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Infection patterns and molecular data reveal host and tissue specificity of *Posthodiplostomum* species in centrarchid hosts

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Abstract

Posthodiplostomum minimum utilizes a three-host life cycle with multiple developmental stages. The metacercarial stage, commonly known as 'white grub', infects the visceral organs of many freshwater fishes and was historically considered a host generalist due to its limited morphological variation among a wide range of hosts. In this study, infection data and molecular techniques were used to evaluate the host and tissue specificity of *Posthodiplostomum* metacercariae in centrarchid fishes. Eleven centrarchid species from three genera were collected from the Illinois portion of the Ohio River drainage and necropsied. *Posthodiplostomum* infection levels differed significantly by host age, host genera and infection locality. Three *Posthodiplostomum* spp. were identified by DNA sequencing, two of which were relatively common within centrarchid hosts. Both common species were host specialists at the genus level, with one species restricted to *Micropterus* hosts and the other preferentially infecting *Lepomis*. Host specificity is likely dictated by physiological compatibility and deviations from *Lepomis* host specificity may be related to host hybridization. *Posthodiplostomum* species also differed in their utilization of host tissues. Neither common species displayed strong genetic structure over the scale of this study, likely due to their utilization of bird definitive hosts.

Introduction

Host specificity is a measure of the number and phylogenetic diversity of hosts a parasite can infect at a particular stage in its life cycle (Poulin *et al.* 2011). Specialists have high host specificity and are only able to infect one or a few hosts, whereas generalists have low host specificity and infect a broad range of species (Poulin *et al.* 2011). The degree of host specificity a parasite displays has a direct bearing on its ability to utilize different hosts if a preferred host becomes difficult to obtain, as well as the parasite's success in utilizing any single host species (Poulin *et al.* 2011). Host specificity was historically difficult to evaluate in larval helminths due to the inability to accurately identify species using morphological characteristics (De León and Nadler, 2010; Perkins *et al.* 2011). To address this problem, molecular data have been increasingly utilized to obtain accurate identifications of larval parasite species, including trematodes within fish hosts (De León and Nadler, 2010; Locke *et al.* 2010; De León *et al.* 2016; Soldánová *et al.* 2017).

The use of molecular data has led to a better understanding of trematode diversity and life cycles as previously considered conspecific or unknown larval forms are often identified as separate 'cryptic species' and/or linked to other larval and adult forms (Poulin, 2011). Improved species identification reveals potential inaccuracies in our understanding of how trematode species utilize hosts. While molecular data are an increasing component of trematode studies, many are at the taxonomic level and few molecular studies examine large numbers of trematodes from different infection sites within hosts (Locke *et al.* 2015; Blasco-Costa *et al.* 2016). Given the prevalence of cryptic species and that larval trematodes often infect multiple locations within their hosts, current estimates of infection site preferences/specificity are likely inaccurate (Hoffman, 1999; Poulin, 2011). Infection site preference is a key aspect of trematode evolution, transmission, and virulence, and accurately assessing infection locale preferences is necessary for understanding host–parasite interactions and how these parasites impact ecosystems (Locke *et al.* 2010; 2015; Mladineo *et al.* 2010; Herrmann and Poulin, 2011).

Posthodiplostomum minimum is a strigeid digenean trematode which utilizes a complex three-host life cycle (Hoffman, 1999). Adult worms occur in fish-eating birds, cercariae develop in physid snails, and metacercariae, commonly known as white grub, encyst in fish tissues (Spall and Summerfelt, 1969; Hoffman, 1999). White grub has been reported from several tissues of multiple species of freshwater fishes and is a concern for fisheries biologists due to infections causing detrimental effects on host health (Klak, 1940; Hoffman, 1958, 1999; Meade and Bedinger, 1967; Grizzle and Goldsby, 1996; Pracheil and Muzzall, 2010). Two

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subspecies have been generally recognized: *P. minimum minimum* which infects fishes of the family Cyprinidae and *P. minimum centrarchi* which infects fishes of the family Centrarchidae (Hoffman, 1999). Historically, larval *P. minimum* were classified as generalists with low host specificity due to the lack of morphological variation among host species (Hoffman, 1999; Locke *et al.* 2010). Molecular-based studies performed to date have revealed that there are at least eight cryptic species of *Posthodiplostomum* metacercariae infecting freshwater fishes (Moszczynska *et al.* 2009; Locke *et al.* 2010; Stoyanov *et al.* 2017), at least six of which can infect centrarchids (Locke *et al.* 2010). One of these, *Posthodiplostomum* sp. 3, has been described as *P. centrarchi* (Stoyanov *et al.* 2017). While these species did not infect all hosts equally, sample sizes were too small ($n \leqslant 6$ for all parasite species except spp. 3 and 4) to infer host-specificity within Centrarchidae (Locke *et al.* 2010). Based on infections in bluegill (*Lepomis macrochirus*) *vs* white crappie (*Pomoxis annularis*) and literature reviews, Lane *et al.* (2015) suggested that *P. minimum* is more of a host specialist than previously recognized and that *P. m. centrarchi* is a *Lepomis* specialist lacking host preferences outside of the genus. Given the molecular evidence for multiple *Posthodiplostomum* 'minimum' species within centrarchids, we refer to metacercariae in this study as *Posthodiplostomum* and identify specific species when appropriate.

Within fish hosts, *Posthodiplostomum* metacercariae can infect several organs and tissues. Lane *et al.* (2015) detected higher kidney infections in *L. macrochirus* than *P. annularis* and attributed it to potential 'spill over' from saturated livers. Given the evidence for multiple *Posthodiplostomum* species infecting freshwater fishes, it is possible that infection site preferences also differ. For example, Locke *et al.* (2010) recorded different *Posthodiplostomum* spp. from 'viscera' and 'musculature'. Careful examinations of infection sites combined with molecular data are currently needed to determine if infection site preferences differ among *Posthodiplostomum* species.

Given how widespread and common *Posthodiplostomum* infections are within freshwater fishes, their potentially damaging effects on hosts, and the ecological and recreational importance of many of their hosts, it is important to understand the infection dynamics of this species complex parasitizing centrarchid fishes. The purpose of this study was to determine host and tissue specificity of *P. minimum* in 11 species of sympatric centrarchids using necropsy and molecular data. We compared three measures of parasitism (prevalence, intensity and abundance) to determine differences in host infection rates and the structuring of infections within the family Centrarchidae. Molecular data were used to determine the number of *Posthodiplostomum* species present and their distributions within host tissues and confirm host species identifications. In addition, the geographic genetic structure of the two most common *Posthodiplostomum* species was investigated.

Materials and methods

Host collections and measures

Eleven species of centrarchid fishes from three genera were collected from May to October in 2014 and 2015 throughout the Illinois portion of the Ohio River Drainage, including the main channel of the Ohio River and seven of its tributaries (Table 1, Fig. 1). Fish were collected during pre-existing long-term monitoring surveys. Ohio River and Wabash River sites were sampled using pulsed DC electrofishing and collections in the remaining six tributaries used AC electrofishing. Site locations within the Ohio River and the Wabash River were selected using the create random points tool in ArcGIS (ESRI, 2015). All other tributaries were sampled at fixed locations approximately one mile from the confluence with the Ohio River. In the field, fish were identified using morphological characteristics described by Pflieger (1997). All fish were measured and weighed, sacrificed in the field and frozen individually until dissection in the laboratory.

Host samples were allowed to thaw for at least 12 h at 4 °C before dissection. Sagittal otoliths were removed from the neurocranium, cleaned in a deionized water bath, and placed in a 1.5 mL microcentrifuge tube to dry. After a drying period of at least 3 weeks, otoliths were embedded in epoxy and multiple transverse sections were cut from each using a Buehler Isomet® low-speed saw (Buehler Limited, Lake Bluff, Illinois; Quist *et al.* 2012). Cross-sections were placed in immersion oil on a contrasting background and viewed under a stereomicroscope (Leica Microsystems Inc., Buffalo Grove, Illinois). In a blind fashion, two independent readers estimated the age of each fish by counting the number of annuli on each section. Readers resolved discrepancies with a consensus age. Host sex was determined by examination of the gonads. Often sex in juvenile hosts could not be identified, so sex was classified as unknown. Five *Lepomis* hosts (one *L. megalotis*, one *L. cyanellus*, two *L. gulosus* and one *L. macrochirus*) were likely sexually mature (greater than age 1), but due to the status of the reproductive tissue after spawning, sex was unknown.

Posthodiplostomum collections and measures

Visceral organs (heart, kidney, liver and spleen), and tissue next to the neurocranium and the first two vertebrae (head) were removed from the body cavity. Metacercarial cysts from each of the five infection sites were counted by compressing tissues in saline between two slides and viewed with a dissection microscope. Visceral organs >0.10 g were sectioned into multiple sections to ensure visibility. A random subset of metacercriae from each of the infected anatomical locations was placed in a small dissection dish with saline where they were released from their cysts and then stored in 70% ETOH for DNA analyses. Dissection equipment was cleaned between organ necropsies to prevent contamination of genetic material.

Prevalence (percentage of hosts infected with *Posthodiplostomum* at any locality), mean abundance (average number of *Posthodiplostomum* metacercariae in all hosts, uninfected and infected) and mean intensity (average number of *Posthodiplostomum* metacercariae per infected host) were calculated for each fish host, following Bush *et al.* (1997). Due to small sample sizes in some host species, infection analyses were performed at the taxonomic level of genus. Overall prevalence (*Posthodiplostomum* at any locality) and tissue-specific prevalence were analysed using logistic modelling and chi-square analyses with host genera and host age as factors. *Pomoxis* hosts were excluded from all remaining analyses due to the absence of infected hosts. Intensity data were $\log_{10}$ transformed to meet the assumption of normality and analysed using an ANOVA with Tukey's *post hoc* analyses with host genera and infection locality as factors. To visualize the structure of infections within each host genera, relative abundance within each infection locality was plotted for each host using the Bray–Curtis distance metric with non-metric multidimensional scaling (NMDS) in the metaMDS function. Differences in the structure of *Posthodiplostomum* infections between host genera were quantified using a permutational MANOVA in the *Adonis* function. All analyses were conducted in R with an α-value of 0.05 (R Development Core Team, 2016).

Molecular methods

Genetic confirmation of host fish species whose *Posthodiplostomum* were used for molecular analyses was conducted by extracting

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Table 1. Common name, scientific name, sample size, range of host total length (mm), range of host age (year) and host sex (F – female, M – male, U – unknown) for the 11 centrarchid species collected from the Illinois portion of the Ohio River drainage

Species common name	Scientific name	Host *n*	Host TL range (mm)	Host age range (year)	Host sex
Lepomis spp.					
Bluegill	*L. macrochirus*	44	35–164	0–3	F – 8, M – 17, U – 19
Green sunfish	*L. cyanellus*	26	51–110	0–2	F – 5, M – 3, U – 18
Longear sunfish	*L. megalotis*	50	58–140	0–3	F – 14, M – 18, U – 18
Orangespotted sunfish	*L. humilis*	36	41–82	0–2	F – 1, M – 1, U – 34
Redear sunfish	*L. microlophus*	17	71–186	0–3	F – 6, M – 9, U – 2
Warmouth	*L. gulosus*	13	71–135	0–3	F – 3, M – 5, U – 5
Micropterus spp.					
Largemouth bass	*M. salmoides*	18	80–308	0–4	F – 4, M – 10, U – 4
Smallmouth bass	*M. dolomieu*	8	84–144	0–2	F – 0, M – 6, U – 2
Spotted bass	*M. punctulatus*	126	34–399	0–7	F – 52, M – 53, U – 21
Pomoxis spp.					
Black crappie	*P. nigromaculatus*	15	69–316	0–3	F – 4, M – 9, U – 2
White crappie	*P. annularis*	6	71–200	0–2	F – 3, M – 0, U – 3

DNA from approximately 0.5 cm^2 of host fin tissue and amplifying a portion of the cytochrome *c* oxidase subunit I (COI) barcode gene using the PCR primers VF2_t1 and FR1d_t1 for *Lepomis* or FishF2-t1 and FishR2_t1 for *Micropterus* and *Pomoxis* (modified from Walsh *et al.* 1991; Ivanova *et al.* 2007) (see Supplementary File 1 for details of molecular methods). To further investigate potential *Micropterus punctulatus* misidentification, one additional mitochondrial gene, NADH dehydrogenase subunit 2 (ND2), and two nuclear regions: internal transcribed spacer second intron (ITS2) and fourth intron of the calmodulin gene (CaM) were analysed following Breden *et al.* (1999), Presa *et al.* (2002) and Chow and Takeyama, (2000), respectively. Purified PCR products were sequenced using PCR primers at the DNA Analysis Facility at Yale University. NCBI BLASTN searches were conducted to determine if morphological identifications of hosts matched resources available in GenBank.

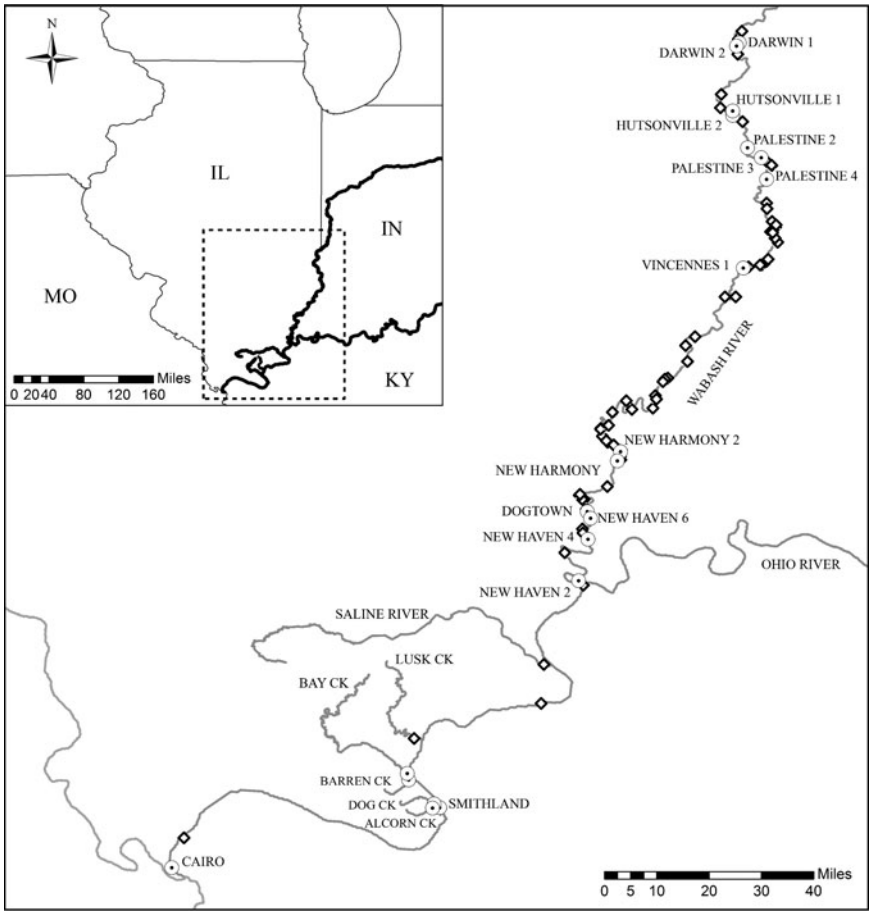

Fig. 1. Map of the Illinois portion of the Ohio River drainage with sampling localities. Labelled sampling localities denoted by a white circle with a black dot on the map are locations where *Posthodiplostomum* metacercariae were utilized for genetic analyses. Unlabelled sites denoted by a diamond are locations where hosts were collected and necropsied for infection analyses only.

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Posthodiplostomum metacercariae were selected for DNA analyses based on host species and infection site. Our goal was to analyse several metacercariae from each host and infection site, when possible. We were not able to identify *Posthodiplostomum* species prior to DNA analyses. DNA was extracted from individual metacercariae and a portion of the COI gene was amplified using the forward primer Plat-diploCOX1F with either the reverse primer Plat-diploCOX1R (Moszczynska *et al.* 2009) or the reverse primer RevComp-JB3: 5′-ATAAACCTCAGGATGCCCAAAAAA-3′ (Keeney unpublished, the reverse complement of primer JB3, Bowles *et al.* 1995). In addition, the ribosomal internal transcribed spacer region 1 (ITS1) was amplified from a subset of white grub representing all major COI clades using the primers BD1 and 4S (Bowles and McManus, 1993).

Clustal W (Thompson *et al.* 1994) as implemented in MEGA7 (Kumar *et al.* 2016) was used to align *Posthodiplostomum* DNA sequences for both COI and ITS1. NCBI BLASTN searches were conducted to determine if confirmation of hosts and identification of *Posthodiplostomum* species were possible based on resources available in GenBank. Bayesian phylogenetic analyses were conducted for COI and ITS1 separately using MrBayes 3.2 (Ronquist *et al.* 2012). Maximum-likelihood analyses were conducted for COI and ITS1 separately using MEGA7 (Kumar *et al.* 2016). For each analysis, 1000 bootstrap replicates were conducted and phylogenetic trees were visualized using FigTree v1.3.1 (Rambaut, 2009). Sequences from previously identified *Posthodiplostomum* species that most closely matched those in the present study were included in phylogenetic analyses for species identification (see Supplementary File 1 for sequence and outgroup information). Uncorrected *p* distances were calculated within and among major *Posthodiplostomum* clades using MEGA7 with all insertion/deletions treated as single nucleotide differences for ITS1.

Genetic population structure was examined with COI haplotypes for *Posthodiplostomum* spp. 3 and 8 using Bayesian clustering and Analysis of Molecular Variance (AMOVA) methods. Spatial Bayesian clustering of individual haplotypes was performed using BAPS v6.0 (Corander *et al.* 2003; Cheng *et al.* 2013) with the estimated number of populations (*k*) allowed to vary from 1 to 20. AMOVA analyses at the sample site level were performed using Arlequin v3.5.2.2 (Excoffier and Lischer, 2010) and incorporated the most appropriate model of sequence evolution available (TN93 + G, with gamma shape parameter = 0.22). Because clonal replicates from the same first intermediate host can accumulate within second intermediate host fish, additional AMOVA analyses were performed with identical haplotypes from the same host fish removed. While identical COI haplotypes do not necessarily indicate identical genetic clones, comparison of results with and without within-host identical haplotypes provides insight as to whether or not any genetic differentiation is influenced by identical haplotypes within hosts.

Results

Host demographics

We collected and necropsied a total of 359 centrarchids from three genera and 11 species. This included 186 *Lepomis* hosts, 152 *Micropterus* hosts and 21 *Pomoxis* hosts. Sample sizes and demographic characteristics of the host species are in Table 1. Age structures were similar among species, with 91% of the hosts being younger than age 3. Largemouth bass and spotted bass were the only two species with age estimates equal to or greater than age 4, but these cohorts only accounted for 2% of the host sample. Because 34% of the hosts were young of year, sex was classified as unknown for 128 of 359 hosts.

Posthodiplostomum infections

A total of approximately 32 000 *Posthodiplostomum* metacercariae were recovered. Prevalence, mean intensity ± S.E., and infection localities for each host species are found in Table 2. Prevalence was greater than 55% for all host species, except black crappie, green sunfish and white crappie. Total mean intensity exceeded 150 in bluegill, largemouth bass and spotted bass, but was <15 for all remaining host species. Metacercariae were recovered from all five tissues in bluegill, largemouth bass, longear sunfish, orangespotted sunfish, redear sunfish and spotted bass. Black crappie, green sunfish and smallmouth bass were the only species that were not infected in all localities. The liver was the only infection site that was infected in all species. Metacercariae were not found in the mesentery surrounding the gonads or the gastrointestinal tract in any of the examined hosts.

Overall prevalence varied with host genera ($\chi^2 = 48.80$; df = 2, 358; $P < 0.001$) and host age ($\chi^2 = 105.63$, df = 5, 358 $P < 0.001$). There was no significant genus × age interaction effect in the model. Overall prevalence was higher in *Micropterus* and *Lepomis* hosts compared with *Pomoxis* hosts. Only 5% (1/21) of all *Pomoxis* hosts were infected with *Posthodiplostomum*, whereas the other two genera displayed a prevalence >65%. Host age had a significant effect on *Posthodiplostomum* prevalence, with younger fishes less likely to be infected. Of all uninfected hosts, 86% were young of year or yearlings. Conversely, <5% of fish from all age classes greater than age 2 were uninfected. Similar to the first logistic model, tissue prevalence varied significantly by host genera ($\chi^2 = 58.04$; df = 1, 1689; $P < 0.001$) and infection locality ($\chi^2 = 149.89$; df = 4, 1689; $P < 0.001$). There was a significant genus × locality interaction effect in the model ($\chi^2 = 68.50$; df = 4, 1689; $P < 0.001$). The liver displayed the highest prevalence of all infection localities, followed by the heart, then the kidney, then the head, and finally the spleen. *Micropterus* hosts displayed higher prevalences in all sites except the heart when compared with *Lepomis* hosts.

Host genus and host tissue also had significant effects on transformed intensity data (Genera: $F_{1,674} = 62.90$, $P < 0.001$; Locality: $F_{4,674} = 9.34$, $P < 0.001$). There was a significant genus × locality

Table 2. Prevalence, mean intensity ± standard error and infection localities of *Posthodiplostomum* from 11 centrarchid species from the Illinois portion of the Ohio River Drainage.

Host species	% Prevalence	Mean intensity ± S.E.	Infection localities
Lepomis spp.			
L. macrochirus	86	156.61 ± 48.80	L, K, H, HD, S
L. cyanellus	15	1.25 ± 0.25	L, H
L. megalotis	62	12.42 ± 3.96	L, K, H, HD, S
L. humilis	56	6.00 ± 2.15	L, K, H, HD, S
L. microlophus	100	8.35 ± 2.79	L, K, H, HD, S
L. gulosus	100	12.92 ± 2.94	L, K, H, HD
Micropterus spp.			
M. salmoides	89	152.00 ± 75.00	L, K, H, HD, S
M. dolomieu	63	2.20 ± 0.49	L, H, HD
M. punctulatus	79	231.34 ± 46.58	L, K, H, HD, S
Pomoxis spp.			
P. nigromaculatus	7	1.00 ± 0	L
P.annularis	0	NA	NA

L, liver; K, kidney; H, heart; HD, head; S, spleen.

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interaction effect in the model ($F_{4,674} = 11.52$, $P < 0.001$). Overall, *Micropterus* hosts were infected with higher total intensities than *Lepomis* hosts. *Micropterus* hosts displayed significantly higher mean intensities in the head (*Micropterus* – 112.38 ± 21.84, *Lepomis* – 6.97 ± 1.84), liver (*Micropterus* – 99.42 ± 18.44, *Lepomis* – 27.88 ± 8.12) and spleen (*Micropterus* – 21.58 ± 4.08, *Lepomis* – 2.61 ± 0.66) than *Lepomis* hosts. Conversely, mean intensities in the heart (*Micropterus* – 9.08 ± 2.33, *Lepomis* – 14.90 ± 3.63) and kidney (*Micropterus* – 30.84 ± 6.24, *Lepomis* – 38.75 ± 13.47) were higher in *Lepomis* hosts than *Micropterus* hosts, but differences were not significant in *post hoc* analyses.

The structure of *Posthodiplostomum* infections varied with host genus. Host genus explained 8.0% of the variation in the dataset ($F_{1,242} = 20.92$, $P < 0.001$). In the NMDS plot, *Micropterus* and *Lepomis* clusters separated spatially indicating different *Posthodiplostomum* infection structures within each host genera (Fig. 2). The majority of *Micropterus* infections were characterized by infections in the spleen and head, while the majority of *Lepomis* infections were characterized by infections in the kidney and heart. Few infections were uniquely identified by infections in the liver because this was the most commonly infected locality in both genera.

Host species genetic confirmation

COI sequences of host fish supported morphological identifications for all *Lepomis* species, *Pomoxis nigromaculatus*, *Micropterus salmoides* and *Micropterus dolomieu* (99–100% similarity). Three out of four *M. punctulatus* COI sequences showed the highest similarity to *M. salmoides* (100% similarity; next closest species 97% similarity; *M. cf. punctulatus* 95% similarity), and one individual showed potential heteroplasmy for *M. salmoides* and *M. punctulatus*/*dolomieu* COI haplotypes by having double peaks for the appropriate nucleotides at all sites distinguishing these species. Identical *M. punctulatus* results were obtained with ND2. Three out of four *M. punctulatus* matched *M. salmoides* with 100% similarity and several *Micropterus* species with 99% similarity for ITS2 and the fourth *M. punctulatus* was heterozygous at four of the 11 nucleotides that differed between our *M. salmoides* and *M. dolomieu*. Per cent similarities cannot be accurately assessed for this individual without haplotype information for the mutations. The additional nuclear genes lacked divergence among species. Taken together, these results support the identification of all host species, with the possible exception of *M. punctulatus*. Given the presence of tooth patches on their tongues and mitochondrial association with *M. salmoides*, the *M. punctulatus* in our study are potentially hybrids (Godbout *et al.* 2009).

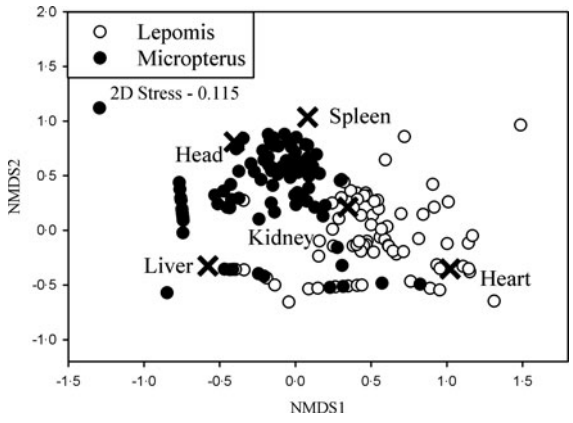

Fig. 2. NMDS plot of relative *Posthodiplostomum* abundances plotted by the five infection localities and separated by host genera. Organ scores (indicated by an ×) represent the influence of infection locality on the ordination.

Posthodiplostomum *species identification and genetic structure*

Posthodiplostomum from a total of 34 host fish representing 10 host species were sampled for DNA sequencing. One to 13 individual *Posthodiplostomum* were analysed from each fish and parasites were analysed from one to five infection sites per fish species (Table 3). A portion of the COI up to 514 nucleotides long was sequenced for 148 *Posthodiplostomum* metacercariae, producing 87 unique COI haplotypes (GenBank Accession #s MG873355-MG873441). Maximum-likelihood and Bayesian analyses produced identical tree topologies with three relatively well-supported clades. Initial NCBI Blast results for COI identified members of the three clades as belonging to species *Posthodiplostomum* sp. 3 (100% similarity), sp. 2 (100% similarity) and sp. 8 (99% similarity) and were supported by phylogenetic analyses (Fig. 3). While divergence within species was typically minimal, it is noteworthy that the single individual analysed from *Pomoxis* was divergent from sp. 2 recovered from *Lepomis* (denoted with * on Fig. 3). An approximately 680 bp sequence including portions of the ITS1 region and 5.8S rRNA was analysed from 20 *Posthodiplostomum* representing the major clades identified with COI, producing 10 different sequences (GenBank accession #s MG857103-MG857112). For ITS1, maximum-likelihood and Bayesian tree topologies were identical, resolving three clades. These clades matched those found for COI. Support for each clade was relatively high, with the exception of Bayesian support for the 'species 2' clade (0.58). This clade was well supported with maximum-likelihood analysis (99% bootstrap support). The relationship between sp. 2 and 8 was not well supported with maximum-likelihood analyses for COI and ITS1 and with Bayesian analysis for ITS1 (Figs. 3 & 4). Pairwise *p*-distances ranged from 0.00 to 3.91% for COI and 0.00 to 0.44% for ITS1 within species and 15.22 to 19.57% for COI and 1.63 to 3.55% for ITS1 among species (Table 4).

The optimal number of spatial clusters recovered by BAPS was $k = 1$ for *Posthodiplostomum* species 3 and 8, suggesting a lack of geographic structure. AMOVA analyses at the sample site level including all haplotypes produced $\Phi_{ST} = 0.060$, $P = 0.042$ and $\Phi_{ST} = 0.003$, $P = 0.409$ for species 3 and 8, respectively. Removal of identical haplotypes within the same host fish ($n = 13$) produced $\Phi_{ST} = 0.029$, $P = 0.206$ and $\Phi_{ST} = -0.021$, $P = 0.658$ for species 3 and 8, respectively.

Host and infection site distribution

Out of the 148 *Posthodiplostomum* analysed, 82 were species 3, 61 were species 8, and 5 were species 2. All 61 *Posthodiplostomum* sp. 8 were recovered from *Micropterus* hosts and all three *Micropterus* species were infected with this parasite (Table 3). Seventy-six (93%) of the *Posthodiplostomum* sp. 3 analysed were from *Lepomis* hosts and six (7%) were recovered from *M. punctulatus*. All six *Lepomis* species examined were infected with *Posthodiplostomum* sp. 3. Four (80%) out of the five *Posthodiplostomum* sp. 2 were from *Lepomis* hosts and one was found in *Pomoxis nigromaculatus*. Looking at hosts, 95% of the parasites analysed from *Lepomis* were species 3 and 5% were species 2, 91% of the parasites analysed from *Micropterus* were species 8 and 9% were species 3 (all from *M. punctulatus*) and the only white grub recovered from *P. nigromaculatus* was a single species 2. *Posthodiplostomum* sp. 3 was recovered from all five tissues sampled but was rare in the spleen and the head. It was common in the heart (Table 3). *Posthodiplostomum* sp. 8 was recovered in approximately equal numbers from all infection sites except the heart and was common in the head (Table 3).

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Table 3. Host species and *Posthodiplostomum* sampled for genetic analyses

Host species	Host *n*	Sample site(s)	Infection site	*Posthodiplostomum* species
L. macrochirus	3	Darwin 2	Head	sp. 3 (*n* = 2)
		Vincennes 1	Heart	sp. 3 (*n* = 2)
		New Haven 2	Kidney	sp. 3 (*n* = 2)
			Liver	sp. 3 (*n* = 5)
			Spleen	–
L. cyanellus	4	New Haven 2	Head	–
		Palestine 2	Heart	sp. 3 (*n* = 1)
		Cairo	Kidney	–
			Liver	sp. 2 (*n* = 2), sp. 3 (*n* = 1)
			Spleen	–
L. megalotis	2	Darwin 2	Head	sp. 3 (*n* = 2)
		Alcorn Ck.	Heart	sp. 3 (*n* = 3)
			Kidney	sp. 3 (*n* = 2)
			Liver	sp. 3 (*n* = 4)
			Spleen	sp. 3 (*n* = 2)
L. humilis	3	New Harmony	Head	–
		Palestine 4	Heart	sp. 3 (*n* = 6)
		Dog Ck.	Kidney	sp. 2 (*n* = 1)
			Liver	sp. 2 (*n* = 1), sp. 3 (*n* = 6)
			Spleen	–
L. microlophus	4	Dogtown	Head	sp. 3 (*n* = 2)
		Alcorn Ck.	Heart	sp. 3 (*n* = 6)
			Kidney	sp. 3 (*n* = 6)
			Liver	sp. 3 (*n* = 7)
			Spleen	–
L. gulosus	6	New Haven 6	Head	–
		Alcorn Ck.	Heart	sp. 3 (*n* = 11)
		Barren Ck.	Kidney	sp. 3 (*n* = 4)
			Liver	sp. 3 (*n* = 1)
			Spleen	–
M. salmoides	3	New Haven 4	Head	sp. 8 (*n* = 7)
		Smithland	Heart	–
			Kidney	sp. 8 (*n* = 5)
			Liver	sp. 8 (*n* = 6)
			Spleen	sp. 8 (*n* = 7)
M. dolomieu	4	Palestine 2	Head	sp. 8 (*n* = 1)
		Palestine 3	Heart	–
		Palestine 4	Kidney	–
			Liver	sp. 8 (*n* = 5)
			Spleen	–
M. punctulatus	4	Darwin 1	Head	sp. 8 (*n* = 10)
		New Harmony 2	Heart	sp. 3 (*n* = 2)
		Hutsonville 1	Kidney	sp. 8 (*n* = 7), sp. 3 (*n* = 2)
		Hutsonville 2	Liver	sp. 8 (*n* = 6), sp. 3 (*n* = 3)
			Spleen	sp. 8 (*n* = 7)
P. nigromaculatus	1	Bay Ck.	Head	–

(*Continued*)

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Table 3. (*Continued.*)

Host species	Host *n*	Sample site(s)	Infection site	*Posthodiplostomum* species
			Heart	–
			Kidney	–
			Liver	sp. 2 (*n* = 1)
			Spleen	–

The number of hosts sampled (Host *n*), sample site, sites of infection, and *Posthodiplostomum* analysed from each infection site are listed for each host species. Sample sites refer to Fig. 1.

Four of the five *Posthodiplostomum* sp. 2 were found in the liver and one was recovered from the kidney.

Discussion

We combined infection data and molecular species identification to determine the diversity, host specificity and tissue site specificity of *Posthodiplostomum* species in centrarchid fishes from the Ohio River drainage. Prevalence of *Posthodiplostomum* infection was relatively high (>50%) in all species examined, except *L. cyanellus* and the two *Pomoxis* species. It is unclear whether the low prevalence in *Pomoxis* was due to host specificity or small sample size. Lane *et al.* (2015) reported >50% prevalence of white grub in *P. annuaris* from a eutrophic lake, but this

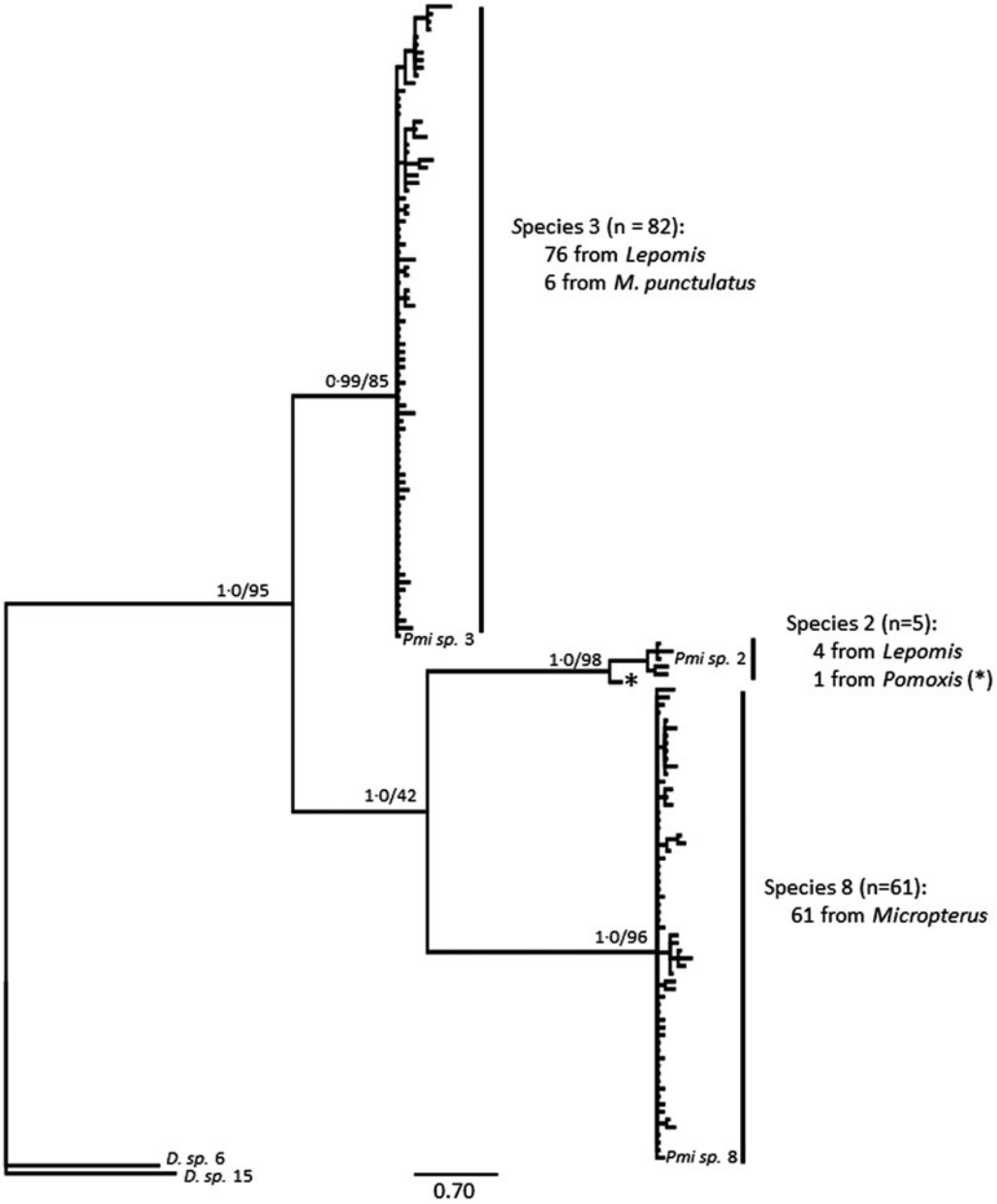

Fig. 3. Bayesian topology of phylogenetic relationships of *Posthodiplostomum* COI sequences. Nodal support is Bayesian support values/maximum-likelihood bootstrap values. Outgroups are *Diplostomum* spp. 6 (GenBank Accession # KX037901.1) and 15 (# KR271125.1), and *Posthodisplostomum* sp. 2 (# HM064797.1), sp. 3 (# HM064800.1) and sp. 8 (# HM064876.1) are included as references. The *Posthodiplostomum* sp. 2 recovered from *Pomoxis nigromaculatus* is indicated with an '*'.

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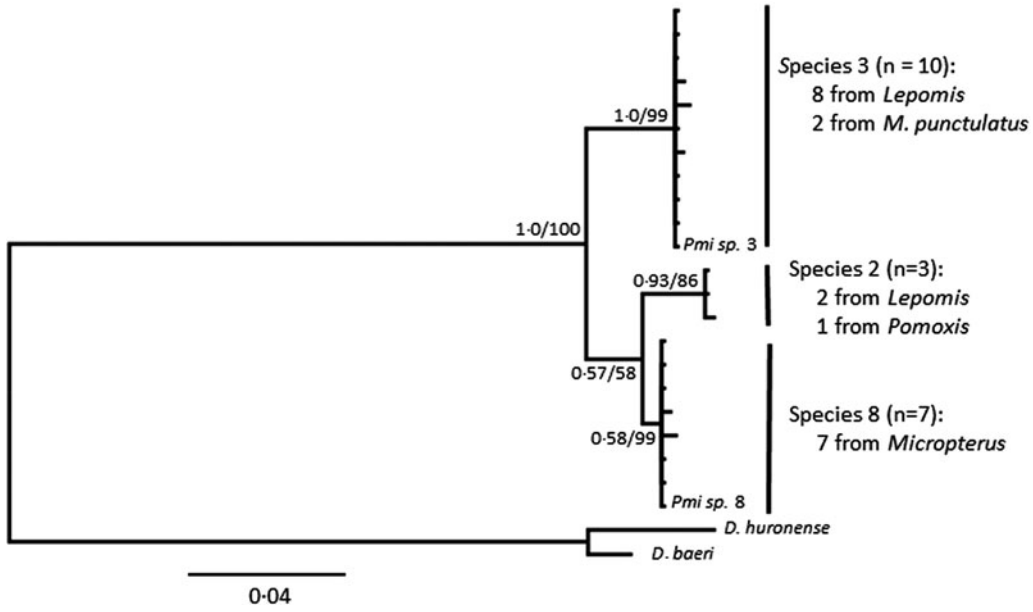

Fig. 4. Bayesian topology of phylogenetic relationships of *Posthodiplostomum* ITS1 sequences. Nodal support is Bayesian support values/maximum-likelihood bootstrap values. Outgroups are *Diplostomum huronese* (GenBank Accession # AY123044.1) and *D. baeri* (# AY123042.1) and *Posthodisplostomum* sp. 3 (# HM064951.1) and sp. 8 (# HM064962.1) are included as references.

species was rare in the lotic habitats sampled in this study. *Lepomis cyanellus* was common in our study system but prevalence was much lower than in other *Lepomis* species, implying a species-specific effect. This pattern was consistent with the summary of Lane *et al.* (2015) and findings from our studies in the Sangamon River in Illinois in which prevalence in bluegill (*L. macrochirus*) was over 90% but *L. cyanellus* were rarely infected (Boone, unpublished data).

Highest mean intensities were seen in *L. macrochirus*, *M. salmoides* and *M. punctulatus* (>150 *vs.* <15 in all other hosts). Differences in intensity between *Lepomis* and *Micropterus* are likely influenced by differences in age structures. *Micropterus* hosts displayed higher mean intensities in the liver, head and spleen compared with *Lepomis* spp., which is likely explained by the older age structure and larger organs in the *Micropterus* host sample. However, despite having smaller visceral organs and a younger age structure, *Lepomis* hosts displayed larger mean intensities in the heart and the kidney compared with *Micropterus* spp., suggesting genera-specific infection preferences.

Prevalence increased with fish host age, consistent with older fish having greater opportunities to encounter cercariae and long-lived infections (Hoffman, 1958). Fish size may have been a factor, but was not included in our model (Lane *et al.* 2015). *Micropterus salmoides* and *M. punctulatus* samples contained older fish than *M. dolomieu* but >90% of individuals from all species were less than 3. High prevalence but very low mean intensity in *M. dolomieu* is consistent with comparable exposure rates among *Micropterus* species, at least early in life, but differential levels of resistance. Although the sample size was small, it is possible that *M. dolomieu* mounts an effective immune response that can prevent superinfections but does not eliminate encysted metacercariae. Likewise, the higher mean intensity in *L. macrochirus* was not due to age differences among *Lepomis* hosts. In fact, both *L. microlophus* and *L. gulosus* had higher prevalence values than *L. macrochirus*, but much lower mean intensity. High prevalence in *L. microlophus* is consistent with their feeding extensively on snails (Pflieger, 1997) creating high exposure rates to free-swimming ceracariae, but low mean intensity argues for resistance in this host as well. Infection rates in *L. gulosus* are not as easily explained by diet influencing exposure. We do not know when these fish were infected, so they may be infected at a high rate when young and then not accumulate additional parasites. Overall, our findings confirm that *Posthodiplostomum* is quite successful at parasitizing *Lepomis* and *Micropterus* in our study area. Differential levels of infection among fish hosts suggest that *Posthodiplostomum* varies in its ability to parasitize individual host species within each genus and/or fish hosts vary in their immunologic resistance (Poulin *et al.* 2011).

Table 4. COI and ITS1 *p*-distance values (%) within (diagonal) and among *Posthodiplostomum* species

	sp. 2	sp. 3	sp. 8
COI			
sp. 2	0.43–2.17		
sp. 3	15.22–18.26	0.00–3.91	
sp. 8	16.52–19.57	15.22–19.13	0.00–3.48
ITS1			
sp. 2	0.00–0.15		
sp. 3	3.11–3.55	0.00–0.44	
sp. 8	1.63–2.07	2.37–2.96	0.00–0.44

Molecular analyses of parasites identified three species separated by a minimum of 15% COI divergence corresponding to *Posthodiplostomum* spp. 2, 3 and 8 (Vilas *et al.* 2005; Moszczynska *et al.* 2009; Locke *et al.* 2010; De León *et al.* 2016). While we only examined a subset of the metacercariae utilized for prevalence analyses, strong patterns emerged that likely reflect general trends. *Posthodiplostomum* sp. 3 and 8 are specialists for the host genera *Lepomis* and *Micropterus*, respectively, but infect multiple species within each genus. Despite limited sample sizes, Locke *et al.* (2010) also recovered these *Posthodiplostomum* species from the same host genera further supporting the high degree of host specificity detected. They also recovered sp. 3 from *Ambloplites rupestris*, demonstrating that this species can infect additional host genera. This species appears to specialize on *Lepomis* in our study region, but may utilize other hosts throughout its range

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and may use centrarchid hosts that were not included in our study (bantam sunfish *Lepomis symmetricus*, flier *Centrarchus macropterus*, pumpkinseed *Lepomis gibbosus*). Its presence in *A. rupestris* (Locke *et al.* 2010) and exclusion from *Pomoxis* in the present study suggests that this species' host-specificity does not reflect host phylogeny (Near *et al.* 2005); a pattern consistent with the literature (Lane *et al.* 2015). As parasites can utilize different hosts throughout their range (Hoberg and Brooks, 2008; Lane *et al.* 2015), a more thorough geographic investigation of host utilization by *Posthodiplostomum* spp. is warranted. However, it is clear in our study that species 3 utilizes *Lepomis* hosts preferentially over *Micropterus* hosts.

The only non-*Lepomis* host species infected with *Posthodiplostomum* species 3 in our study was *M. punctulatus*. Despite sequencing a small subset of white grub, this species was identified from three out of the four *M. punctulatus* examined. These were also the only hosts whose species identification was not positively supported with genetic data. These individuals possessed tooth patches on their tongues and lower jaw lines not extending past their eyes, characters typical of *M. punctulatus* and hybrids, but rare in *M. salmoides* (Godbout *et al.* 2009). The presence of these characters and genetic identification of likely *M. salmoides* maternal ancestry and ambiguous paternal ancestry suggest that these fish are *M. salmoides* × *M. punctulatus* hybrids. If these were misidentified *M. salmoides*, it would be an exceptional coincidence that they are the only *M. salmoides* from which species 3 was recovered. Hybridization in fishes can decrease host specificity by potentially altering immune mechanisms specific to each parental strain (Šimková *et al.* 2013). In our system, hybridization may have allowed for parasitism by species that are not common in at least one of the host species (*M. salmoides*) and potentially both given its overall lack in *Micropterus*. Ecological differences could also produce differences in parasite–host specificity between *M. punctulatus* and other *Micropterus* (Dupont and Crivelli, 1988; Le Brun *et al.* 1992). If *M. punctulatus* utilize different habitats, prey on different organisms, etc. that are more similar to *Lepomis* spp., they could be more likely to be exposed to *Posthodiplostomum* sp. 3. However, given that both *M. punctulatus* and *M. salmoides* are ecologically similar and often co-occur (Godbout *et al.* 2009), it is likely that they are exposed to similar parasites, supporting an altered host physiology. Host-specificity of Diplostomatid metacercariae in fishes is the result of physiological compatibility restraints between hosts and parasites and there is growing support for the importance of this in other fish metacercariae (Locke *et al.* 2010; De León *et al.* 2016).

A single species 2 was the only white grub individual recovered from *Pomoxis*, with the remaining four individuals infecting *Lepomis* spp. It should be noted that two of the four metacercariae sequenced from *L. cyanellus* were also species 2 rather than the *Lepomis* specialist species 3 that dominated all other *Lepomis* infections. Species 2 may be a generalist but rare in our geographic area. Alternatively, it may be a *Pomoxis* parasite but not well adapted for transmission in a lotic system. Metacercariae species were not determined in the study by Lane *et al.* (2015), so we cannot compare with lentic systems.

Differences in infection site specificity were observed between the heavily infected host genera *Micropterus* and *Lepomis* and are consistent with the detection of different species being common in each genus. While the liver was heavily infected in both genera, white grub from *Micropterus* were more common in the spleen and head while *Lepomis* infections were more common in the kidney and heart. Therefore, both species 3 and 8 utilize the liver but differ in their utilization of other host tissues. *Posthodiplostomum* '*minimum*' infect their host by burrowing through the skin. Once in the circulatory system, they preferentially travel to the liver and potentially utilize other organs as the liver becomes heavily parasitized (Hoffman, 1958; Lane *et al.* 2015). Our data suggest that since each species would have equal access to host tissues, infection site differences may be based on some undetermined factors related to *Posthodiplostomum* species preferences and/or host-specific tissue susceptibility differences between *Micropterus* and *Lepomis*. Different *Posthodiplostomum* species often utilize different tissues within their hosts (Hoffman, 1999; Kvach *et al.* 2017).

We did not detect strong evidence of genetic structure among geographic locations with either of the two common *Posthodiplostomum* species. This is not surprising given the relatively small geographic scale of our study and the utilization of avian definitive hosts by *Posthodiplostomum* spp. Trematodes typically lack innate mechanisms for distant geographic dispersal but can be dispersed by their hosts. Often, the most vagile host utilized will determine the extent of a trematode's genetic structure (Blasco-Costa and Poulin, 2013). While initial sample site level AMOVA results for species 3 did suggest a very low but significant level of genetic differences ($P = 0.042$), results contradicted Bayesian analyses and genetic differences were not detectable when identical haplotypes were removed from individual hosts. *Posthodiplostomum* reproduces asexually within snail hosts producing large numbers of genetically identical cercariae (Hoffman, 1958; Lane *et al.* 2015). While second intermediate fish are likely accumulating different cercariae from different snail hosts, some may occasionally be infected by multiple identical clones from a single snail as has been detected in other trematode second intermediate hosts (Rauch *et al.* 2005; Keeney *et al.* 2007). Their inclusion provides an incorrect estimate of the degree of genetic differences among sites. However, identical haplotypes are not necessarily genetic clones and species 3 did show larger Φ_{ST} value than species 8 after removal of identical haplotypes. Having now identified the host and tissue specificity of these species, further work focusing on their population genetics could reveal that differences in life histories, such as utilization of different hosts, effective population sizes, etc., are influencing their evolution.

In conclusion, our study contributes to the growing body of evidence that *Posthodiplostomum* infecting centrarchid fishes are a complex of several species. We have provided direct evidence that two different species are common in the genera *Lepomis* and *Micropterus* and utilize different tissues within these hosts. Utilization of a single host species in the non-targeted genus by one *Posthodiplostomum* species may be an example of a paratenic host or the result of host hybridization altering infection dynamics. Neither of the common *Posthodiplostomum* species displayed strong genetic structure likely due to their use of vagile bird hosts and the small geographic scale of our study, but differences may exist between them. Data from additional *Posthodiplostomum* species and study regions will shed further light on the transmission dynamics of this common and economically important species complex.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018000306

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