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Morphological and molecular characterization of *Quinqueserialis* (Digenea: Notocotylidae) species diversity in North America

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Abstract

Estimates of trematode diversity are inaccurate due to unrecognized cryptic species and phenotypic plasticity within species. Integrative taxonomy (genetics, morphology and host use) increases the clarity of species delineation and improves knowledge of parasite biology. In this study, we used this approach to resolve taxonomic issues and test hypotheses of cryptic species in a genus of trematode, *Quinqueserialis*. Specimens from throughout North America were field collected from hosts and obtained from museums. We found three morphologically distinct groups and successfully sequenced specimens from two of these groups. DNA sequencing at the 28S and CO1 gene regions revealed that two of the three groups were genetically distinct. One genetic group included two morphological clusters demonstrating host-induced phenotypic plasticity within *Quinqueserialis quinqueserialis*. The other unique genetic group is a novel species, *Quinqueserialis kinsellai* n. sp., which is described herein. Our study illustrates the importance of integrating multiple sources of evidence when investigating trematode diversity to account for the influence of cryptic species or phenotypic plasticity. However, further sampling is needed to understand *Quinqueserialis* spp. diversity as some species have no genetic information associated with them.

Research Article

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Introduction

Trematode species diversity is underestimated, and one reason for this may be unrecognized cryptic species (Pérez-Ponce de León and Poulin, 2017). Species are deemed cryptic when there are no perceivable diagnostic morphological traits, and genetic analyses reveal more than one distinct lineage (Pérez-Ponce de León and Nadler, 2010). While many cryptic species complexes have been reported, relatively few studies have investigated the extent of morphological similarity among cryptic species complexes. For instance, there is a paucity of investigations that have linked the genetic differences among cryptic lineages with detailed analyses of the morphology and life history of the cryptic lineages (Blasco-Costa *et al.*, 2010). This lack of integrative analysis has led to poor species-level taxonomic resolution, which ultimately biases estimates of parasite species diversity, and can mask differences in the ecology and life history of cryptic species (Poulin and Leung, 2010).

Quinqueserialis Harwood, 1939 (Digenea: Notocotylidae) is typical of other trematodes in that the number of species recognized within this genus has fluctuated over time. Up to four nominal species have been recognized in North America, *Quinqueserialis quinqueserialis* Barker and Laughlin, 1911, *Quinqueserialis hassalli* McIntosh and McIntosh, 1934, *Quinqueserialis floridensis* Rausch 1952 and *Quinqueserialis zibethicai* Gupta 1962. Two of the species were originally described as *Notocotylus* Diesing 1839 species but were reassigned to *Quinqueserialis* due to the presence of five rows of ventral papillae, as opposed to three rows among *Notocotylus* species, and a cirrus armed with heavy spines (Harwood, 1939). Currently, only two of the four taxa are considered nominal species: *Quinqueserialis floridensis* and *Quinqueserialis quinqueserialis* (Barker and Laughlin, 1911; Rausch, 1952*a*; Kinsella, 1971). These two species can be distinguished morphologically based on the placement of the vitelline follicles and overall size, with *Q. floridensis* specimens being smaller overall and having vitelline follicles posterior to the uterine coils (Rausch, 1952*a*; Kinsella, 1969). The two *Quinqueserialis* species infect rodent hosts such as the round-tailed muskrat (*Neofiber alleni* True 1884) for *Q. floridensis* and voles (*Microtus pennsylvanicus* Ord 1815) and muskrats (*Ondatra zibethicus* Linnaeus 1766) for *Q. quinqueserialis* (Barker and Laughlin, 1911; Rausch, 1952*a*, 1952*b*). The two *Quinqueserialis* species do not overlap in geographic range, with *Q. quinqueserialis* distributed from the North American Arctic to parts of the southern United States and *Q. floridensis* restricted to Florida where *Q. quinqueserialis* has not been reported (Rausch, 1952*a*). Thus, there is evidence to support that *Q. floridensis* and *Q. quinqueserialis* are distinct species. However, there is still ambiguity within this genus due to similarities in morphology and host use.

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A comparative morphological study suggested that two formerly recognized species, *Quinqueserialis hassalli* and *Quinqueserialis zibethicai*, are synonymous to *Q. quinqueserialis* (Kinsella, 1971). Further evidence for synonymy among these species was overlap in definitive host use (the former infects meadow voles and the latter infects muskrats). However, some of the perceived morphological differences described in *Q. hassalli* are attributed to host-induced phenotypic plasticity. Adult *Q. quinqueserialis* parasites in voles tend to be larger than adult stages from the muskrats (Kinsella, 1971). In the case of *Q. zibethicai*, the difference in microhabitat (duodenum *vs* caecum for *Q. quinqueserialis*) within the definitive host was used to delineate this species (Kinsella, 1969). However, there are no voucher specimens of *Q. zibethicai;* therefore, morphological and genetic characteristics of this taxon cannot be evaluated (Kinsella, 1969).

Relative to the other species in this genus, *Q. quinqueserialis* has a broad geographic distribution, ranging from the North American Arctic to southern United States (Rausch, 1952*b*; Detwiler *et al*., 2012). Across this broad geographic range, differences in host use and spatial distance among populations could potentially result in isolation, leading to diverged populations (Lively, 1999). Unlike parasites that use hosts (e.g. birds) that disperse and mix parasites from different populations, *Q. quinqueserialis* infects a variety of cricetid rodent hosts that generally have limited home ranges, so dispersal by these hosts would be unlikely to promote gene flow of parasites across large distances (Rausch, 1952*b*; Getz, 1961; Marinelli and Messier, 1993). The broad distribution and host use of *Q. quinqueserialis* evokes the question of whether this species comprises a complex of cryptic species.

The uncertainty surrounding species diversity of *Quinqueserialis* in North America revolves around morphological descriptions from few specimens and limited geographic sampling. For instance, the species *Q. quinqueserialis* was described from a single specimen collected from a muskrat in Nebraska, USA (Barker and Laughlin, 1911). Thus, the extent of intraspecific variation among species in this group remains unknown. Further, only one species in this genus, *Q. quinqueserialis*, has had their life cycle described and several intermediate hosts identified (Herber, 1942; Gagnon and Detwiler, 2019). There are many gaps in the knowledge of the biology and diversity of this genus and most of the research on this group was completed before the advent of modern molecular technology. Here we use adult morphological and adult and larval DNA sequence data of the 28S nuclear and CO1 mitochondrial genes to determine whether cryptic species are present in the genus *Quinqueserialis* and to elucidate the taxonomic status of *Quinqueserialis* spp. in North America.

Materials and methods

Specimen collection

Muskrats, and vole definitive hosts (*Microtus* spp. Schrank 1798), were field collected in six areas throughout five Canadian provinces and US states in North America: Northern Northwest Territories, Canada; Northern Manitoba, Canada; Southern Manitoba, Canada; Minnesota, USA, Virginia, USA; and Alabama, USA (Table 1). Muskrat carcasses were salvaged from licensed trappers at each location, excluding Northern Manitoba, from 2015 to 2018. Voles were either live-trapped or snap-trapped at three locations where muskrats were collected (Table 1; Government of Manitoba Scientific Collection permit No. WB18783 and WB23398, Government of Northwest Territories Wildlife Collection permit No. WL500642, Virginia Game and Inland Fisheries Scientific Collection permit No. 061288). Upon recovery, voles (*Microtus* spp.) were euthanized in the field *via* anaesthetic overdose (Animal Use Protocol 2016-0023, Texas A&M University) followed by cervical dislocation (Animal Use Protocol AC11347, University of Manitoba). Voles were sorted into individual bags and transported to the lab for necropsy. The intestinal tract, from the duodenum to colon, of each muskrat and vole host was sectioned, separated in Petri dishes and opened longitudinally. The contents of each section of intestinal tract were searched for *Quinqueserialis* spp. adults under a stereomicroscope.

Live parasites were heat killed in 70° C distilled water, then transferred to warm ethanol. The parasites were preserved in 80% EtOH and stored at 4° C for molecular and morphological analysis. Adult worms were initially identified as *Quinqueserialis* spp. by the presence of five rows of ventral papillae (Harwood, 1939). The holotype specimen for *Q. hassalli* and a paratype of *Q. floridensis* were borrowed from the U.S. National Museum of Natural History (NMNH) and included in the morphological analyses. As there is neither a holotype nor a paratype of *Quinqueserialis quinqueserialis* available, 17 additional specimens identified as *Quinqueserialis* spp. were also loaned from the NMNH to assess intra- and interspecific morphological variation (Supplementary Table 1).

A voucher image was captured of each worm using an Axio Cam ICcI digital camera connected to an Axio Imager M2 compound microscope (Zeiss Canada Ltd., Toronto, Canada). Drawings were made with the aid of a drawing tube on an Olympus MT5310L compound microscope. Tissue samples were collected from gravid worms prior to staining and mounting to create hologenophore-type vouchers (Pleijel *et al*., 2008). Once the tissue sample was retrieved, adult worms were stained with acetocarmine, dehydrated in ethanol, cleared in xylene and mounted in Canada Balsam. An adult worm recovered from the same host as molecular voucher worms was also stained and permanently mounted to create paragenophore-type vouchers (Pleijel *et al*., 2008). Voucher and holotype parasite materials from this study are deposited in the Smithsonian National Museum of Natural History, Washington, US (Supplementary Table 2). Vouchers of vole hosts from locations where *Q. quinqueserialis* was detected are deposited in the Canadian Museum of Nature, Ottawa, ON (Supplementary Table 3).

Multivariate analysis

Thirteen morphological measurements were analysed for a total of 99 specimens identified as *Quinqueserialis* spp.: 27 museum specimens (7 museum lots had multiple individuals measured), 15 field-collected paragenophores and 57 field-collected hologenophores. Two museum specimens were excluded from the multivariate analyses because accurate measurements could not be obtained. All morphological features were normally distributed with the exception of the width of uterine coils and width of the left testes, which were log-transformed to meet assumptions of normality prior to analysis. Principal component analysis (PCA) was performed to determine if specimens clustered according to nominal species. This analysis included specimens that were field collected and genetically identified, as well as museum specimens that were morphologically identified by other researchers. The resulting clusters from the PCA were used to assign specimens to three *a priori* groups that were then analysed in a linear discriminant analysis (LDA). The LDA was applied to 99 specimens to evaluate the morphological differences between them and to identify the morphological features that yield optimal group separation. A random 75% subset of the adult parasite morphological measurements were used to train a parasite species assignment model, which was then used to predict the parasite species identity of the remaining 25% of the data. This process

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Table 1. Field collection locations and definitive hosts sampled

Location	GPS coordinates	Host species (N)	Parasite species	Prevalence % (range)
NT, Canada	68.295, −133.612	*Ondatra zibethicus* (10)	*Q. quinqueserialis*	100 (228–1006)
		Microtus pennsylvanicus (8)		38 (1–7)
		Microtus oeconomus (1)		100 (12)
		Myodes rutilus (45)		0
	68.623, −135.369	*O. zibethicus* (12)	*Q. quinqueserialis*	100 (69–669)
		M. pennsylvanicus (3)		67 (1–44)
Northern MB, Canada[a]	58.739, −93.818[a]	*M. pennsylvanicus* (35)	*Q. quinqueserialis*	8.6 (3–14)
			Q. kinsellai n. sp.	5.9 (1–4)
	58.759, −93.94	*M. pennsylvanicus* (2)	*Q. quinqueserialis*	50 (1)
Southern MB, Canada	49.129, −97.302	*O. zibethicus* (25)	*Q. quinqueserialis*	68 (2–170)
		M. pennsylvanicus (10)		10 (NA)
		Myodes gapperi (12)		0
		Zapus hudsonius (1)		0
	50.339, −97.657	*O. zibethicus* (34)	*Q. quinqueserialis*	82 (1–325)
	50.176, −97.083	*O. zibethicus* (17)	*Q. quinqueserialis*	100 (11–211)
	49.073, −100.989	*O. zibethicus* (18)	*Q. quinqueserialis*	100 (36–701)
MN, USA[b]	43.839, −93.84	*Peromyscus leucopus* (1)	*Q. quinqueserialis*	0
		O. zibethicus (NA)	*Q. quinqueserialis*	NA
VA, USA	37.2, 80.565	*M. pennsylvanicus* (3)		0
	38.59, −77.357	*O. zibethicus* (NA)	*Q. quinqueserialis*	NA
	37.257, −80.442	*O. zibethicus* (NA)	*Q. quinqueserialis*	NA
	38.383, −78.836	*O. zibethicus* (NA)	*Q. quinqueserialis*	NA
	37.92, −78.327	*O. zibethicus* (NA)	*Q. quinqueserialis*	NA
AL, USA	34.47, −86.304	*O. zibethicus* (7)	*Q. quinqueserialis*	100 (13–291)

Prevalence of adult *Quinqueserialis quinqueserialis* parasites reported at each location and prevalence of adult *Quinqueserialis kinsellai* n. sp. reported from the type locality
[a]Voles (*Microtus* spp.) were trapped at this location in two separate years, 2016 and 2019.
[b]Adult *Q. quinqueserialis* specimens from muskrats (*Ondatra zibethicus*) were donated from collections by Dr. R. Sorensen (Minnesota State University-Mankato). No prevalence data available.

was repeated 1000 times; each with a randomly selected set of training data, and mean prediction success was calculated. The PCA was conducted with the package 'vegan' and the LDA was conducted with the package 'MASS' in Rstudio 1.1.463 (Venables and Ripley, 2002; Oksanen *et al.*, 2019).

Molecular data

To have both a molecular and stained morphological voucher for a single specimen, the anterior third of the worm was removed and used for DNA extraction. Tissue samples were soaked in MilliQ water to remove the EtOH prior to being incubated in 200 μL 5% Chelex solution with 0.2 mg/mL proteinase K at 56 C for 2 hr. DNA samples were then vortexed, boiled at 100 C for 8 min and vortexed again after cooling. Extracted DNA samples were stored at −20° C until polymerase chain reaction (PCR) could be performed. If amplification was unsuccessful with chelex-extracted samples, an adult worm from the same host was used for whole-worm extraction using the Qiagen DNeasy Blood & Tissue kit following a modified manufacturer protocol. These samples were eluted in a total volume of 30 μL of millipore water.

Partial (~1000 base pairs (bp)) 28S rDNA sequences were amplified and sequenced using forward primers LSU-5 (5′-TAG GTC GAC CCG CTG AAY TTA AGC A-3′) or 300 F (5′-CAA GTA CCG TGA GGG AAA GTT G-3′) when LSU-5 failed to amplify and 1500 R (5′-GCT AGG GAA ACT TCG-3′) reverse primer (Olson *et al.*, 2003). The 28S RNA gene region was targeted because it is commonly utilized to investigate interspecific variation among trematodes and sequences are available for a larger number of trematode species on public databases, including *Quinqueserialis quinqueserialis* (Olson *et al.*, 2003; Detwiler *et al.*, 2012; Blasco-Costa *et al.*, 2016, Pérez-Ponce de León and Hernández-Mena, 2019). We amplified ~500 bp of the cytochrome c oxidase subunit I (CO1) mtDNA gene with primers DICE 1F (5′-TTW CNT TRG ATC ATA AG-3′; Moszczynska *et al.*, 2009) and DICE 11R (5′- GCW GWA CHA AAT TTH CGA TC −3′; Van Steenkiste *et al.*, 2015). The 5′ end of the CO1 mitochondrial gene region is commonly used in DNA barcoding of trematodes (Moszczynska *et al.*, 2009). Mitochondrial DNA accumulates substitutions at a higher rate than nuclear DNA and can therefore distinguish congeneric trematodes more clearly (Vilas *et al.*, 2005). The 28S amplifications were carried out in 25 μL reactions containing 2 μL of extracted DNA, 2.5 μM of 10 × buffer, 1.5 μM of $MgCl_2$, 0.5 μM of each primer, 0.5 μM of dNTP and 0.05 μL^{-1} units Taq polymerase. The CO1 amplifications were carried out in 25 μL reactions containing 5 μL of extracted DNA, 2.5 μM of 10 × buffer, 3.5 μM of $MgCl_2$, 0.5 μM of each primer, 0.5 μM of dNTP and 0.5 μL^{-1} units Taq polymerase (Omega Bio-Tek, Georgia, USA). The following thermocycling conditions were used for 28S rDNA amplification with primers LSU-5 and 1500R: 94° C for 3 min, once; 94° C for

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1 min, 56° C for 45 s, 72° C for 2 min, 35 times; 72° C for 7 min, once. Amplification of the 28S gene region using primers 300F and 1500R consisted of 95° C for 3 min, once; 95° C for 45 s, 56° C for 30 s, 72° C for 2 min, 40 times; 72° C for 7 min, once. Amplification of CO1 mtDNA was 95° C for 2 min, once; 95° C for 30 s, 50° C for 30 s, 72° C for 1 min, 35 times; 72° C for 10 min, once. PCR products were purified with a MO Bio Laboratories Inc. PCR clean up kit and sequenced in both directions at the Hospital for Sick Children, Toronto, ON.

Contigs were constructed and sequences were assembled using Sequencher 4.1 (Gene Codes Corp., Ann Arbor, MI, USA) and were aligned in MEGA 7.0 (Kumar *et al*., 2016). Genetic distances between specimens were calculated at both gene regions with uncorrected p-distance in MEGA. A genetic benchmark of >1% different at the 28S gene region and >5% different at the CO1 gene region was interpreted as evidence of distinct species (Vilas *et al*., 2005). These cut offs were used to assign specimens to species groups, and genetic distances between and among species was calculated with uncorrected p-distance in MEGA.

Results

Specimens identified as *Q. quinqueserialis* were field collected from at least one host within the six sampling areas (Table 1). Prevalence of *Q. quinqueserialis* infection was 83% (114/123) in muskrats and 27% (35/128) in voles (*Microtus pennsylvanicus, M. oeconomus* Pallas 1776 and *Myodes* spp. Pallas 1811) and jumping mice (*Zapus* sp. Coues 1875). Notably, only meadow voles (*M. pennsylvanicus*) and a single tundra vole (*M. oeconomus*) were infected with *Q. quinqueserialis*.

Morphological data

Thirteen morphological characters were measured and analysed for the field-collected and museum *Quinqueserialis* sp. specimens (Table 2). The first and second principal components accounted for 82% of the total observed morphological variation. The first principal component (PC1) accounted for 71% of the total variation (eigenvalue = 9.2). PC1 was interpreted as describing overall body size, meaning all morphological variables contributed significantly (cut-off value of factor scores was 0.8, determined following Abdi and Williams, 2010). The second principal component (PC2) accounted for 12% of the total variation (eigenvalue = 1.5). PC2 was interpreted as describing the width and length of the oral sucker, which both contributed positively, and the width of the uterine coils, which contributed negatively.

Among the specimens identified as *Q. quinqueserialis*, those from voles were generally larger than those from muskrats (Fig. 1). The museum specimens identified as *Q. floridensis* formed a separate cluster along PC2 from those identified as *Q. quinqueserialis*. The *Q. floridensis* specimens were smaller than the specimens in the other clusters and had wider uterine coils (Fig. 1). There was a third cluster consisting of museum specimens identified as *Q. quinqueserialis* from voles and specimens from field-collected voles. This cluster separated from the *Q. floridensis* specimens along PC1 and from the *Q. quinqueserialis* specimens along PC2 (Fig. 1). Specimens within this cluster were larger than *Q. quinqueserialis* specimens, had wider uterine coils, but had relatively smaller oral suckers. The three clusters formed by the PCA were used to assign specimens to groups for the LDA. These groups are subsequently referred to as: *Q. quinqueserialis*, *Q. floridensis* and *Q. quinqueserialis* morphotype 2.

The trained LDA run on 13 morphological characters (Table 2) separated the three species with an average 95.3% accuracy. The first canonical function clearly discriminated *Q. floridensis* specimens from the other two groups, whereas the second canonical function discriminated *Q. quinqueserialis* morphotype 2 from *Q. quinqueserialis* (Fig. 2). The specimens of *Q. floridensis* and *Q. quinqueserialis* morphotype 2 were all assigned to their *a priori* groups, whereas one *Q. quinqueserialis* specimen was misclassified as *Q. quinqueserialis* morphotype 2 (4.7% misclassification). These misclassifications could be attributed to the higher dispersion and overlap among specimens of *Q. quinqueserialis* and *Q. quinqueserialis* morphotype 2 (Fig. 2). Five variables were associated with the discriminatory power of the model: oral sucker length, oral sucker width, left testes width, left vitellaria length and width of uterine coils (Table 2).

Molecular analysis

For the mitochondrial gene, we generated 85 CO1 sequences (510–606 bp, MW853836-MW853920) from both larval and adult *Quinqueserialis* parasites from 61 individual hosts (muskrats, voles and snails), including 56 adult sequences of which 25 are from vole hosts, and 31 are from muskrat hosts. For the nuclear gene region, we generated 91 partial 28S sequences (912–1271 bp, MW934276- MW934366) from both larval and adult *Quinqueserialis* parasites, including 61 adult sequences of which 19 are from vole hosts and 42 sequences are from muskrat hosts. There were two unique 28S haplotypes.

The mean genetic distance at the CO1 gene region between *Q. quinqueserialis* and *Q. quinqueserialis* morphotype 2 was 10% (over 452 bp), which is two times the p-distance difference for species suggested by Vilas et al. (2005). The mean genetic distance at the CO1 gene region within *Q. quinqueserialis* was 0.76% (over 452 bp), while there was no genetic variation at the CO1 gene region within *Q. quinqueserialis* morphotype 2. The mean genetic distance at the 28S gene region between *Q. quinqueserialis* and *Q. quinqueserialis* morphotype 2 was 1.6% (over 810 bp), which is above the 1% p-distance difference for species suggested by Vilas *et al.* (2005). There was no intraspecific genetic variation at the 28S gene region for both *Q. quinqueserialis* and *Q. quinqueserialis* morphotype 2 specimens. Genetic comparisons of *Q. quinqueserialis* and *Q. quinqueserialis* morphotype 2 to *Q. floridensis* specimens could not be completed as DNA could not be extracted from the museum specimens.

Examination of morphological data and genetic sequencing of *Q. quinqueserialis* from the same host species (meadow vole) and locality (Churchill, MB, Canada) confirmed the distinctness of *Q. quinqueserialis* morphotype 2. Thus, we propose erecting this morphotype as a species and propose the name *Quinqueserialis kinsellai* n. sp.

Quinqueserialis kinsellai n. sp.

Type-host: *Microtus pennsylvanicus*, the meadow vole (Cricetidae). Two hosts vouchered at the Texas A&M Biodiversity Research and Teaching Collections, College Station, TX, USA (TCWC 66632 and TCWC 66647).

Type-locality: Churchill Northern Studies Centre, Churchill, Manitoba, Canada (58.739218 N. -93.817874W).

Site: Small intestine and caecum of intestine.

Type-material: Holotype (USNM 1643534) and paratypes (USNM 1643530-1643533) deposited at the Smithsonian National Museum of Natural History, Washington, D.C., United States.

Representative DNA sequences: COX1 (MW853846, MW853847, MW853848, MW853855), 28S (MW934288, MW934289).

Etymology: The species is named for Dr J. Michael Kinsella in recognition of his contributions to the knowledge of *Quinqueserialis* spp. taxonomy and biology.

Description ×Fig. 3A–D

The morphological description is based on four partially mounted adult specimens and one whole-mounted adult

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Table 2. Comparative morphological data for three *Quinqueserialis species*

Species	*Quinqueserialis quinqueserialis* (*N* = 83)			*Quinqueserialis floridensis* (*N* = 5)			*Quinqueserialis kinsellai* n. sp. (*N* = 8)		
Morphological features	Mean	± S.E.	Range	Mean	± S.E.	Range	Mean	± S.E.	Range
Total length	3615	78	1922–5232	1817	192	1354–2299	3614	196	2897–4386
Total width	1251	35	601–1967	1018	92	729–1236	1739	111	1142–2036
OS length	352	8	182–508	169	19	132–239	287	32	164–474
OS width	387	8	229–585	171	16	141–222	331	21	233–442
Left testes length	470	15	180–823	225	41	106–305	596	42	425–778
Left testes width	294	9	154–499	177	24	91–220	472	36	346–635
Right testes length	483	16	187–880	260	42	121–338	640	50	431–858
Right testes width	302	10	134–521	179	31	99–237	461	37	297–598
Ovary length	320	10	123–576	179	29	108–237	383	41	299–635
Ovary width	230	8	107–394	173	26	107–239	297	23	217–376
Width of uterine coil	647	18	271–1072	774	86	557–962	1219	89	707–1488
Left vitellaria length	899	23	499–1452	236	38	123–318	1112	76	789–1415
Right vitellaria length	892	23	488–1377	322	59	120–428	1024	82	580–1300

Measurements (μm) of 13 morphological variables of three species of *Quinqueserialis* found in North America: *Quinqueserialis quinqueserialis*, *Quinqueserialis floridensis*, *Quinqueserialis kinsellai* n. sp.

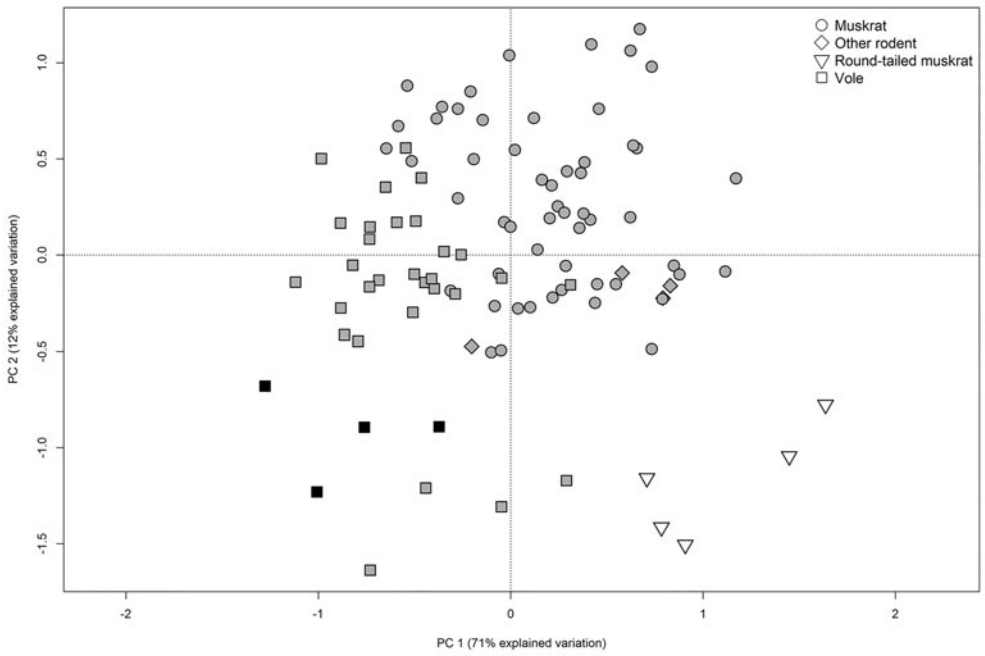

Fig. 1. PCA of *Quinqueserialis* spp specimens. PCA of 13 morphological measurements of 99 *Quinqueserialis* spp. specimens. Three species of *Quinqueserialis* demarcated by colour: *Q. quinqueserialis* (grey), *Q. floridensis* (white) and *Q. kinsellai* n. sp. (black), first referred to as *Q. quinqueserialis* morphotype 2 in text and described as a novel species herein. *Quinqueserialis* spp. specimens demarcated by shape according to host species.

specimen. Measurements are presented in μm (mean ± S.D.). Body oblong, and slightly attenuated anteriorly, with concave ventral surface, 3535–4385 long (4051 ± 362) by 1437–2006 (1716 ± 232) in greatest width (Fig. 3). Ventral surface with five rows of glands with an average of 13 papillae in the lateral rows, 15 papillae in the proximal rows and 15 papillae in the medial row (Fig. 3A). Oral sucker is 319–441 (358 ± 57) in diameter (Fig. 3C). The oesophagus is short, and typical for genus. Intestinal caeca pass medial to vitellaria and testes and end blindly posterior to the posterior margins of the testes. Excretory pore median and situated at level just posterior to ends of intestinal caeca. Testes 613–857 (719 ± 77) long by 384–608 (482 ± 78) wide, lobed and immediately posterior to vitellaria. Cirrus armed with conical spines is 496 long with a cirrus sac that is 665–838 (763 ± 89) long by 104–224 (155 ± 62) wide (Fig. 3D). Ovary is lobed, intercaecal and situated at the same level as testes. Ovary is 362–635 (460 ± 120) long by 241–355 (333 ± 63) wide. Mehlis gland is anterior to the ovary. Uterus with >10 convoluted transverse loops, which extend laterally beyond margins of intestinal caeca. Uterine coils 1073–1414 (1297 ± 160) in maximum width. Metraterm is strongly developed, 416–718 (585 ± 118) long by 180–247 (207 ± 24) wide. Vitellarium consists of two

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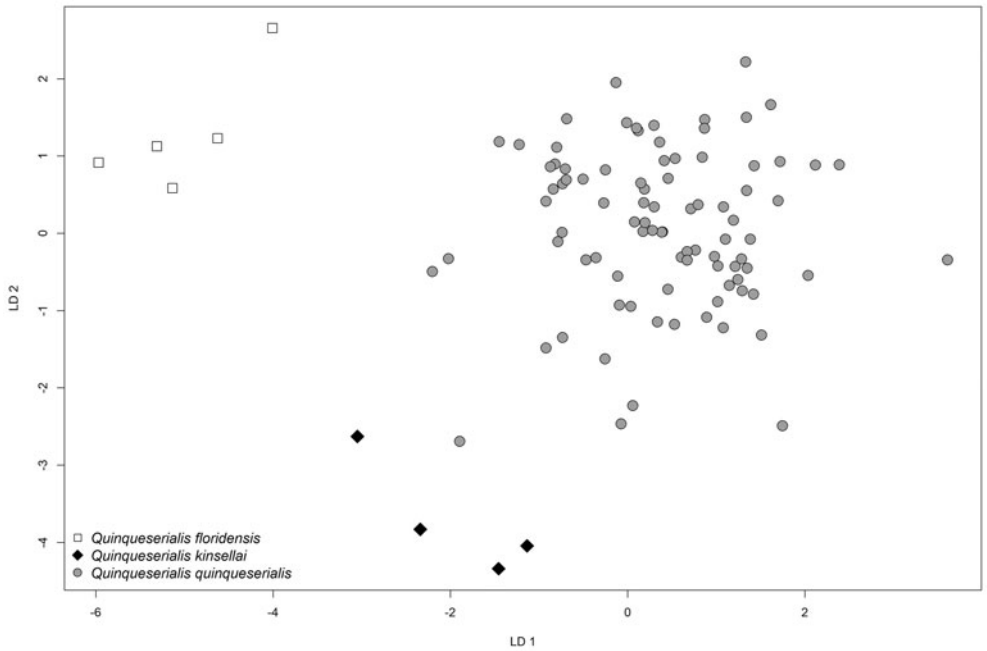

Fig. 2. LDA of three Quinqueserialis species. LDA of 13 morphological measurements of 99 specimens assigned to three taxa: *Quinqueserialis quinqueserialis*, *Quinqueserialis floridensis* and *Quinqueserialis kinsellai* n. sp., first referred to as *Q. quinqueserialis* morphotype 2 in text and described as a novel species herein.

lateral groups of numerous follicles arranged in clusters that extend from the anterior margin of the testes to the base of the metraterm, 925–1415 (1200 ± 152) in maximum length (Fig. 3B, C). Eggs are ovoid with polar filaments 17 (17.4 ± 0.2) long by 11 (11.5 ± 0.7) wide.

Remarks

This material exhibits some diagnostic characteristics of *Q. quinqueserialis*, i.e. five rows of ventral papillae, armed cirrus, lateral groups of vitellaria arranged in clusters (Harwood, 1939; Kinsella, 1971). However, *Q. kinsellai* n. sp. is characterized by the convoluted transverse loops of the uterus that extend beyond the margins of the intestinal caeca while the loops of the uterus are not convoluted and only extend past the intestinal caeca in certain hosts among *Q. quinqueserialis* specimens. On average, the testes and uterus of *Q. kinsellai* n. sp. are larger than those of *Q. quinqueserialis*, however the upper limits of the ranges of these features in *Q. quinqueserialis* specimens overlap with the ranges of these features in *Q. kinsellai* n. sp. Despite overlap in the size range of many morphological features, both multivariate approaches (PCA and LDA) clearly indicate that each species is a distinct morphological cluster. Further, *Q. kinsellai* n. sp. has a larger body size than *Q. floridensis* and does not exhibit the diagnostic feature of vitelline clusters restricted to a position posterior to the uterine coils as *Q. floridensis* exhibits. However, as multivariate analyses are required to differentiate *Q. kinsellai* n. sp. from *Q. quinqueserialis* and *Q. floridensis,* we consider this species functionally cryptic. A larger sample size of *Q. kinsellai* n. sp. is required to determine whether this species has diagnostic morphological traits. The observed genetic divergence support the distinct species status of *Q. kinsellai* n. sp.

Life cycle investigation

Currently, only the adult stage of this species has been described. Specimens from two individual meadow voles were collected from Churchill, MB, Canada in 2016. In 2019, potential first intermediate host gastropod snails were collected and screened for trematode parasite stages in Churchill, MB, where the infected definitive hosts were collected. In total, 817 snails (*Gyraulus* spp., *Lymnaea* spp., *Physella* spp. *Planorbdella trivolvis, Planorbula armigera, Promenetus exacuous*) were screened and 36 snails were infected with redia that produced cercariae with the monostome morphotype. Of the infected snails, rediae from 27 snails were sequenced (CO1 MW853894-MW853912, and MW853916-853920; 28S MW934341-MW934355). None of the DNA sequences from rediae were genetically similar to the DNA sequences from the adult specimens of the novel species *Q. kinsellai* n. sp. (CO1: 7–11% different at 454 bp; 28S: 2% different at 885 bp). Instead, the CO1 sequences from the rediae were similar to adult and larval *Q. quinqueserialis* (1–3% different at 454 bp) sequences from the field-collected specimens in the other four sampling regions (MW853836-853845, MW853849-853854, MW853856-853893, MW853913-853915). Redia sequences that were not genetically identical to either *Q. kinsellai* or *Q. quinqueserialis* were queried using the blast algorithm on GenBank. All sequences with e-value of 0 and percent identity >95% were retained in an alignment in MEGA 7 (Kumar *et al.*, 2016). Additionally, the GenBank sequences included within the alignment had query coverage of at least 90%. The alignments were trimmed to compare regions where all sequences were of equal length. The trimmed alignments were used to calculate p-distance using MEGA 7 (Kumar *et al.*, 2016). Rediae from five snails (*Gyraulus* sp. and *Physella gyrina*) were genetically similar at the CO1 gene region to unidentified *Notocotylus* sp. sequences (CO1: 2–5% different at 446 bp, MW853916-MW853920). The 28S sequences from the rediae were genetically identical to *Q. quinqueserialis* adult and larval (0% different at 885 bp) sequences from the field-collected specimens in the other four sampling regions (MW934276-934287, MW934290-934340, MW934356-934365). In addition, a redia from an infected *Gyraulus* sp. snail was genetically similar to sequences from an unidentified *Notocotylus* sp. and *Pseudocatatropis dvoryadkini* (1% different at 785 bp), and were identical to a redia from a *Lymnaea elodes* snail field collected in Northwest Territories, Canada (MW934366). The snail hosts found to be infected with *Q. quinqueserialis* were consistent with those

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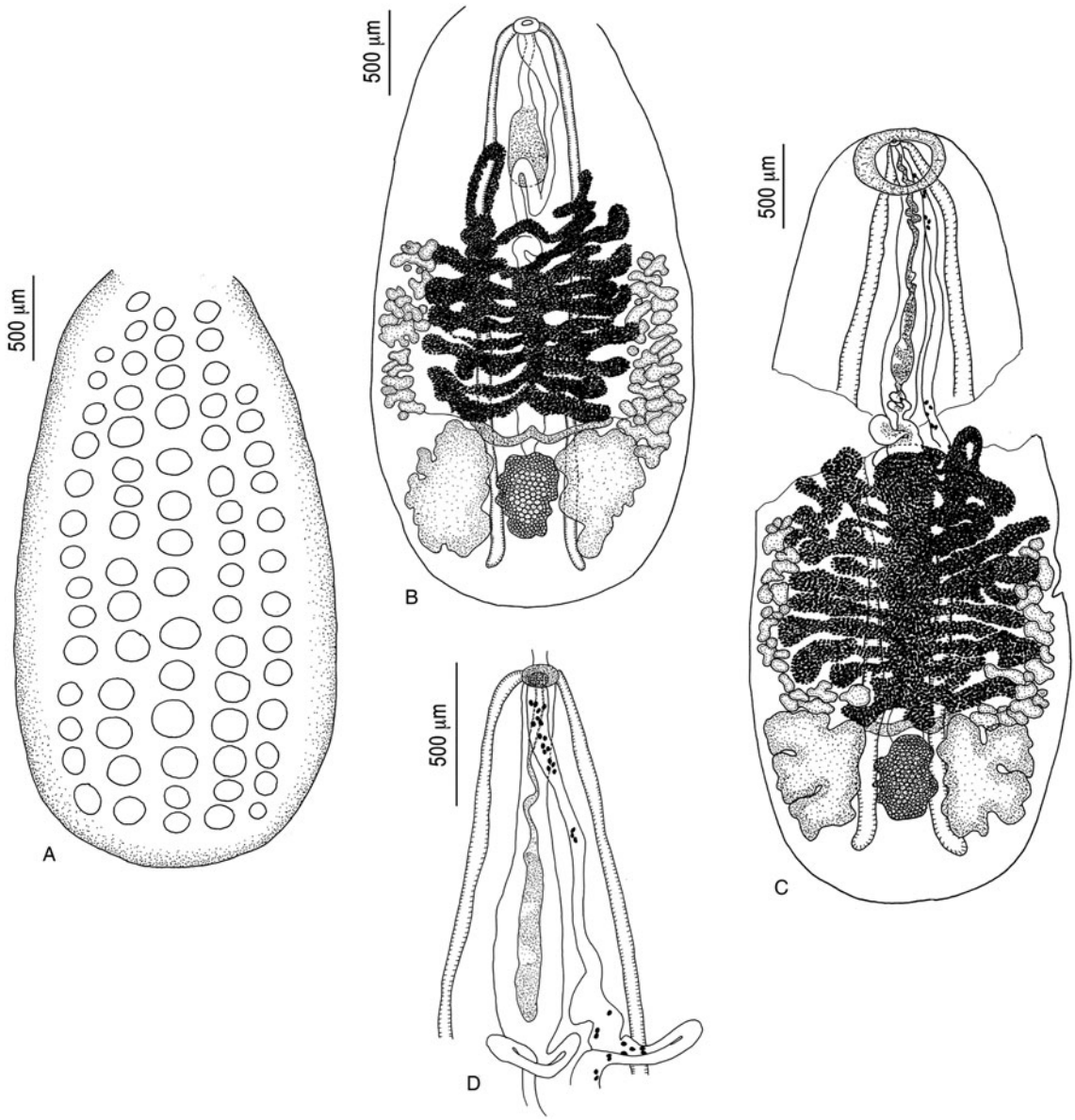

Fig. 3. *Quinqueserialis kinsellai* n. sp., ventral views. (A) Ventral view of the holotype (USNM 1643534) showing distribution of glands. Scale = 500 μm. (B) Ventral view of the holotype showing internal features, ventral glands omitted, lacking oral sucker. Scale = 500 μm. (C) Ventral view of paratype (USNM 1643530) showing oral sucker. Oral sucker folded over genital pore and specimen torn at posterior margin of the cirrus. Scale = 500 μm. (D) Ventral view of paratype (USNM 1643532) showing terminal portion of genital ducts. Scale = 500 μm.

identified in other locations throughout Canada (Gagnon and Detwiler, 2019). Vouchers of snail hosts from locations where *Q. quinqueserialis* was detected in Manitoba are deposited in the Manitoba Museum, Winnipeg, MB, Canada (Supplementary Table 4). Presently, the intermediate host(s) of *Q. kinsellai* n. sp. remains to be identified.

Discussion

This study is the first to incorporate genetic and morphological data focused on characterizing species in the genus *Quinqueserialis*. We hypothesized that cryptic species may be detected, and found a novel species that is functionally cryptic, *Q. kinsellai* n. sp. However, both PCA and LDA analyses demonstrated the presence of three morphologically distinct *Quinqueserialis* species. Two of the three species were also genetically distinct, while the remaining species, *Q. floridensis*, requires additional sampling as all known specimens cannot be sequenced (i.e. permanent slides or stained and stored in ethanol). Our study confirmed that *Quinqueserialis* spp. diversity was underestimated because a novel species, *Q. kinsellai* n. sp., was discovered. Importantly, if using morphology alone, this new species could be misclassified as *Q. quinqueserialis* due to morphological similarity and host-induced phenotypic plasticity in specimens from vole hosts of the latter species. Thus, we demonstrate that by integrating morphology, genetics and host use, we can make sense of inter- and intra-specific variation to clarify taxonomy and host use of groups with a long history of inadequate descriptions, poor specific diagnoses and extensive synonymy (Smith, 1954; Kinsella, 1971).

The multivariate analyses of 13 measurements of seven morphological traits revealed three clusters representing separate *Quinqueserialis* species. However, overlap in measurements of morphological trait ranges, and overlap among specimens of *Q. quinqueserialis* and *Q. kinsellai* n. sp. in the LDA support the conclusion that the two species are functionally cryptic. Cryptic species are hypothesized to occur more frequently among trematodes than any other helminth taxa (Pérez-Ponce

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de León and Poulin, 2017). However, in our study, the underestimated diversity of trematode parasites may be more attributed to limited geographic sampling and a lack of studies of *Quinqueserialis* that integrate molecular and morphological analyses. For *Quinqueserialis*, most studies used either morphology or genetics to characterize parasites (e.g. Kinsella, 1971; Detwiler *et al.*, 2012). The latter study is typical of the 'molecular prospecting' approach and does not include in-depth morphological analyses (Blouin, 2002). In the case of *Quinqueserialis*, the size of the oral sucker and the width of the uterine coils were among the morphological traits that contributed to the discriminatory power of the LDA between species. However, the width of the uterine coils has been shown to be subject to host-induced phenotypic plasticity among *Q. quinqueserialis* specimens (Kinsella, 1971). The intraspecific variation observed in this trait could explain the 4.7% misclassification rate of the LDA. Thus, while the three species form separate clusters they remain functionally cryptic, as 4.7% of *Quinqueserialis* specimens collected could be misidentified even when analysing morphology with multivariate analyses, leading to erroneous diversity estimates. The possibility of morphological misidentification demonstrates the importance of integrating morphology and genetic data, as gene sequencing informs species boundaries.

Our study increases the number of nominal species in North America from two to three. Before our study, four species of *Quinqueserialis* were described in North America, though only two were considered valid by Kinsella (1971). Our results genetically confirm the presence of one of the nominal species, *Q. quinqueserialis*, throughout North America, but also reveal a novel species occurring in meadow voles in Churchill, MB, Canada. While this species is genetically different from *Q. quinqueserialis* at both the nuclear 28S and mitochondrial CO1 gene regions, it is functionally morphologically cryptic. According to the criteria for the genetic species concept, these two parasite groups could be designated as different species as they are two distinct evolutionary units (Baker and Bradley, 2006). However, in the case of *Q. floridensis*, we only have evidence to support the morphological species concept because no genetic data could be obtained from the museum specimens. *Quinqueserialis floridensis* specimens can be distinguished from the other two species by its overall smaller body size, the distribution of the vitellaria and the wide, lateral extent of the uterine coils. Due to its distinguishing morphological characters, and its unique host association with the round-tailed muskrat, this species should remain valid until further sampling and genetic sequencing can be completed.

In conclusion, we found evidence for crypsis among *Quinqueserialis* species, and that diversity within this genus was underestimated likely due to restricted geographic sampling. A novel species, *Q. kinsellai* n. sp., genetically distinct from *Q. quinqueserialis* and morphologically distinct from *Q. floridensis*, was found at the periphery of the known range for this genus in North America. Our data suggests that delimiting species using solely morphology may be imprecise, as 4.7% of specimens in this genus were misclassified. Thus, this study demonstrates the importance of integrating many sources of evidence, such as morphology, genetics, host use and geographic distribution in determining trematode diversity.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021000792.

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Author contribution. DKG and JTD conceived, designed the study and wrote the article. DKG conducted field collections, gathered data and performed statistical analyses. WCP and LKB conducted field collections, and provided critical revisions to the article. ELK created the line drawings for the novel species described herein and provided critical revisions to the article.

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Conflicts of interest. The authors declare there are no conflicts of interest.

Ethical standards. Small mammal trapping was performed to standard of approved Animal Use Protocol AC11347 submitted by DKG and JTD to the Animal Care Committee at the University of Manitoba and approved Animal Use Protocol 2016-0023 submitted by WCP to the Institutional Animal Care and Use Committee at Texas A&M University.

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