

# Cryptic speciation and patterns of phenotypic variation of a highly variable acanthocephalan parasite

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## Abstract

**An investigation of a parasite species that is broadly host- and habitat-specific and exhibits alternative transmission strategies was undertaken to examine intraspecific variability and if it can be attributed to cryptic speciation or environmentally induced plasticity. Specimens of an acanthocephalan parasite, *Leptorhynchoides thecatus*, collected throughout North America were analysed phylogenetically using sequences of the cytochrome oxidase I gene and the internal transcribed spacer region. Variation in host use, habitat use, and transmission were examined in a phylogenetic context to determine if they were more likely phylogenetically based or due to environmental influences. Results indicated that most of the variation detected can be explained by the presence of cryptic species. The majority of these species have narrow host and microhabitat specificities although one species, which also may comprise a complex of species, exhibits broad host and habitat specificity. Alternate transmission pathways only occurred in two of the cryptic species and correlate with host use patterns. Taxa that mature in piscivorous piscine hosts use a paratenic fish host to bridge the trophic gap between their amphipod intermediate host and piscivorous definitive host. One potential example of environmentally induced variation was identified in three populations of these parasites, which differ on their abilities to infect different host species.**

*Keywords:* Acanthocephala, cryptic speciation, host specificity, microhabitat specificity, parasite, phenotypic plasticity, transmission

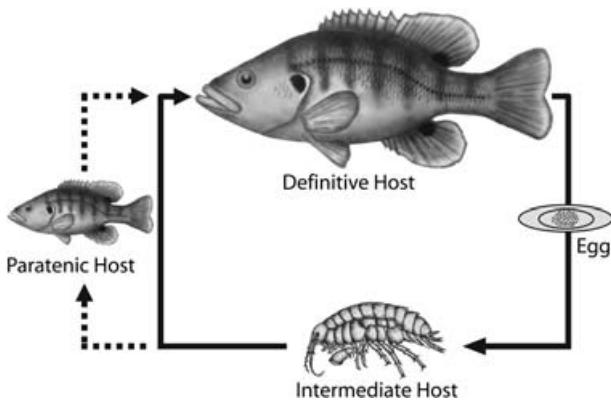
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Genetic studies in almost all taxonomic groups have contributed to the discovery of evolutionarily isolated lineages that were not apparent on the basis of purely morphological features. Cryptic species have been detected in even the most intensely studied vertebrate and insect taxa (e.g. Avise & Walker 1999; Hebert *et al.* 2004). We are only beginning to understand the cryptic structure of multicellular species complexes in organisms that do not exhibit obvious taxonomic features, especially for groups such as fungi (Le Gac *et al.* 2007) and parasites in general (Schmidt 1969; Jousson *et al.* 2000). Intraspecific variation in parasitic organisms often can be attributed to strain formation or host-race formation in which the strains or races diverge as an adaptation to ecological conditions or various host species. However, variation can also be attributed to differences in environmental conditions.

For instance, development, morphology, and infection capability of parasites can vary according to the species of host inhabited and its nutrition status (Haley 1962; Watertor 1967; Manter 1969; Buckner & Nickol 1975; Crompton *et al.* 1982; Mayr & Ashlock 1991; Neves *et al.* 2004; Lambrechts *et al.* 2006). Also, transmission pathways of parasites can be plastic (Vizoso & Ebert 2005), and can depend on chemical cues from host species present in the environment (Lagrue & Poulin 2007) or on the growth rate of the host (Kaltz & Koella 2003). Additionally, plasticity in virulence of a parasite can be influenced by environmental temperature (Mitchell *et al.* 2005).

*Leptorhynchoides thecatus* (Linton, 1891) Kostylew, 1924, is an acanthocephalan (spiny-headed worm) that parasitizes fishes throughout eastern North America. This species has been described as morphologically variable (Lincicome & Van Cleave 1949b) with a broad range of host species, broad microhabitat specificity within the host, and a life cycle with variable transmission pathways. However, a meta-analysis of the literature indicated that these traits

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**Fig. 1** Alternative transmission pathways of a trophically transmitted parasite. Obligate pathway is indicated by solid lines. An amphipod becomes infected by ingesting a parasite egg, which hatches and develops into a juvenile stage. The definitive fish host ingests the amphipod and the parasite matures within the digestive tract. The alternative pathway is indicated by dashed lines. In this pathway, the amphipod is ingested by a paratenic host and the parasite encysts within the body cavity of the fish. When the definitive host ingests the paratenic host, the parasite will become sexually mature within the digestive tract.

differed among populations and detected geographical patterns to this variation, and therefore hypothesized the existence of cryptic species or broad scale environmentally induced variation (Steinauer *et al.* 2006). The life cycle of *L. thecatus* involves two obligate hosts including an amphipod intermediate host and a fish definitive host (Fig. 1) (DeGiusti 1949). Adult worms live and reproduce sexually in the digestive tract of their fish definitive host. Female worms release eggs that are expelled into the environment with the faeces of the host. After ingestion of the eggs by an amphipod (*Hyallela azteca*) intermediate host, the parasite develops to a juvenile or cystacanth stage. If a fish of an appropriate species ingests an infected amphipod, the parasite develops to an adult within the digestive tract of the fish. The alternate transmission pathway includes an additional fish host called a paratenic host that transports the parasite from amphipod to another fish host. After ingestion of the infected amphipod, the parasite burrows out of the digestive tract and encysts in a parenteral location. The parasite will remain in this stage until the paratenic host is ingested by an appropriate definitive host species. It can be determined whether a fish is a paratenic host or a definitive host based on the location of the parasite, the digestive tract or outside of the digestive tract in parenteral locations. Interestingly, it is possible for the same host individual to be a paratenic and definitive host for different individual worms. The stimuli that cause a parasite to undergo this alternate transmission pathway are unknown and apparently not all populations are capable of altering their life cycle in this manner (Steinauer *et al.* 2006).

Host specificity and habitat specificity of *L. thecatus* at the level of the definitive hosts is broad, but varies among populations, and is geographically patterned (Steinauer *et al.* 2006). Principle host species that harbour the majority of worms in a population and in which the parasites can fully mature include fishes of the genera *Micropterus*, *Ambloplites*, and *Lepomis* (Lincicome & Van Cleave 1949a; Ashley *et al.* 1989; Steinauer *et al.* 2006). Within the definitive host, *L. thecatus* occupies the pyloric ceca and the intestine; however, populations vary concerning the narrowness of this niche. Survey data and experimental evidence suggests that some populations are restricted to the pyloric ceca and that maturation cannot occur in the intestine (Ashley 1980; Uznanski & Nickol 1982; Ashley & Nickol 1989). However, other studies have found that some populations of *L. thecatus* are restricted to the intestine, and other populations are capable of inhabiting both locations of the same individual fish (Lincicome *et al.* 1949a; Muzzall & Gilliland 2004; Steinauer *et al.* 2006).

Variation detected in the presumably single species of *L. thecatus* may be induced by environmental variance or it may have a genetic basis, with independent genetic lineages carrying alternative phenotypes. The former explanation would indicate that this species is a broad generalist that can exploit a variety of environments or host species, and that population variation is due to different ecological or physiological environments. The latter explanation would reveal genetic structure, indicating that variation has been induced by selection (host or external environment) or genetic drift in different geographical populations or host species. The aim of this study is to use a phylogenetic approach based on mitochondrial DNA and nuclear DNA markers to test for the presence of cryptic species and to examine several parasite-related phenotypes. Although environmentally induced variation is best established through experimental means, manipulation of such an extensive number of populations of parasitic organisms is not practical. The association between genetically isolated lineages and parasite phenotypes would support a genetic basis of the observed phenotypic variation. This approach aims to identify the phylogenetic component of variation so that groups potentially exhibiting environmentally induced variation can be identified.

## Materials and methods

To determine the genetic diversity and systematic status of *Leptorhynchoides thecatus*, sequences of the internal transcribed spacer regions 1 and 2 (*ITS1* and *ITS2*) and part of the cytochrome oxidase 1 gene (*cox1*) were obtained from worms collected throughout the range of this species. Specimens were obtained from fishes collected from various locations in North America (Table 1, Fig. 2). Collections and host use analyses focused on species of *Micropterus*,

Site	C	I	County/Parish	State/Province	North	West	
1	Whitemouth Falls	5	5	Whiteshell	Manitoba	50.1000	95.9400
2	Atkinson Reservoir	4	4	Holt	Nebraska	42.1667	99.0333
3	Swan Lake	3	0	Holt	Nebraska	42.5392	99.0011
4	Pickeral Lake	6	5	Becker	Minnesota	46.8052	95.8475
5	Connor Lake	4	3	Sawyer	Wisconsin	45.7595	90.7265
6	Flambeau River	2	2	Price	Wisconsin	45.8647	90.5492
7	Lake Winnebago	4	2	Winnebago	Wisconsin	44.0500	88.5167
8	Shawano Lake	8	7	Shawano	Wisconsin	44.8184	88.4680
9	Oconto River	12	6	Oconto	Wisconsin	44.8583	88.0583
10	Gull Lake	3	5	Kalamazoo	Michigan	42.3885	85.3938
11	Mansfield Hollow	7	5	Tolland	Connecticut	41.7689	72.1745
12	Henderson Swamp	5	3	St Martin	Louisiana	30.3069	91.7394
13	I55 Canal	8	0	Tangipahoa	Louisiana	30.2635	90.4003
14	Pearl River	11	5	St Tammany	Louisiana	30.2562	89.6500
15	Escambia River	7	4	Escambia	Florida	30.6667	87.2667
16	Apalachicola River	6	4	Gulf	Florida	29.7820	85.0466
17	Ochlocknee River	13	3	Leon	Florida	30.3833	84.6559
18	Suwannee River	14	8	Gilchrist, Levy	Florida	29.7011	82.9386
19	Withlacoochee River	17	11	Marion	Florida	29.0466	82.4614
20	St Mary's River	5	6	Nassau Camden	Florida Georgia	30.7582	81.7411
21	Altamaha River	6	1	Glynn	Georgia	31.4279	81.6056

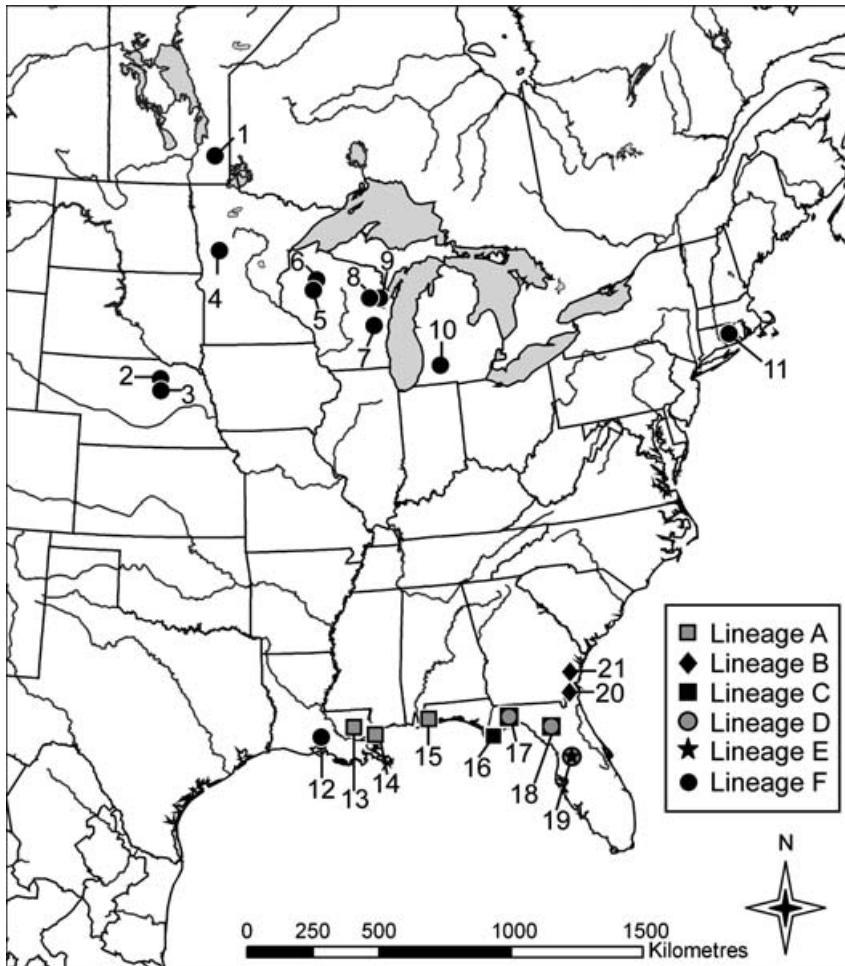
**Table 1** Collection localities and sample sizes of individuals of *Leptorhynchoides* sequenced at *cox1* (C) and ITS (I) markers for phylogenetic analysis. GPS coordinates are projected in North American Datum 1983 decimal degrees. Sites are numbered according to Fig. 2

Lineage	<i>Micropterus</i>	<i>Lepomis</i>	<i>Ambloplites</i>	Worms per host	Adult	Juvenile
A	18	105	0	1.56 (1–7)	24	1
B	3	62	0	1.10 (1–2)	10	1
C	10	131	0	1.44 (1–5)	23	0
D	11	116	0	1.29 (1–2)	6	12
E	4	22	0	2.25 (1–4)	9	0
F	63	129	28	1.75 (1–7)	55	9

**Table 2** Collection and sampling data for six lineages of *Leptorhynchoides* including the number of fish individuals of three genera (*Micropterus*, *Lepomis*, and *Ambloplites*) examined, the mean and range of worms sampled per host for the *cox1* phylogeny, and the number of each developmental stage of the worms sampled

*Ambloplites* and *Lepomis* because they are the primary definitive hosts of *L. thecatus* (Ashley *et al.* 1989; Steinauer *et al.* 2006). Species of *Ambloplites* could not be collected at all sites due to its limited range. Most specimens were collected and processed by the primary author in the following manner. Fish were killed immediately prior to dissection and within 48 h of collection. Worms were removed from the digestive tract or body cavity of the fishes, and the location of the worms, sex (if obvious), and general appearance of the worms were noted. Worms were either preserved in 95% ethanol and stored below 0 °C until DNA extraction, or genomic DNA was extracted immediately in the field with the AquaPure genomic DNA isolation kit (Bio-Rad). Sampling details concerning the number of fish sampled for each major lineage of *Leptorhynchoides* and the number of worms sampled per population, lineage, and host are given in Tables 1 and 2. Full collection data is available (Steinauer 2004) or can be obtained from the primary author.

Polymerase chain reaction (PCR) was used to amplify the *ITS1*, *ITS2* and *cox1* regions. The *ITS1* and *ITS2* regions were amplified in one continuous fragment (*ITS*) using the primers and methods described by Král'ová-Hromadová *et al.* (2003). Part of the *cox1* gene was amplified using a combination of one of two forward primers 506F 5'-TTAGTGTATTATTAGTGTGCTTCGT-3' and 509F 5'-GTGTTTATTAGTGTGCTTCGTAG-3' along with a reverse primer RCOI 5'-TGAAAATGAGCCACAATAATAAG-3'. These primers were designed from the complete mitochondrial genome sequence (Steinauer *et al.* 2005) from *Leptorhynchoides thecatus*. Template DNA (approximately 20–50 ng) was amplified in a 15 µL reaction containing 2 mM MgCl<sub>2</sub>, 1.67 mM of each deoxynucleotide triphosphate (dNTP), 0.4 µM of each primer, and 0.75 U of *Taq* polymerase in 1× reaction buffer (Invitrogen). The thermocycling protocol for the *cox1* PCR amplification was 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54–56 °C for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C



**Fig. 2** Collection localities of *Leptorhynchoides*. Symbols correspond to the lineages detected with the phylogenetic analysis as shown on Fig. 3. Overlapping symbols indicate the sympatric distribution of multiple lineages. Collection sites are numbered according to Table 1.

for 7 min. PCR products were visualized with a 0.8% agarose gel containing ethidium bromide and prepared for sequencing by adding 1  $\mu$ L of shrimp alkaline phosphatase (SAP) and 0.2  $\mu$ L of exonuclease (*ExoI*) to 5  $\mu$ L of PCR product and incubating at 37 °C for 30 min and then 80 °C for 15 min. Approximately 1.5–2.5  $\mu$ L of the SAP/exonuclease-prepared product was used for sequencing with the BigDye terminator kit version 3.1 (Applied Biosystems) and a BaseStation automated sequencer (MJ Research). Sequences were obtained for both forward and reverse PCR primers for *ITS* and *cox1*, and additional sequences for *cox1* were obtained using internal primers that varied among populations. Sequences were deposited in GenBank Data Libraries under Accession nos AY690487–AY690595.

The 5.8S ribosomal gene was invariant among taxa; therefore, it was excised from the *ITS* sequences and the *ITS1* and *ITS2* sequences were concatenated for analysis. Polymorphic sites within an individual were considered ambiguous, and coded with the appropriate IUB code. Sequence alignment was performed using CLUSTAL\_X (Thompson *et al.* 1997). Default gap penalties were used for *cox1* alignment, and a gap opening penalty of 7 and gap

extension penalty of 0.5 were used in the *ITS* alignment. The resulting alignment was adjusted by eye in cases where multiple gaps divided by only one or two nucleotides occurred. Adjustments minimized the number of gaps and maximized the gap size. Potential saturation of transitions at third codon positions in the *cox1* gene was assessed by comparing the number of transition and transversion substitutions at these positions with sequence divergence. Sequence statistics were obtained using the program MEGA version 2.1 (Kumar *et al.* 2001).

Phylogenetic analyses using maximum parsimony (MP), minimum evolution (ME), maximum likelihood (ML), and Bayesian optimality criteria were performed on the *ITS* and *cox1* data separately and concatenated. Congruence among data partitions (*cox1* vs. *ITS*) was assessed with a partition-homogeneity test (Farris *et al.* 1994; 1995) with 100 iterations using heuristic searches with 100 random stepwise addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping as implemented in PAUP\* 4.0b10 (Swofford 2001). Nucleotide substitution models were selected using the likelihood ratio test implemented in MODELTEST 3.0 (Posada & Crandall 1998). These

models were applied to the phylogenetic analyses using ME, ML, and Bayesian optimality criteria.

Unweighted MP and ME tree searches were done heuristically using PAUP\* 4.0b10 (Swofford 2001) with TBR branch swapping on initial trees that were obtained by random stepwise addition of taxa, replicated 1000 times. To reduce computation time for the large *cox1* data set, the number of rearrangements allowed per addition sequence replicate was limited to 100 million. Node support was assessed by bootstrap analysis (Felsenstein 1985) using a heuristic search with TBR branch swapping, and random stepwise addition of taxa with 1000 pseudoreplicates. The *ITS* and combined data set used 100 stepwise addition replicates while the *cox1* data set used one stepwise addition replicate.

The ML search for the *ITS* data set was executed with PAUP\* 4.0b10 using a heuristic search, 1000 replications of a random stepwise addition of taxa, and TBR branch swapping. Bootstrap analysis with 1000 pseudoreplicates of a heuristic search with 100 replicates of random stepwise addition and TBR branch swapping was used to assess node support. Maximum-likelihood tree searches for the *cox1* and concatenated data sets were executed with METAFIGA 1.0.2b (Lemmon & Milinkovitch 2002). METAFIGA uses a metapopulation genetic algorithm, involving several populations of trees that are forced to cooperate in the search for the optimal tree. This approach is accurate and much faster than existing heuristic searches, allowing large data sets to be analysed in practical computing times (Lemmon & Milinkovitch 2002). Each search was replicated 100 times, and was begun with four populations of four individual random trees. One best tree was kept per population per replicate. Default settings were used for other options as recommended in the manual. A majority-rule consensus tree was constructed from the 400 best trees found during the search.

Bayesian analyses were conducted on all three data sets using MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001). Nucleotide substitution models were the same as those applied in the likelihood analysis and a mixed model analysis was used for the combined data. Four chains were run for 5 million generations, trees were sampled every 100 generations, and only the last 5000 trees were used to build a majority-rule consensus tree.

Genetic divergence was calculated using MEGA version 2.1 (Kumar *et al.* 2001). Within-clade divergence and net between-clade divergence were calculated using uncorrected *p*-distances, which is the proportion of sites that differ between two taxa. Bootstrap analysis with 500 pseudoreplicates estimated standard errors of the divergences.

A suitable outgroup to root the tree could not be obtained. The most closely related acanthocephalan genera are *Illiosentis* and *Koronacantha* (Garcia-Varela & Nadler 2006). Both of these genera are represented in GenBank

with partial *cox1* sequences; however, these sequences are extremely divergent (20–25%, uncorrected *p*-distance) from *Leptorhynchooides* even if the third codon position is not considered. The outgroup method of tree rooting can be problematic and possibly misleading if there is great divergence between outgroups and the ingroup (Huelsenbeck *et al.* 2002). Therefore, a molecular clock was used to root the tree instead. Following the method of Huelsenbeck *et al.* (2002) and Jennings *et al.* (2003), the *cox1*, *ITS* and combined data sets were analysed with MRBAYES version 3.0 (Huelsenbeck & Ronquist 2001) as described above, but with a molecular clock enforced. The clock-enforced trees were tested against the nonclock trees with the likelihood ratio test (Goldman 1993) by comparing the log-likelihood scores obtained from PAUP\* 4.0b10. The test statistic  $\delta$ , was calculated as the difference between the two likelihood scores multiplied by 2 and its statistical significance was determined with the chi-square table and using  $N-2$  ( $N$  = number of taxa) degrees of freedom.

Habitat use, development, and host use were examined with three separate contingency table analyses with Pearson's chi-squared goodness-of-fit to determine if these characters were independent of the major lineages (A–F) that were identified with the phylogenetic analysis. Habitat was considered as three categorical variables including the number of hosts per lineage that contained worms only in the ceca, only in the intestine, or in both habitats. Paratenic host use was scored as the number of hosts from a lineage that contained juvenile parenteral worms vs. the number of hosts that contained worms in the digestive tract, but no parenteral juveniles (uninfected fish were not included). Host use was considered as two major categories and calculated per lineage as the prevalence (percentage of infected hosts) in fishes of the genus *Lepomis* vs. *Micropterus*. Prevalence was used instead of raw numbers because the number of hosts collected per genus was not equivalent.

One lineage, named lineage F, was investigated further due to its wide geographical distribution, substructure within the phylogenetic tree, and broad host and habitat ranges. We examined this lineage for evidence of environmentally derived variation by comparing population characteristics within the sublineages. Only sublineage F2 was considered because the sampling was most complete among multiple host taxa. Host use, habitat use, and development were compared across three populations within sublineage F2. To test for correlation between genetic distance and geographical distance within this lineage, a permutation test (Mantel 1967) was performed on the matrices of genetic (*cox1* sequences) and geographical distances between pairs of collection sites using MATLAB (The Mathworks Inc.). Tamura-Nei (1993) distances calculated with MEGA version 2.1, and geographical distances calculated from GPS coordinates with GENALEX (Peakall & Smouse 2006) were used for this analysis. Confidence

intervals for the test were obtained by randomization techniques, running 1 million iterations.

## Results

Alignment of the *cox1* sequences resulted in 972 bp of sequence, and consisted of 90 unique haplotypes from 150 individuals sequenced. The alignment contained no gaps and the translation of the sequences matched the translation of *cox1* from the complete mitochondrial genome of *Leptorhynchoides thecatus* in GenBank (NC 006892). Of 303 variable sites, 267 were parsimony informative. Neither transitions nor transversions at the third codon position of the *cox1* sequences were saturated. The alignment of the *ITS1* and *ITS2* sequences combined was 547 bp total length, and it consisted of 295 bp of *ITS1* and 252 bp of *ITS2*. The alignment included seven gaps that ranged in length from 1 to 12 bp. A total of seven unique genotypes from 89 individuals were found, and 34 of 67 variable sites were parsimony informative.

The likelihood ratio tests comparing clock and nonclock trees indicated that the *ITS* data did not meet the clock assumption ( $\delta = 25.064$ ,  $\chi^2_{[0.05]} = 11.070$ ,  $P < 0.001$ ); however, the *cox1* ( $\delta = 32.898$ ,  $\chi^2_{[0.05]} = 110.90$ ,  $P > 0.999$ ) and the combined data sets ( $\delta = 15.702$ ,  $\chi^2_{[0.05]} = 67.505$ ,  $P > 0.995$ ) did meet the clock assumption. The clock-enforced trees were rooted at the branch-dividing clades A–C and D–F. The clock-enforced *ITS* tree was rooted at the branch dividing lineage C from A and B.

All of the phylogenetic analyses yielded trees with similar topologies regardless of what data or optimality criteria were used. Two major clades that are each subdivided into three distinct lineages are evident in all trees (Figs 3–5). The amount of divergence among the major lineages ranged from 6.3 to 11.6% in *cox1* sequences, and 1.0–8.7% among *ITS* sequences (Table 3). The amount of

divergence within the six lineages was 0.4–2.8% in *cox1*, and 0% in *ITS* of most lineages except lineage F which differed by 0.3%. The phylogenetic analyses differed in the arrangement of the terminal taxa, which is expected for an intraspecific phylogeny, as well as the relationships between the lineages within the two major clades. The nodes that conflict have low bootstrap support or posterior probabilities (lineages A, B, and C and the three groups within lineage F in Fig. 3). One of the lineages, named lineage F in Fig. 3, is further subdivided into two groups in the *ITS* analysis (Fig. 4) and three groups in the *cox1* (Figs 3 and 5) and concatenated analyses; however, the relationships between these groups are not well resolved.

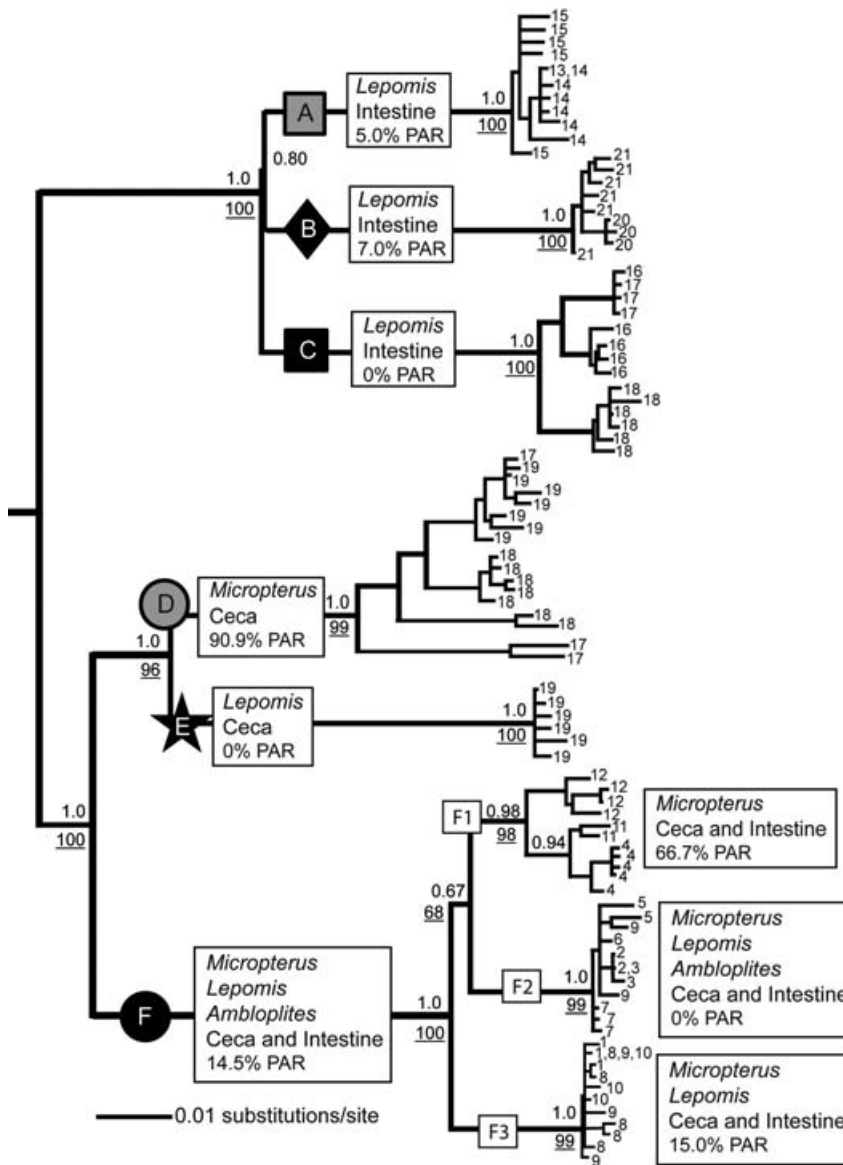
For the *cox1* data set, all analyses yielded consensus trees with similar topologies (Fig. 3). The HKY + I +  $\Gamma$  model of nucleotide substitution was selected by the likelihood ratio test applied by MODELTEST 3.0. Base frequencies were A = 0.24, C = 0.10, G = 0.20, and T = 0.46, the transition to transversion ratio was 10.0611, the proportion of invariable sites was 0.5298, and the gamma shape parameter was 0.945. Parsimony searches yielded 28 211 most parsimonious trees (length = 672, CI = 0.4992, RI = 0.9494, RC = 0.5001, HI = 0.4732). Minimum evolution searches yielded 72 shortest trees with a length of 1.02327.

For the *ITS* data set, MP, ML, and ME tree searches all yielded trees with similar topologies (Fig. 4), which differ from Fig. 3 in that lineages E and F were sister taxa; however, the bootstrap value of this node was 61%. The Bayesian analysis yielded a tree similar to Fig. 3; however, the relationship between lineages D and E was supported by a posterior probability of 0.51. The Hasegawa, Kishino, Yano (HKY) model of nucleotide substitution was selected by the likelihood ratio test applied by MODELTEST 3.0. Base frequencies were A = 0.26, C = 0.17, G = 0.27, and T = 0.30, and the transition to transversion ratio was 2.62. The scores of the ME tree and the ML tree were 0.13554 and 1154.9806, respectively. Parsimony searches found a single most parsimonious tree [length = 71, consistency index (CI) = 0.972, retention index (RI) = 0.966, rescaled consistency index (RC) = 0.938, homoplasy index (HI) = 0.028].

The partition-homogeneity test comparing the *cox1* and *ITS* data sets failed to reject the null hypothesis that the gene partitions are different from random partitions of the data ( $P = 0.96$ ) and indicated that the phylogenetic signal of the two data sets were not significantly different. For the concatenated *cox1* and *ITS* data set, all analyses produced trees with similar topologies to that of Fig. 3; however, the relationship between lineages A, B, and C varies among analyses and was not well supported in any of the analyses according to bootstrap proportions and posterior probabilities (Fig. 5). The trees from the concatenated analyses also differed from Fig. 3 in the relationship between the three groups within lineage F. The parsimony tree search yielded six most parsimonious trees (length = 584, CI = 0.661,

**Table 3** Net average distance in *ITS* (upper matrix) and *cox1* (lower matrix) sequences between six clades (A–F) calculated by:  $dA = dXY - (dX - dY)/2$ , where  $dXY$  is the average distance between clades X and Y and  $dX$  and  $dY$  are the mean within groups distances. Intraclade distance is the mean genetic distance for *cox1* sequences calculated as the arithmetic mean of all individual pairwise distances between taxa within clades. Uncorrected  $p$ -distances are expressed as percentages

	A	B	C	D	E	F	Intraclade $p$ -distance
A	—	1.6	1.8	7.2	8.2	8.7	0.7
B	7.0	—	1.0	6.4	7.4	7.9	0.5
C	6.3	7.9	—	6.2	7.2	7.8	1.3
D	9.6	10.2	9.5	—	1.8	2.5	2.3
E	10.8	11.6	10.9	6.6	—	3.6	0.4
F	10.7	10.9	11.1	7.4	10.4	—	2.8

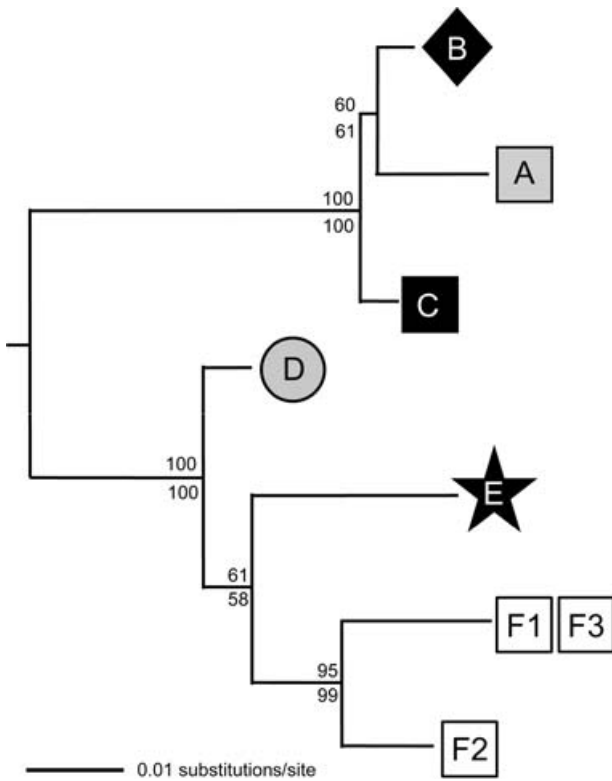


**Fig. 3** Consensus tree from Bayesian analysis of 972 bp of the cytochrome oxidase I gene of *Leptorhynchoides*. Posterior probabilities are given above the branches and bootstrap values for the maximum likelihood analysis are given below the branches for the major nodes of the tree. Tree was rooted using a molecular clock. Numbers on the terminal branches indicate collection localities where specimens were collected and refer to the numbers given in Table 1. Major lineages are labelled from A–F and the symbol given refers to the symbols on Fig. 2. Sub groups of lineage F are labelled F1, F2, and F3. Boxes indicate host genera and the specific habitat that individuals from each clade occupy, and the percentage of hosts that were paratenic hosts (PAR).

RI = 0.947, RC = 0.626, HI = 0.339) and the ME search yielded one shortest tree with a length of 0.61335.

Contingency table analyses indicated that there was an association between lineage and habitat use ( $\chi^2 = 402.776$ ,  $P < 0.001$ ), paratenic host use ( $\chi^2 = 239.579$ ,  $P < 0.001$ ), and adult host use ( $\chi^2 = 225.520$ ,  $P < 0.001$ ) (Table 4, Fig. 3). Most lineages do not use both the intestinal tract and the ceca making the 'both' category of the habitat-use analysis rare and creating an excess of cells with expected frequency  $< 5$ , which is a violation of the assumptions of the analysis. Therefore, the both category was discarded and the analysis rerun to be sure that this category was not biasing the results and this change did not influence the interpretation of the results ( $\chi^2 = 360.980$ ,  $P < 0.001$ ). For most lineages, habitat use was narrow and worms from

lineages A–C mainly inhabited the intestine only, D and E mainly inhabited the ceca only, and lineage F often occurred in both habitats, but was more frequently found in the ceca. The use of paratenic hosts mostly occurs in clades D and F, particularly subclades F1 and F3. Host use also was narrow for most lineages and lineages A–C mainly inhabited fishes of the genus *Lepomis* and lineage D mainly inhabited *Micropterus*. The host use statistics may be problematic for some lineages for which small numbers of *Micropterus* were sampled. Lineage E in particular seems to suffer from sample size bias. The prevalence statistics indicate that *Lepomis* is a somewhat better host genus than *Micropterus* (45.5 to 25%); however, worm burden or intensity data indicate that *Lepomis* fishes are far better hosts since only one individual worm was found in a single



**Fig. 4** Maximum-likelihood tree from 547 bp of the internal transcribed spacer regions 1 and 2 of various species of *Leptorhynchooides*. Maximum likelihood bootstrap proportions are given above each branch and maximum parsimony proportions are given below each branch. Tree was rooted using a molecular clock. Lineages are labelled according to Fig. 3.

*Micropterus* while the worm burdens in *Lepomis* ranged from 3 to 34 worms with a mean of 14 worms per host.

All populations of sublineage F2 did not incorporate paratenic hosts in their life cycle. Interspecific differences existed mainly in host use patterns (Fig. 6). Although

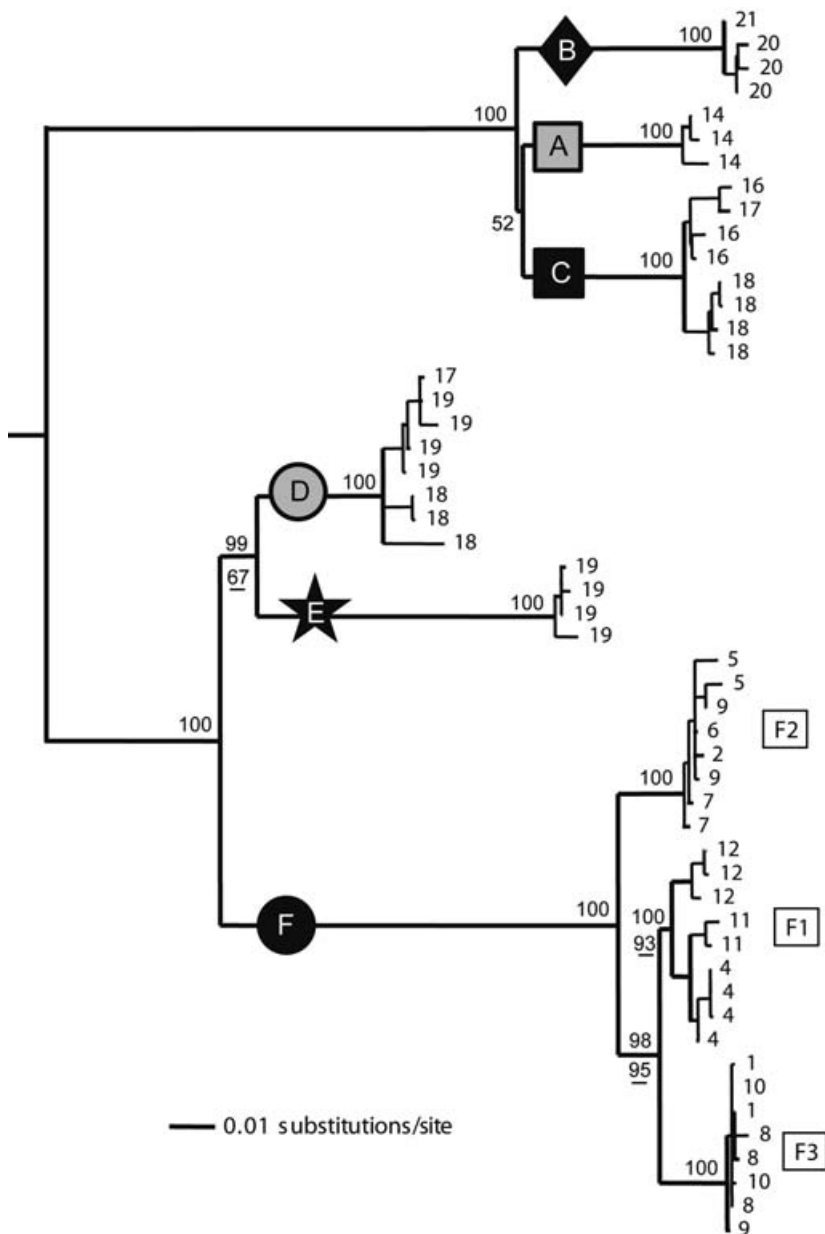
all host species do not occur at all three sites, differences in the infection rates of three fish species among populations could be detected. *Lepomis macrochirus* occurred at all three sites and was the principle definitive host at the Oconto site due to its high prevalence of 75%, mean intensity of 5.1 worms per host, and possession of 94% of the gravid worms in the population. In contrast, at the Connor site, worms were much less common in this host compared to other hosts at the site (Fig. 6) and mainly contained immature worms. This host species was not infected at the Atkinson site. Our sample size of *Lepo. macrochirus* was low at this site; however, a previous survey of this area by Ashley & Nickol (1989) supports our findings and indicated that of four centrarchid species, *Lepo. macrochirus* was the least suitable host, and with prevalence less than 20%, a relative density of 0.4, and very few gravid females. The principle hosts at Connor Lake included *Micropterus dolomieu* and *Ambloplites rupestris*. These two host species accounted for 89% of all worms found in this population. In contrast, these two species were rarely infected and harboured only 11% of the worms at the Oconto site. Neither of these fish species occurs at the Atkinson site. Habitat use varied little among populations and most fish contained worms only in the ceca except for three of 31 fish from the Connor Lake population and two of 28 fish from the Oconto population which contained worms in both the intestine and ceca. Mantel's test did not detect geographical structure within lineage F ( $r^2 = 0.1753, P = 0.1085$ ).

**Discussion**

The results of these analyses indicate that most of the observed differences among populations of *Leptorhynchooides thecatus* in terms of host use, habitat use, and transmission pathways can be attributed to phylogenetic divergence within this species. Nuclear and mitochondrial DNA sequences congruently indicated that *L. thecatus* comprises at least six highly divergent and independent lineages

Lineage	Habitat			Paratenic host			Definitive host		
	Intestine	Ceca	Both	Yes	No	%	<i>Lepomis</i>	<i>Micropterus</i>	<i>Ambloplites</i>
A	60	0	0	3	57	5.0	74.3	5.6	ND
B	43	0	0	3	40	7.0	66.1	0.0	ND
C	67	1	2	0	70	0.0	54.2	0.0	ND
D	0	10	0	70	7	90.9	5.3	70.0	ND
E	0	11	0	0	10	0.0	45.5	25.0	ND
F	2	93	17	19	112	14.5	46.5	66.7	46.4
F1	1	5	2	16	8	66.7	0.0	87.5	ND
F2	1	82	4	0	87	0.0	61.2	56.4	42.3
F3	0	6	11	3	17	15.0	0.0	100.0	100.0

**Table 4** Descriptive statistics of the habitat use, development, and host use of lineages A–F and sublineages F1–F3 of *Leptorhynchooides*. Habitat indicates the number of hosts harbouring adults in the intestine only, ceca only, or both locations. Paratenic host indicates the number of hosts that either harbour juveniles in parenteral locations for paratenic transmission or only worms in the digestive tract (no parenteral juveniles). Definitive host for each lineage is summarized by the prevalence of infection in three host genera. ND indicates that data is not available because the fish were not collected



**Fig. 5** Maximum-likelihood tree inferred from 972 bp of the cytochrome oxidase I 547 bp of the internal transcribed spacer regions 1 and 2 of various species of *Leptorhynchoides*. Maximum likelihood bootstrap proportions are given above each branch. Tree was rooted using a molecular clock. Lineages are labelled according to Fig. 3.

that should be recognized as separate species. Some of the lineages are geographically sympatric, but are isolated in different host species. Further subdivision within lineage F also indicates more cryptic diversity present in the system although phylogenetic patterns were not congruent between nuclear and mitochondrial markers.

Character states were associated with the lineages detected within *L. thecatus*, and most lineages (A–E) have narrow host and habitat ranges, and limited transmission pathways. In contrast, lineage F was highly variable and also genetically divergent comprising two or three sublineages

as detected by the *ITS* and *cox1* data, respectively. However, even if the sublineages of F are viewed individually, the taxa still have broad host and habitat ranges, although differences can be detected among them. Sublineage F2 differed relative to F1 and F3 in that this lineage did not incorporate paratenic hosts into its life cycle, infected fishes of the genus *Lepomis* as well as other species, and had more specific habitat requirements within the pyloric ceca of the host. Therefore, some of the variation among populations within lineage F can be explained by phylogenetic divergence, but not all. Within sublineage F2, parasite populations varied in their host use of three species, *Lepomis*

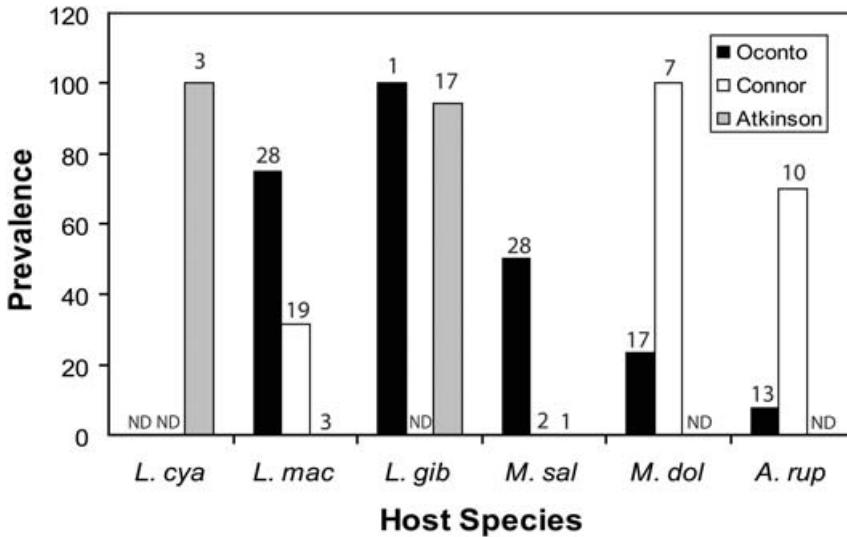


Fig. 6 Prevalence of *Leptorhynchoides thecatus* in various host species in three different populations: Oconto River, Connor Lake, and Atkinson Reservoir. Host species include *Lepomis cyanellus* (*L. cya*), *L. macrochirus* (*L. mac*), *L. gibbosus* (*L. gib*), *Micropterus salmoides* (*M. sal*), *M. dolomieu* (*M. dol*), and *Ambloplites rupestris* (*A. rup*). Numbers above bars indicate sample sizes and ND indicates no data were available because the fish species was not present at that location.

*macrochirus*, *Ambloplites rupestris* and *Micropterus dolomieu*, and this variation did not correspond to phylogenetic divergence. Difference in host use among these populations could be due to factors intrinsic to the hosts and parasites involved, but could also be determined by trophic ecology of the hosts or community structure (Olson & Nickol 1996).

Prior to this study, *L. thecatus* was thought to inhabit a wide range of fish taxa, but our results show that most lineages are narrower in their host specificity and are restricted to a single genus. Lineage F is the exception and is capable of infecting fish from three different genera. Broad host specificity is hypothesized to be a strategy to avoid 'getting lost' due to predation by incompatible hosts (Combes 2001). Undoubtedly, many immature individuals of *Leptorhynchoides* within their amphipod hosts are lost from the population due to predation by an inappropriate host. If the parasite has no control over what fish species ingests it, the ability to infect multiple species of hosts would be advantageous and allow a greater probability of transmission. The factors that determine host specificity in this system are unknown, but a combination of ecological, physiological, and immunological factors play a role. Our field data give us an excellent insight into what hosts are infected in nature, but can only suggest what hosts a parasite is not capable of infecting. For instance, the lack of a parasite in a certain host species may occur because that species never encounters the parasite or because when it does, the parasite does not develop within it. The data do show, however, that lineages D, F1, and F3, are not capable of developing within fishes of the genus *Lepomis*, because these species do ingest the parasites, but they do not establish in the digestive tract, and instead move to parenteral locations for paratenic transmission.

Alteration of the life cycle to include a paratenic host was evident in only two of the lineages of *Leptorhynchoides*, D and F. In other lineages, worms were rarely found in the body cavity of fishes and are likely to be lost worms that have moved to ectopic sites and will not be transmitted further (Elkins & Nickol 1983; Muzzall & Gilliland 2004). Paratenic host use has only evolved in lineages of *Leptorhynchoides* that use large piscivorous fishes (*Micropterus*) as definitive hosts. With the exception of small individuals, it is unlikely that these fishes prey on enough amphipods to become infected through the standard transmission route and support the parasite population. Paratenic hosts bridge the trophic gap and transmit the parasite from amphipod to definitive hosts. Without a paratenic host, transmission would be restricted to only small *Micropterus* or, in the case of lineage F, other host genera. The exception to this pattern includes the population at Connor Lake where *M. dolomieu* and *A. rupestris* are the principle definitive hosts and there is no evidence of paratenic transmission. Transmission across this trophic gap remains a mystery and could indicate that the unusual feeding behaviour of these fishes or that an alternative transmission pathways exist in this population such as paratenic transmission through an alternate host or post-cyclic transmission, which is the transmission of adult parasites from one fish to another through predation.

Divergence of the two major clades of *Leptorhynchoides* is associated with a change in the specific microhabitat of these organisms. Habitat specificity is common in the Acanthocephala, and most species inhabit a specific portion of the digestive tract (Kennedy 2006). Aggregation to find mates is suspected to drive habitat specificity since chemical attractants have never been identified for any acanthocephalan species and some species attach permanently to the host

mucosa, become immobile, and are restricted to mates only in the immediate proximity (Richardson *et al.* 1997). Therefore, it is surprising that lineage F, and sublineages F1 and F3 in particular, occupy a broad region of the digestive tract. It appears, however, that these worms have an affinity for the ceca since worms were rarely found only in the intestine, but often found only in the ceca and in both habitats. It is possible that there is a density-dependent effect that forces worms into less optimal habitats or perhaps habitat specificity depends on developmental stage of the worm (Uznanski *et al.* 1982; Leadabrand & Nickol 1993) although gravid worms were found in both the intestine and ceca of their hosts.

Host and microhabitat specificity have been theorized to play a large role in the speciation of parasitic and free living organisms because specialization promotes speciation by reducing gene flow (Futuyma & Moreno 1988; Brooks & McLennan 1993). Host specificity is associated with the radiation of these species since two pairs of lineages are geographically sympatric, but are partitioned into different host taxa: lineages C and D, and lineages D and E. Two of these lineages, D and E, may be sister taxa as suggested by the *cox1* data, and therefore, may have evolved in sympatry through the process of host race formation or host-switching. However, the alternative explanation of divergence in allopatry followed by secondary contact cannot be ruled out. Whichever the case, patterns of host specificity play a role in reinforcement of speciation because they cause complete reproductive isolation of taxa that overlap geographically.

Lineage F was the most geographically widespread, genetically divergent and generally variable clade. There were no geographical patterns to the diversity as analysed by the Mantel test. This lack of pattern greatly contrasts with the other lineages that typically inhabit two to three neighbouring watersheds and their intralinesage diversity is generally partitioned by watersheds, which is expected for a parasite of freshwater fishes that cannot easily move among drainages. We suspect that fish stocking of *M. salmoides*, *M. dolomieu*, and *Lepo. macrochirus* may be responsible for this observation. Stocking of these species would influence lineage F more than the others because these fish species are principle hosts for lineage F, and not for the other lineages. Also, most of the locations where lineage F was collected are impounded waterbodies as opposed to rivers where most of the other lineages were collected. Fish stocking more commonly occurs in closed water systems from which the fish cannot leave. A further possibility may be the degree of specificity of these lineages to intermediate hosts. It is possible that lineage F is less specific at this level and is capable of invading more habitats.

Traditionally, *L. thecatus* was considered to be a widely distributed, highly variable parasite in terms of morphology,

host specificity, habitat specificity, and transmission pathways. However, the results of these analyses indicate that the presence of cryptic species can explain much of the variation among populations, although one lineage of this complex exhibits population level differences in host use. In the future, this complex of closely related species could serve as a model system for examination of the factors that drive speciation and also the mechanisms of host and habitat specificity and alternate transmission pathways.

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This work was completed as part of Michelle Steinauer's Ph.D. thesis at the University of Nebraska-Lincoln under the advisement of Brent Nickol and Guillermo Ortí. Michelle's research interests include the evolution, ecology, and biogeography of parasitic organisms. She is currently a Postdoctoral Fellow at the University of New Mexico working on the evolutionary epidemiology of *Schistosoma mansoni* in western Kenya. Brent Nickol is an Emeritus Professor at the University of Nebraska-Lincoln and specializes on the Acanthocephala, with a particular interest in transmission dynamics and systematics. Guillermo Ortí is an Associate Professor at the University of Nebraska-Lincoln and his research focuses on using molecular markers to reconstruct gene genealogies ranging from paternity analysis to deep phylogenies, focusing mainly on the molecular systematics of fishes (and their parasites!). For more information, see <<http://golab.unl.edu/>>.

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