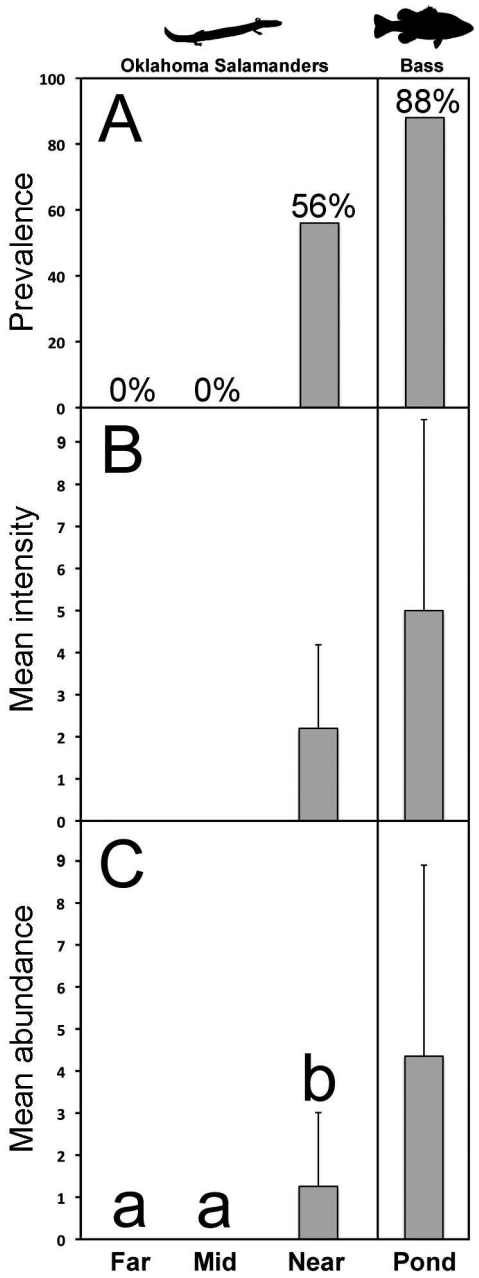

FIGURE 3. Prevalence, intensity, and abundance of *Clinostomum* spp. metacercariae infection in Oklahoma salamanders and largemouth bass in different areas of our study site. (**A**) Prevalence of infection is the percentage of individuals with at least one *Clinostomum* sp. individual. (**B**) Mean intensity of infection is the average number of *Clinostomum* metacercariae found per parasitized individual. (**C**) Mean abundance of infection is the average number of *Clinostomum* metacercariae found per individual examined, including both infected and uninfected individuals. Bars on graphs B and C represent standard deviations. Far, mid, and near refer to the 3 main stream sections downstream from the pond (Fig. 1). Lowercase letters (a and b) represent the results of the Fisher's LSD test from 1-way ANOVA comparing differences in the total number of *Clinostomum* spp. found in the different stream sections per salamander examined. There were significantly more *Clinostomum* spp. in the near-downstream section.

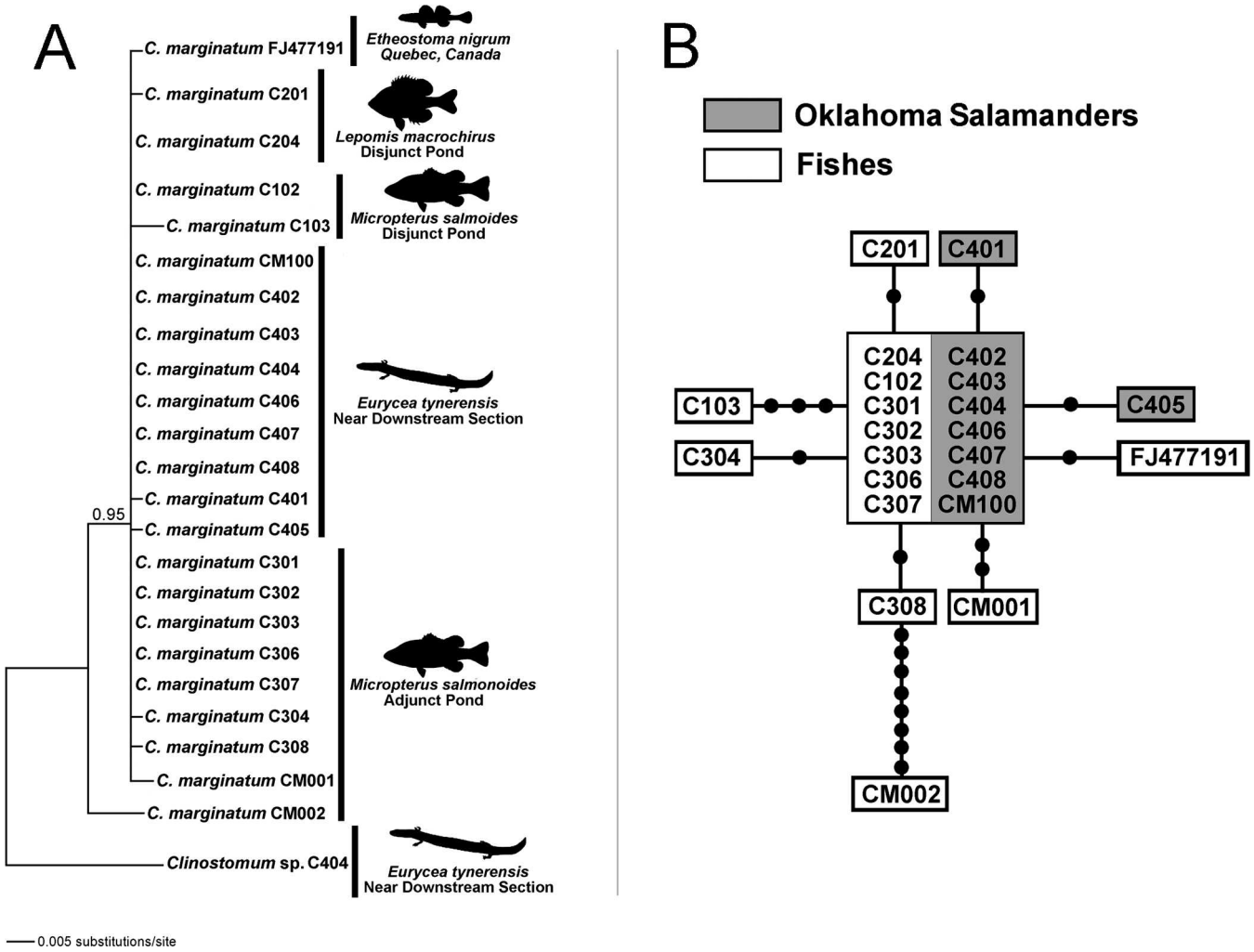

FIGURE 4. Genetic relationships of *Clinostomum marginatum* metacercariae from our study site based on the mitochondrial gene *Co1*. (**A**) Bayesian phylogram with host species and location indicated on the right. Posterior probability of the significant node is shown above the internal branch. (**B**) The minimum spanning network of *C. marginatum.* Metacercariae collected from salamanders are highlighted in gray, and those from fishes are in white. Each dot in the network represents 1 mutational step.

Largemouth bass from the adjunct pond were inspected for *Clinostomum* spp. during December and March. During the December survey, 7 of 8 (88%) of the *M. salmoides* individuals sampled from the adjunct pond were infected (Fig. 3). The infected individuals had a mean intensity of 5 metacercariae (±4.7), and the mean abundance of the population was 4.4 metacercariae (±4.7). Results from the March survey were comparable, i.e., 24 of 26 (92%) of the *M. salmoides* individuals sampled from the adjunct pond were infected. The infected individuals had a mean intensity of 6.3 metacercariae (±5.1), and the mean abundance of the population was 5.9 metacercariae (±5.2).

Phylogenetics and genetic diversity

Phylogenetic analyses of *Co1* showed strong support (BAPP = 0.95) for a clade including 21 haplotypes of *Clinostomum* sp. from our study site (Fig. 4A). The *Co1* uncorrected pairwise genetic divergence within this clade ranged from 0.00% to 0.61%. Samples included in this clade are metacercariae from every host species that we sequenced in our study area, and a *Clinostomum* sp. individual (GenBank FJ477191) sampled from a Johnny darter (*Etheostoma nigrum*) from the Saint Lawrence River, Quebec. Based on morphological analyses (and *18S*; see following section), we consider metacercariae in this clade to be *Clinostomum marginatum*. However, we found 2 other more divergent *Co1* haplotypes at this location. One metacercaria from the adjunct pond (CM002) was on average 1.88% ± 0.08% divergent from haplotypes in the common clade. The other divergent haplotype was from a metacercaria (C404) on an Oklahoma salamander and was on average 5.19% ± 0.12% divergent from all other haplotypes. Interestingly, this individual metacercaria also had a *Co1* haplotype from the common clade. The divergent haplotype translates well, most of its mutations are at the third codon positions, and it has no frame shift mutations. Accordingly, it does not appear to be an artifact of PCR or a mitochondrial pseudogene. The level of divergence in *Co1* suggests that this haplotype may be from another species of *Clinostomum*, but without comparative *Co1* sequences of other species of yellow grub, we will refer to it as *Clinostomum* sp.

TABLE I. Genetic diversity parameters for *Co1* of *Clinostomum* spp. sequenced at our study site. Parameters were estimated based on all individuals, salamanders only, bass from the adjunct pond, and fishes from disjunct pond. The unique haplotypes listed here refer to those that are unique to our analysis.

Parameter	All samples	Salamanders	Bass adjunct pond	Fishes disjunct pond
n	22	9	9	4
Unique haplotypes	9	2	4	2
Different haplotypes	9	3	5	3
Gene diversity	0.606 ± 0.123	0.417 ± 0.191	0.722 ± 0.159	0.833 ± 0.222
Nucleotide diversity	0.003 ± 0.002	0.009 ± 0.001	0.006 ± 0.004	0.004 ± 0.003
π	1.710	0.444 ± 0.434	2.833 ± 1.645	2.000 ± 1.405

The 4 samples that we sequenced for a fragment of *18S* were identical and were also identical to *C. marginatum* (AY245760) and *Clinostomum* sp. USA (AY222095) from GenBank, further supporting our specific identification. The samples that we selected for *18S* sequencing spanned the range of mitochondrial divergence, including the putative heteroplasmous sample (C404) and, therefore, did not help us further resolve the identity of the divergent *Co1* haplotype. Based on the approximately 7 species of *Clinostomum* included in the *18S* phylogeny, we found strong support for a monophyletic *C. marginatum* (BAPP = 0.98) and *C. complanatum* (BAPP = 1.00), but only weak support for a sister relationship between these taxa (BAPP = 0.66). The *18S* analysis confirmed the divergence between *C. complanatum* and *C. marginatum* (2.6% divergent for this highly conserved fragment). This is significant since the *C. marginatum* sample used by Dzikowski et al. (2004) was from Israel (not North America), which left this as an open question (McAllister et al., 2010).

The minimum spanning network shows 14 of the 22 (64%) metacercariae of *C. marginatum* from our site had the common *Co1* ancestral haplotype, while the other 8 had unique haplotypes that are 1 to 10 mutational steps from the ancestral sequence (Fig. 4B; Table I). Nine unique haplotypes in 22 samples is a high number (gene diversity = 0.606 ± 0.123), but nucleotide diversity is low (0.003 ± 0.002), indicating that most unique haplotypes are similar to one another. We observed the same overall pattern even when we analyzed *C. marginatum* metacercariae harvested from different hosts and/or locations separately (Table I). The high value for the mean number of *Co1* haplotypes in *C. marginatum* from largemouth bass in the adjunct pond is primarily driven by sample CM002, which was 10 mutational steps from the ancestral haplotype. The pairwise F_{st} test showed no significant difference in any comparison of metacercariae from different host and/or location groups ($P > 0.05$). Therefore, there is no evidence that *C. marginatum* metacercariae from the largemouth bass in the adjunct pond differ from metacercariae infecting *E. tynerensis* in the near-downstream section ($P > 0.43$).

DISCUSSION

Land-use changes and *Clinostomum* secondary host expansion

Metacercariae of *Clinostomum* spp. encyst in a broad range of larval and adult amphibian taxa (see recent review by McAllister et al., 2010). It is notable that most of the infection records of *Clinostomum* spp. for North American amphibians are from species that primarily live, or breed, in lentic ecosystems (lakes, ponds, swamps). Infections by *Clinostomum* spp. are much less common in amphibian species that inhabit lotic environments (rivers and streams), and they are especially rare in species that inhabit headwater springs (McAllister, 1990; McAllister et al., 2007). Here, we report 2 new host records for metacercariae of *C. marginatum* in cysts in permanently aquatic Oklahoma salamanders (*E. tynerensis*) and a larval grotto salamander (*E. spelaea*). What makes these records unique is that *E. tynerensis* and larval *E. spelaea* salamanders inhabit headwater springs and small streams, and we have observed hundreds of individuals of these 2 species all across the Ozark Plateau (McAllister et al., 1995; Bonett and Chippindale, 2004, 2006; McAllister et al., 2006) and have never observed an infection of *Clinostomum* spp. for them in any other locations. Why are *Clinostomum* spp. encysted in aquatic *Eurycea* at this site (Sawmill Hollow)? We hypothesize that the man-made pond that partially impounds the natural spring brings lentic habitats and their host-parasite fauna (planorbid snails and *Clinostomum* sp.) into close contact with otherwise lotic salamander species. Metacercariae of *Clinostomum* spp. were prevalent in the lining of the mouth, gills, and opercula of ~90% of *M. salmoides* inhabiting the adjunct man-made pond. Our survey from throughout Sawmill Hollow showed a significant increase in prevalence (56%) of *C. marginatum* metacercariae infecting *E. tynerensis* in a section of stream (near downstream) immediately below the outlet of the man-made pond. Comparatively, none (0%) of the *E. tynerensis* surveyed in the mid- or far-downstream sections had metacercariae (Figs. 1, 3). Furthermore, within the 190 m of the near-downstream section, >80% of the infected salamanders were found within ~5 m of 1 of the 2 pond outlets.

Although there is a substantial amount of mitochondrial haplotype diversity at this site, the minimum spanning haplotype network and F_{st} show no significant difference between *C. marginatum* infecting the largemouth bass in the pond and the Oklahoma salamanders immediately downstream. Also consistent with this finding (but with analytical limitations), none of the *E. spelaea* surveyed in the Pond Inlet had metacercariae; however, 1 of the 2 *E. spelaea* in the near-downstream section had a metacercaria of *C. marginatum*. Taken together, our data show a close geographic and genetic association between the *C. marginatum* metacercariae encysting in *Eurycea* and those infecting the largemouth bass in the man-made pond.

Fish are extremely uncommon in the near-downstream section, and we never found Oklahoma salamanders in the pond, suggesting that the *M. salmoides* and *E. tynerensis* individuals at this site are largely restricted to their lentic and lotic habitats, respectively. We found the planorbid snail *Helisoma* cf. *anceps* (a putative first intermediate host; Hunter and Hunter, 1935) to be

common in both of the aforementioned habitats. The snails could potentially move bidirectionally between these habitats, whereas eggs, free-swimming miracidia, and cercariae of *Clinostomum* could likely only disperse from the pond downstream. The artificial development of a pond habitat within this stream system may have introduced a typically lentic amphibian parasite to new lotic amphibian hosts through the dispersal of first intermediate host snails or multiple free-living aquatic stages of the life cycle of *Clinostomum*.

The partial or complete impoundment of lotic habitats can have a broad range of ecological impacts, including altering temperature, water chemistry, nutrient cycling, sedimentation, and species distributions (Baxter, 1977). Permanently aquatic (paedomorphic) populations of Oklahoma salamanders typically live in streams with well-sorted Silurian/Ordovician chert gravel that has large interstitial spaces that the salamanders use to access subsurface water during dry summers (Tumlison and Cline, 2003; Bonett and Chippindale, 2006). Chert bottom streams inhabited by *E. tynerensis* typically have low levels of sedimentation, but much of the near-downstream section is embedded with overflow sediment from the pond. In contrast to the situation in playa lakes of the southern Great Plains, where sedimentation reduces hydroperiod and limits the infection of *C. attenuatum* (Gray et al., 2007), in our site the sedimentation may effectively have converted portions of the stream into a pond. The most important consequence of this may be to provide habitat for the proliferation of *Helisoma* spp. Sediment may also increase buildup of fecal matter from piscivorous birds. The fine-scale distributions of sedimentation, *Helisoma* spp., *Clinostomum* spp., and secondary hosts at this site are the subjects of an ongoing study.

Genetic diversity and heteroplasmy in *Clinostomum*

A by-product of our genetic isolation test is the first estimate of genetic diversity of *Clinostomum* spp. collected from a single site. The level of haplotype diversity is relatively high given that the metacercariae were collected from a relatively small area (<4 km). However, the haplotype divergence was very low, indicating that most of the unique haplotypes were less than a few mutational steps from the common ancestral haplotype. A surprising finding was that a *Clinostomum* specimen collected from a darter in the St. Lawrence River more than 1,800 km away was only 2 mutational steps from the ancestral haplotype, and was phylogenetically nested among individuals from our study site (Fig. 4). The striking similarity between *C. marginatum* in northeast Oklahoma and Quebec is indicative of long-distance dispersal or range expansion. Lentic fishes, amphibians, snails, and eggs, miracidia, and cercariae of *Clinostomum* spp. probably have limited dispersal capabilities; therefore, local- and broad-scale genetic patterns in *Clinostomum* spp. are likely shaped by their most vagile host, i.e., piscivorous birds.

Multiple divergent copies of mitochondrial genes have been detected within single individuals of several different types of parasitic flatworms (van Herwerden et al., 2000; Le et al., 2002). These have been explained as either nuclear pseudogenes or mitochondrial heteroplasmy. Here, we document the first case of 2 divergent mitochondrial DNA copies (*Co1* haplotypes) from a single metacercaria of *Clinostomum* sp. (C404). One of the haplotypes was identical to the common *C. marginatum* haplotype, while the other was 5.19% divergent from the rest of our *C. marginatum* haplotypes. Given the level of divergence, we suspected that this could be the haplotype of another species of *Clinostomum*, but analysis of the nuclear gene *18S* shows that this specimen was homozygous for alleles of *C. marginatum* and, therefore, was not an F_1 hybrid. The divergent haplotype completely translated with no stop codons or length variation, so there is no direct evidence that it is a nuclear pseudogene. Furthermore, we did not find this copy in any of the other samples that we sequenced, indicating that, if it is a pseudogene, it has limited presence in the nuclear genomes of related individuals. A species-level phylogeny of the genus *Clinostomum* based on mitochondrial DNA (particularly of *Co1*) will be necessary to understand if the putative heteroplasmy is the result of interspecific hybridization.

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