Investigating taxonomy and speciation of *Quinqueserialis* (Digenea: Notocotylidae) parasites with an integrative taxonomic approach

By: Demi Gagnon

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Department of Biological Sciences

University of Manitoba, Winnipeg, MB.

Abstract

Accurate parasite diversity estimates are essential to understanding their infection dynamics and life history. However, current estimates of parasite diversity may be inaccurate due to unrecognized cryptic species and phenotypic plasticity. If cryptic species are present, parasite species diversity is underestimated and variation in host use and geographic distributions may be masked. If phenotypic plasticity is present, parasite species diversity may be overestimated because intraspecific host-induced morphological variation may be interpreted as new parasite species. Trematode parasites have the highest rates of cryptic species (genetically distinct species within nominal species that were assumed to be morphologically indistinguishable). However, few studies have tested the hypothesis of crypsis with detailed morphological comparisons of these genetic lineages. To avoid these problems, we performed integrative taxonomy (using genetics, morphology and host use) to delineate parasite species, which in turn resolved gaps in our knowledge of life cycles, host specificity, and evolution. Our work focused on improving the taxonomic resolution of one genus of trematodes, *Quinqueserialis* Harwood 1939. This parasite group is typical of other trematodes in that species diversity within this genus has fluctuated over time. For instance, it expanded due to unrecognized phenotypic plasticity and then contracted with a synonymy of two species. Like most trematodes, their life cycles are poorly understood, particularly for first intermediate hosts, because of limited geographic sampling especially in the northern extent of its range. Further, there are no comparative studies examining the genetics of this group, so it is unknown whether cryptic species are affecting estimates of species diversity. In Chapter 1, we used integrative taxonomy (gene sequences, morphology and host use) to accurately delineate *Quinqueserialis* spp. We field-collected larval and adult parasites from freshwater snails (Planorbidae and

Lymnaeidae), muskrats (*Ondatra zibethicus* Linnaeus 1766), and voles (*Microtus* spp. Schrank 1798), from throughout the northern and southern extent of their range in North America. Multivariate analyses of adult morphology combined with genetic sequences from nuclear (28S) and mitochondrial (cytochrome c oxidase subunit I) revealed three genetically and morphologically distinct species of *Quinqueserialis* in North America. We demonstrated that parasite species diversity of *Quinqueserialis* was underestimated, but not due to crypsis or phenotypic plasticity. A novel species was discovered by sampling new locations in the northern part of the parasites' range. In addition, three novel intermediate hosts of *Q. quinqueserialis* were genetically identified from hosts collected in the northern extent of the parasites' range: the snails *Gyraulus circumstriatus*, *Gyraulus crista* and *Promenetus exacuous*. After improving the taxonomy of this group and other fundamental aspects of its biology, we investigated the influences of host specificity and geography on speciation within *Quinqueserialis* (Chapter 2). Multivariate analyses of morphology combined with genetic identifications were used to test whether specimens clustered according to host or geographic location. In addition, genetic analyses were used to determine the effect of host and geography on the genetic divergence and haplotype diversity for the two species that were sequenced. We found that only one of the species, *Q. floridensis*, was isolated by host use and geography implicating both factors in the vicariance of this species. In contrast, *Q. quinqueserialis,* exhibited host-induced morphological variation with parasites in one host species being larger than in another host species, but there was no genetic divergence between these clusters. This species was widely distributed and showed a latitudinal cline of genetic dissimilarity from northern Canada to the southern USA suggesting a northern origin. The novel species *Quinqueserialis* n. sp. was not isolated by geography or host use as it co-occurred with *Q. quinqueserialis* in the same host species

(*Microtus pennsylvanicus* Ord 1815) and geographic location (Churchill, MB). Further sampling of this novel species and species outside of North America is needed to determine whether these two species diverged in sympatry or from a past vicariance event. The broader significance of our work is to show that an integrative approach can resolve issues in trematode taxonomy that can lead to inaccurate estimates of parasite species diversity. Further, it demonstrates the sampling in all parts of the parasites' range can expand our knowledge of host specificity and life cycles and provide insights into parasite speciation.

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Thesis Introduction

Parasite taxonomy is at a crossroad. On one hand, sequencing technologies have made it easier to identify genetic variation in parasites (Nadler, 1990). On the other hand, this genetic variation is not often linked to morphology, which was historically the basis for the taxonomic classification in most parasite groups (Nadler and Pérez-Ponce de León, 2011). As a result, the proportion of parasite taxa identified to species in parasite studies has declined over the past decade (Poulin and Leung, 2010). This trend is concerning because species-level resolution is critical for understanding the ecological role and evolutionary history of parasites (Poulin and Leung, 2010). Therefore, parasite taxonomy is at a point where we must integrate genetic sequencing with morphology and other parasite life history characteristics to define and delineate species boundaries.

Traditionally, parasite species have been delimited based on morphological characters. However, among parasites, there is often morphological similarity between closely related species, rendering this method unreliable (Andrews and Chilton, 1999). This problem is exemplified by cryptic species complexes, which are defined as distinct species that have previously been recognized as a single species (Bickford et al, 2007). Gene sequencing has suggested that a growing number of parasites species are cryptic species complexes (Pérez-Ponce de León and Poulin, 2017). Further, among parasitic helminths, trematodes have the highest occurrence of cryptic species, suggesting that species have been misidentified and diversity is underestimated (Pérez-Ponce de León and Poulin, 2017). The reasons for higher rates of crypsis in trematodes are unclear. There are some technical aspects that can influence whether or not subtle morphological differences can be/are observed (Pérez-Ponce de León and Nadler, 2010). Some of these reasons will be explored in more depth in a later paragraph. However, assuming

that parasites are truly cryptic, several hypotheses relating to the evolutionary ecology of parasites have been proposed. Parasites may experience strong selection on physiological or behavioural characters for adaptation to their hosts, and thus may not show morphological changes when speciation occurs (Bickford et al, 2007). However, this hypothesis has only been tested on parasitic arthropods, and has not been tested in endoparasitic helminths. It has also been hypothesized that because trematode parasites lack hard structures, there may be a time lag between genetic speciation and morphological change (Poulin, 2011). To our knowledge, this hypothesis has not been tested either. It is important to distinguish between these hypotheses as both could explain why cryptic species use the same host species. For example, three potential cryptic species previously classified as *Echinostoma trivolvis* Cort 1914 co-occur in muskrat hosts from the same geographic location (Detwiler et al, 2012). "Potential" is used deliberately here as the molecular studies that find cryptic species often do not test the hypothesis of crypsis with detailed morphological analyses (Herrmann et al, 2014). For the *E. trivolvis* complex, the hypothesis of crypsis was initially proposed after three genetically distinct groups were found to be morphologically similar at a few characters (Detwiler et al, 2010). Later, detailed morphological analyses combined with genetic analysis confirmed two of the three lineages to be cryptic (Sultana, 2018). Whether the third lineage is also morphologically similar is unknown due to a lack of properly prepared specimens. This example illustrates that for most trematodes, we are still at the stage of testing the hypothesis of crypsis rather than determining the evolutionary and ecological reasons for cryptic speciation.

While the presence of cryptic species can lead to underestimation of diversity, phenotypic plasticity can also affect estimates of parasite species diversity. Phenotypic plasticity occurs when the environment or host induces changes in parasite behaviour, morphology, or

physiology (Price et al, 2003). If phenotypic plasticity goes unrecognized, it could lead to overestimates of parasite species diversity. For example, a series of experimental infections in 14 host species demonstrated that the trematode *Quinqueserialis quinqueserialis* Barker and Laughlin 1911 was prone to host-induced phenotypic plasticity (Kinsella, 1971). One of the traits that was demonstrated to be plastic, whether or not uterine coils crossed the ceca, had been used to discriminate *Q. quinqueserialis* from another species, *Quinqueserialis hassalli* McIntosh and McIntosh 1934. However, the specimens from the lab exposures demonstrated that vole (*Microtus* spp. Schrank 1798) derived worms usually had uterine coils crossing the ceca while specimens from muskrats (*Ondatra zibethicus* Linnaeus 1766) rarely did. As a result, Kinsella (1971) synonymized the two species and reduced the number of species in this group. There is also evidence of host-induced phenotypic plasticity in field-collected trematodes. *Plagiorchis noblei* Park 1936 adults collected from two closely related and ecologically similar hosts, the Eastern kingbird (*Tyrannus tyrannus* Linnaeus 1758) and the Western kingbird (*Tyrannus verticalus* Say 1823), at the same locality were compared morphologically, and found to differ significantly (MacKenzie and MacKenzie, 1980). These specimens were morphologically similar to another species, *P. gonzalchavezi* Zerecero 1949, identified from another *Tyrannus* spp. host. Thus, it was concluded that *Plagiorchis noblei* was exhibiting host-induced phenotypic plasticity and that *P. gonzalchavezi* was synonymous with *P. noblei* (MacKenzie and MacKenzie, 1980). These two examples demonstrate that unrecognized phenotypic plasticity can lead to overestimates of parasite species diversity and that it is essential to compare specimens from different host species when characterizing intraspecific morphological variation.

Confirming whether parasites are cryptic or plastic requires a detailed assessment of morphology. Relative to the number of trematode species, there are a limited number of experts

familiar with how to obtain and properly prepare and examine specimens for morphological study. The process of preparing specimens for morphological assessment begins even before the hosts are collected. One must consider how the condition of the host (recently euthanized versus salvaged and frozen) will affect the condition of their intestinal parasites. Helminths collected from frozen hosts may have lower quality DNA and may lose important morphological structures for identification, such as spines on trematodes (Sepulveda and Kinsella, 2013). If helminths are collected from recently euthanized hosts, one must then consider how the helminths will be killed and fixed. There are many options for fixatives, and thus it is important to consider whether specimens will also be used for genetic analysis. If morphological and genetic work will be conducted on the same specimen, then diluted ethanol is a popular fixative because it preserves DNA and does not shrink and harden tissue like absolute ethanol does (Kennedy, 1979; Sepulveda and Kinsella, 2013). Another critical decision is the choice of staining agent, as different stains may highlight different organs more effectively (Kennedy, 1979). Further, the staining procedure is another step that may affect morphological analyses, as many stains require a regressive approach, with a clearing stage after staining. The clearing agent of choice and the amount of time the specimens spend in this agent can affect what internal structures become stained (Kennedy, 1979). In addition to the long and tedious process of preparing specimens, few trematode species have hard structures or obvious species-specific morphological characters (Poulin, 2011). Further, because trematodes are soft-bodied, the relative position of their internal organs may be subject to individual variation (Wallace, 1935). Thus, not only does morphological analyses require technical expertise, but it also requires large sample sizes to account for intraspecific variation, which may not be feasible to obtain depending on the trematode system of study.

The lack of defining morphological characters among trematodes also contributes to difficulty in linking larval stages to their adult counterparts (Blasco-Costa et al, 2016). There is abundant literature describing species from cercariae (free-swimming larval trematode), however most cercariae are only identified to morphotype, and these morphotypes are shared by many taxa. For example, three different trematode families, each with several genera, produce the monostome morphotype (Schell, 1985). In the past, cercariae could only be linked to the adult stage through a series of experimental infections, which for many parasite species are not feasible to carry out in the laboratory (Gonchar and Galaktionov, 2017). As a result, life cycles remain unknown for the majority of trematode parasites (Bolek et al, 2016). It is essential to elucidate the life cycles to understand the epidemiology, ecology, and evolution of these parasites.

Because of these problems with morphological identification, the use of molecular markers in parasite identification has become increasingly popular. Gene sequence analysis offers an efficient approach to characterizing distinct species and intraspecific variation (McManus and Bowles, 1996). The DNA sequence of a given region in the genome can be used as a marker of identity shared by individuals in the same species (McManus and Bowles, 1996). As sequencing technology became more accessible, universal helminth barcoding primers were developed to facilitate species identification (Moszczynska et al, 2009; Van Steenkiste et al, 2015). The barcoding approach is advantageous because it uses a standardized marker, ensuring that sequences are comparable across studies (Moszczynska et al, 2009). Selecting an appropriate gene region requires consideration of their rates of evolution and the purpose of the study (Nolan and Cribb, 2005). For example, a more conserved gene region may be targeted to elucidate relationships among parasite families, while relationships among parasite species may be better

resolved using a gene region with a faster rate of evolution, like mtDNA. One barcoding region for trematodes is the 5' end of the cytochrome c oxidase subunit I (CO1) gene in the mitochondrial genome (Moszczynska et al, 2009; Van Steenkiste et al, 2015). This region can distinguish between congeneric helminths more clearly than nuclear genes because of its higher rate of evolution (Vilas et al, 2005). Nuclear genes, such as the large ribosomal RNA subunit (28S), are generally non-coding and highly conserved (McManus and Bowles, 1996). For this reason, nuclear genes are useful in evaluating genetic differentiation in congeneric species and for determining phylogenies (Vilas et al, 2005). Gene sequence comparisons are especially useful for elucidating trematode life cycles. Where in the past trematode life cycles were solved through laboratory experimental infections, we can now field-collect larvae for genetic identification (Blasco-Costa et al, 2016). For example, two species of periwinkle (*Littorina* spp. Férussac 1822) were confirmed as intermediate hosts of the trematode *Tristriata anatis* Belopolskaia 1953 using both nuclear and mitochondrial gene sequences (Gonchar and Galaktionov, 2017). DNA barcoding of larvae presents an efficient method of surveying different geographical localities for parasites and understanding parasite life cycles and host specificity (Gordy et al, 2016).

Although gene sequences appear to provide efficient parasite species identification, there are some caveats to this method. The foremost problem with using gene sequences to identify parasites, is the potential lack of reference sequences for comparison in public databases. This problem may be more concerning for parasites that are not as well studied as those that are of human, veterinary, and agricultural importance. Second, this method relies on species identification of reference sequences to be accurate, which is not always true (e.g. Detwiler et al, 2010). The final problem with the molecular approach to parasite identification and species

delineation is the lack of biologically relevant information associated with gene sequences (Nolan and Cribb, 2005). Sequencing can inform the amount of genetic differentiation between species, but this boundary is not always clear. At conserved gene regions, a single base pair difference between specimens from two separate populations could indicate distinct genetic species. However, confidently establishing that species boundary may require investigating other aspects of the specimen's biology. Thus, to effectively study parasite taxonomy and species diversity, information about morphology, host range, life cycle, and geographic distribution along with gene sequences are essential (Nolan and Cribb, 2005). Integrating data from multiple sources provides stronger evidence to support the delineation of parasite species. Consequently, many researchers are advocating for the integration of morphological vouchers and molecular analysis in taxonomic studies (Perkins et al, 2011; Blasco-Costa et al, 2016).

Characterizing parasite diversity is complex, and thus should be studied from many perspectives that complement each other (Dayrat, 2005). Data from population biology, phylogeography, and gene sequencing can all be used to delimit species boundaries (Dayrat, 2005). While this integrative approach is ideal, it is difficult to execute in practice. In fact, many parasite species descriptions are based off a few parasites collected from few hosts in a single location (e.g. Nùñez et al, 2017). While this traditional approach to species delineation and description remains practical, the small sample sizes of specimens used in descriptions may not permit sufficient examination of intraspecific variation. Thus, when the parasite is encountered in another location, or in a different host species, the extent of morphological variation could lead to an erroneous decision to describe a new species (Dayrat, 2005; Perkins et al, 2011). The interpretation of intraspecific variation as new species leads to synonymous species names, which can affect species diversity estimates (Dayrat, 2005). To avoid creating redundant species

names, species boundaries should be revised and should incorporate information from various sources. When delimiting species, it is critical to consider what species concept is being followed because our interpretation of biodiversity is directly linked to species concepts (Fiser et al, 2017).

Several species concepts have been proposed, however only a select few are applicable to parasites. One of the most popular species concepts among vertebrate taxa is the "Biological Species Concept", where species are defined as a group of interbreeding natural populations that are reproductively isolated from other such groups (Baker and Bradley, 2006). However, this definition of a species is complicated when applied to parasites, as it is difficult to study the mating dynamics of helminths given their endoparasitic nature (Kunz, 2002). The "Morphological Species Concept", where groups of organisms with a certain level of morphological differences are considered separate species, has been used to define parasite species in the past (Kunz, 2002; Baker and Bradley, 2006). However, the occurrence of crypsis, phenotypic plasticity, and the degree of expertise needed to measure parasite morphology may limit the usefulness of this concept for trematodes. Given that a growing number of studies may only have DNA sequences from parasites, the "Genetic Species Concept" is another way to infer species boundaries. This model defines a species as a group of genetically compatible interbreeding natural populations that are genetically isolated from other such groups (Baker and Bradley, 2005). Thus, integrating morphological and genetic data allows one to determine if both the morphological species concept and the genetic species concept suggest the same answer in terms of what is a species. If there is more than one source of data suggesting distinct species, we can be more confident in the designation of parasite species and overall estimates of parasite diversity.

Once species boundaries have been established and parasite species have been accurately identified, questions related to parasite ecology and evolution can be addressed. There are several factors that may influence parasite speciation, such as the number of hosts used, host population dynamics, geographic distribution, and mobility of hosts (Price, 1977). The question of which factors have most influenced parasite speciation has rarely been tested. Two factors, host specificity and geographic distribution may act as isolating mechanisms that drive the speciation of parasites.

Patterns of host specificity can shape parasite speciation, as some parasite populations become specialized on a certain host species and diverge from other populations (Poulin, 2007). Host specificity is often defined as the range of hosts in which a parasite can successfully infect, grow, and reproduce (Lymbery, 1989; Poulin et al, 2011). Trematode parasites have complex life cycles in which they infect multiple hosts to complete their development. Specificity for hosts at each stage in the life cycle may vary. For example, most trematodes infect a single snail family, genus, or even species as their first intermediate host (Lockyer et al, 2004). High specificity for molluscan first intermediate hosts led to the hypothesis that trematodes first evolved using only molluscs in their life cycles. During this long association, there was selection, by physiological and behavioural interactions between molluscs and trematodes, for a high degree of compatibility between specific trematode-host species. High compatibility is advantageous for the parasite because it increases the likelihood of successful establishment and reproduction (Lockyer et al, 2004). In contrast, there tends to be less host specificity for second intermediate and definitive host species. For these hosts, parasites have lower compatibility for specific host species (i.e. may not establish as well) (Lockyer et al, 2004). By using a larger number or more diverse set of second intermediate or definitive host species, the parasite may increase the

likelihood of transmission to the next host in the life cycle (Adamson and Caira, 1994). Higher specificity for first intermediate hosts has suggested the hypothesis that trematode speciation is more likely to be driven by first intermediate host specificity rather than definitive host specificity (McCarthy, 1990; Jousson et al, 2000).

Parasite speciation may also be influenced by geographic isolation as the spatial structure of populations and of interspecific interactions are important factors contributing to ecological and evolutionary processes of parasites (Thompson, 1999). However, because parasites require hosts to complete their life cycles, the influence of host geographic distribution is important to consider. Specificity for hosts can be influenced by the landscape because parasites are exposed to communities of potential host species that vary in composition and density from one locality to the next (Poulin et al, 2011). Geographically isolated parasite populations can undergo adaptive divergence, leading to speciation (Lively, 2018). These geographic mosaics may be common for structured populations of hosts interacting with a variable community of parasites (Lively, 1999). Parasites may continually adapt to infect local host genotypes in some areas, creating a coevolutionary hotspot. For example, snails exposed to trematode eggs from an allopatric parasite source had a low infection frequency compared to those exposed to trematode eggs from the same source lake as the snails (King et al, 2011). Thus, it is important to consider both host use and geography when investigating parasite speciation.

Parasites can exhibit evolutionary trajectories independent from their host's evolutionary history, and in these circumstances biogeographical context and ecological settings are critical in determining patterns of diversity (Hoberg and Brooks, 2008). While it is understood that ecological and geographical associations among species of hosts and parasites are important in diversification, few studies have determined what factors have had the stronger influence on

speciation (Hoberg and Brooks, 2008). The paucity of knowledge regarding the factors influencing parasite speciation across parasitic taxa may be linked to the variation in ecological settings, host use, and biogeographical history between taxa. Thus, to disentangle and understand trematode speciation, we must first understand the basic biology and life history of parasites.

The genus *Quinqueserialis* Harwood 1939 (Platyhelminthes: Digenea) is a model for the investigation of trematode diversity and speciation because it is a small group, and thus may be more tractable to apply an integrative approach and test hypotheses of speciation. There are three nominal species of *Quinqueserialis* in North America and a single species in Europe (Barker and Laughlin, 1911; McIntosh and McIntosh, 1934; Skvortsov, 1935; Rausch, 1952). Parasites in this group use freshwater snails as intermediate hosts (larval development) and mammals, such as muskrats (*Ondatra zibethicus*) and voles (*Microtus* spp.), as definitive hosts (adult development). These parasites occur ubiquitously in muskrats from wetlands throughout North America (McKenzie and Welch, 1979; Detwiler et al, 2012). Each host in the life cycle (snails and muskrats or voles) has a broad geographic distribution and limited dispersal, making it feasible to collect all hosts at a single locality. Invertebrate collection methods and small-mammal trapping protocols are well established, and muskrats are easily collected with the help of local fur trappers. Thus, specimens from this genus are relatively easy to obtain and can therefore allow us to investigate questions regarding their diversity, life cycles and speciation.

Here, we used integrative taxonomy to confirm species diversity within the group *Quinqueserialis* in North America and investigate the factors influencing speciation. Because *Quinqueserialis* species descriptions were based off a few adult specimens from few hosts and limited geographic locations, it has been difficult to identify morphological characters that reliably differ between species. *Quinqueserialis quinqueserialis* was described morphologically

from one specimen, even though over 200 worms were collected from 27 muskrats (Barker and Laughlin, 1911). *Quinqueserialis hassalli* was described from three specimens collected from meadow voles (*Microtus pennsylvanicus* Ord 1815) and distinguished from *Q. quinqueserialis* by the distribution of vitelline follicles and by the uterine coils overlapping the intestinal ceca (McIntosh and McIntosh, 1934; Smith, 1954). However, upon further investigation it was determined that both of these morphological characters were subject to host-induced phenotypic plasticity (Kinsella, 1971). For this reason, the validity of *Q. hassalli* was questioned. Field surveys suggest that *Q. quinqueserialis* primarily uses muskrats as a final host, while *Q. hassalli* uses voles. However, experimental infections have demonstrated that *Q. quinqueserialis* can successfully establish in 14 different host species (Kinsella, 1971). The third species, *Q. floridensis* Rausch 1952 was described from specimens collected from a single round-tailed muskrat (*Neofiber alleni* True 1884) and was distinguished from the other species in this genus by its overall smaller body size and the shape and distribution of the vitelline follicles (Rausch, 1952; Kinsella, 1971).

All the species descriptions in this genus have focused on the adult stage, so life cycles and range of hosts used by larvae remain unclear. Only *Q. quinqueserialis* has had its intermediate hosts identified, the planorbid snail *Gyraulus parvus* Say 1817 in North America and *G. stroemi* Westerlund 1881 in Eurasia (Herber, 1942; Zhaltsanova and Beliakova, 1986). Surveys of freshwater snails are required to understand the geographic range and host specificity of larval *Quinqueserialis* spp. parasites. There are many gaps in the knowledge of the biology of this genus and most of the research on this group was completed before the advent of modern molecular technology. Thus, using an integrative approach will resolve the taxonomy of this genus, and allow us to accurately evaluate diversity, host specificity, and life cycles. In a broader

sense, understanding parasite diversity and life history is critical for research in wildlife disease epidemiology and can provide insights into host ecology as well.

Literature Cited

Adamson, M.L. and Caira, J.N. 1994. Evolutionary factors influencing the nature of parasite specificity. Parasitology **109**: S85-S95.

Andrews, R.H. and Chilton, N.B. 1999. Multilocus enzyme electrophoresis: a valuable technique for providing answers to problems in parasite systematics. International Journal for Parasitology **29**: 213-253.

Baker, R.J. and Bradley, R.D. 2006. Speciation in mammals and the genetic species concept. Journal of Mammalogy **87**: 643-662.

Barker, F.D. and Laughlin, J.W. 1911. A new species of trematode from the muskrat, *Fiber zibethicus*. Transactions of the American Microscopical Society **30**: 261-274.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., and Das, I. 2007. Cryptic diversity as a window on diversity and conservation. Trends in Ecology & Evolution **22**: 148-155.

Blasco-Costa, I., Cutmore, S.C., Miller, T.L., and Nolan, M.J. 2016. Molecular approaches to trematode systematics: "best practice" and implications for future study. Systematic Parasitology **93**: 295-306.

Bolek, M.G., Stigge, H.A. and Gustafson, K.D. 2016. The iron wheel of parasite life cycles: then and now! *In* A Century of Parasitology: Discoveries, ideas and lessons learned by scientists who published in The Journal of Parasitology, 1914-2014. J. Janovy Jr and G.W. Esch (eds.). John Wiley & Sons, Ltd, Chichester, UK, p. 131-147.

Dayrat, B. 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society **85**: 407-415.

Detwiler, J.T., Bos, D.H., and Minchella, D.J. 2010. Revealing the secret lives of cryptic species: examining the phylogenetic relationships of echinostome parasites in North America. Molecular Phylogenetic and Evolution **55**: 611-620.

Detwiler, J.T., Zajac, A.M., Minchella, D.J., and Belden, L.K. 2012. Revealing cryptic parasite diversity in a definitive host: echinostomes in muskrats. Journal of Parasitology **98**: 1148-1155.

Fišer, C., Robinson, C.T., and Malard, F. 2018. Cryptic species as a window into the paradigm shift of the species concept. Molecular Ecology **27**: 613-635.

Gonchar, A. and Galaktionov, K. V. 2017. Life cycle and biology of *Tristriata anatis* (Digenea: Notocotylidae): morphological and molecular approaches. Parasitology Research **116**: 45-59.

Gordy, M.A., L. Kish, M. Tarrabain, and P. C. Hannington. 2016. A comprehensive survey of larval digenean trematodes and their snail hosts in central Alberta, Canada. Parasitology Research 115: 3867-3880.

Herber, E.C. 1942. Life history studies on two trematodes of the subfamily Notocotylinae. Journal of Parasitology **28**: 179-196.

Herrmann, K.K., Poulin, R., Keeney, D.B., Blasco-Costa, I. 2014. Genetic structure in a progenetic trematode: signs of cryptic species with contrasting reproductive strategies. International Journal for Parasitology **44**: 811-818.

Hoberg, E.P. and Brooks, D.R. 2008. A macroevolutionary mosaic: episodic host-switching, geographical colonization and diversification in complex host-parasite systems. Journal of Biogeography **35**: 1533-1550.

Jousson, O., Bartoli, P., and Pawlowski, J. 2000. Cryptic speciation among intestinal parasites (Trematoda: Digenea) infecting sympatric host fishes (Sparidae). Journal of Evolutionary Biology **13**: 778-785.

Kennedy, M.J. 1979. Basic methods of specimen preparation in parasitology. International Development Research Centre, Ottawa, Ontario, Canada. P. 44.

King, K.C., Delph, L.F., Jokela, J., and Lively, C.M. 2011. Coevolutionary hotspots and coldspots for host sex and parasite local adaptation in snail-trematode interaction. Oikos **120**: 1335-1340.

Kinsella, J.M. 1971. Growth, development, and intraspecific variation of *Quinqueserialis quinqueserialis* (Trematoda: Notocotylidae) in rodent hosts. Journal of Parasitology **57**: 62-70.

Kunz, W. 2002. When is a parasite species a species? Trends in Parasitology **18**: 121-124.

Lively, C.M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. The American Naturalist **153**: S34-S47.

Lively, C.M. 2018. Habitat heterogeneity, host population structure, and parasite local adaptation. Journal of Heredity **109**: 29-37.

Lockyer, A.E., Jones, C.S., Noble, L.R., and Rollinson, D. 2004. Trematodes and snails: an intimate association. Canadian Journal of Zoology **82**: 251-269.

Lymbery, A.J. 1989. Host specificity, host range and host preference. Parasitology Today **5**: 298.

MacKenzie, D.I. and McKenzie, C.E. 1980. Morphological variation in *Plagiorchis noblei* Park 1936 (Trematoda: Plagiorchiidae) from *Tyrannus tyrannus* and *T. verticalis* (Aves: Tyrannidae). Journal of Parasitology **66**: 145-149.

McCarthy, A.M. 1990. Speciation of echinostomes: evidence for the existence of two sympatric sibling species in the complex *Echinoparyphium recurvatum* (von Linstow 1873) (Digenea: Echinostomatidae). Parasitology **101**: 35-42.

McIntosh, A. and McIntosh, G. 1934. A new trematode, *Notocotylus hassalli*, n. sp. (Notocotylidae), from a meadow mouse. Proceedings of the Helminthological Society of Washington **1**: 36-37.

McKenzie, C.E., and Welch, H.E. 1979. Parasite fauna of the muskrat, *Ondatra zibethica* (Linnaeus, 1766), in Manitoba, Canada. Canadian Journal of Zoology **57**: 640-646.

McManus, D.P. and Bowles, J. 1996. Molecular genetic approaches to parasite identification: their value in diagnostic parasitology and systematics. International Journal for Parasitology **26**: 687-704.

Moszczynska, A., Locke, S.A., McLaughlin, D., Marcogliese, D.J., and Crease, T.J. 2009. Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. Molecular Ecology Resources **9**: S75-S82.

Nadler, S.A. 1990. Molecular approaches to studying helminth population genetics and phylogeny. International Journal for Parasitology **20**: 11-29.

Nadler, S.A. and Pérez-Ponce de León, G. 2011. Integrating molecular and morphological approaches for characterizing parasite cryptic species: implications for parasitology. Parasitology **138**: 1688-1709.

Nolan, M.J., and Cribb, T.H. 2005. The use and implications of ribosomal DNA sequencing for the discrimination of digenean species. Advances in Parasitology **60**: 101-163.

Núñez, V., Drago, F., Jones, S., and Lunaschi, L. 2018. Helminth parasites of the endangered hooded grebe, *Podiceps gallardoi*, from Patagonia Argentina, with description of two new digenean species. Journal of Helminthology **92**: 563-571.

Pérez-Ponce de León, G. and Nadler, S.A. 2010. What we don't recognize can hurt us: a plea for awareness about cryptic species. The Journal of Parasitology **96**: 453-464.

Pérez-Ponce de León, G. and Poulin, R. 2017. An updated look at the uneven distribution of cryptic diversity among parasitic helminths. Journal of Helminthology **92**: 197-202.

Perkins, S.L., Martinsen, E.S., and Falk, B.G. 2011. Do molecules matter more than morphology? Promises and pitfalls in parasites. Parasitology **138**: 1664-1674.

Poulin, R. 2007. Are there general laws in parasite ecology? Parasitology **134**: 763-776.

Poulin, R. 2011. Uneven distribution of cryptic diversity among higher taxa of parasitic worms. Biology Letters **7**: 241-244.

Poulin, R. and Leung, T.L.F. 2010. Taxonomic resolution in parasite community studies: are things getting worse? Parasitology **137**: 1967-1973.

Poulin, R., Krasnov, B.R., and Mouillot, D. 2011. Host specificity in phylogenetic and geographic space. Trends in Parasitology **27**: 355-361.

Price, P.W. 1977. General concepts on the evolutionary biology of parasites. Evolution **31**: 405-420.

Price, T.D., Qvarnström, A., and Irwin, D.E. 2003. The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society of London, Series B **270**: 1433-1440.

Rausch, R. 1952. Helminths from the round-tailed muskrat, *Neofiber alleni nigrescens* Howell, with descriptions of two new species. Journal of Parasitology **38**: 151-156.

Schell, S.C. 1985. Trematodes of North America. University of Idaho Press, Moscow, Id, USA. P. 263.

Sepulveda, M.S. and Kinsella, J.M. 2013. Helminth collection and identification from wildlife. Journal of Visualized Experiments **82**: e51000.

Skvortsov, A.A. 1935. On a study of the helminth fauna of the water rat, *Arvicola terrestris*. (In Russian). Vestnik Microbiologii Epidemiologii i Parazitologii. **13**: 317-326.

Smith, C.F.1954. Studies on *Quinqueserialis hassalli* and taxonomic considerations of the species of *Quinqueserialis* (Trematoda: Notocotylidae). Journal of Parasitology **40**: 209-215.

Sultana, A. 2018. Integrating morphology and genetics to estimate species diversity, host specificity and life cycles of echinostome trematodes. MSc Thesis, University of Manitoba.

Thompson, J.N. 1999. Specific hypotheses on the geographic mosaic of coevolution. The American Naturalist **153**: S1-S14.

Van Steenkiste, N., Locke, S.A., Castelin, M., Marcogliese, D.J., and Abbott, C.L. 2015. New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). Molecular Ecology Resources **15**: 945-952.

Vilas, R., Criscione, C.D., and Blouin, M.S. 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology **131**: 1-8.

Wallace, F.G. 1935. A morphological and biological study of the trematode, *Sellacotyle mustelae* n.g., n. sp. Journal of Parasitology **21**: 143-164.

Zhaltsanova, D.S., and I. V. Beliakova. 1986. Intermediate host of the trematode *Quinqueserialis quinqueserialis* (Trematoda, Notocotylidae) in the USSR and the morphology of its parthenitae and larvae. (In Russian). Parazitologiia **20**: 323–326.

Chapter 1: Using integrative taxonomy to determine *Quinqueserialis* spp. (Notocotylidae) diversity and life history.

1.1 Introduction

There has been increasing concern that helminth species diversity is underestimated (Poulin, 2011). One reason for this, especially for trematodes, may be unrecognized cryptic species (Pérez-Ponce de León and Poulin, 2017). Cryptic species encompass a set of genetically distinct species that were previously classified as a single species because they were deemed morphologically indistinguishable (Leung et al, 2009). While many cryptic species complexes have been proposed, few studies have tested whether the species are truly cryptic. For instance, few studies 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; Detwiler et al, 2012). 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).

The first and foremost step towards improving estimates of trematode species diversity is accurate species delineation. Over the past century, new parasite species were described based on morphological differences (i.e. Barker and Laughlin, 1911). However, morphological descriptions prove problematic when cryptic species are present. Thus, species diversity can be underestimated if only using morphological characters to identify parasites present. It is also difficult to distinguish between intraspecific variation and interspecific differences when considering morphology alone (Blasco-Costa et al, 2016). One important source of intraspecific variation for parasites is related to host-induced phenotypic plasticity.

Phenotypic plasticity may occur when conspecific parasites use different environments or hosts. Parasites in each place or host may respond to different selection pressures that may

favour divergence from the ancestral population. This can result in changes in the individual's physiology, behaviour, and morphology (Price et al, 2003). Parasite phenotypic plasticity can be related to many host attributes, such as species, age, physiology, and past parasitic infection (Stunkard, 1957). Host-induced phenotypic plasticity has been observed in experimental infections with parasites. For example, Kinsella (1971) observed that *Quinqueserialis quinqueserialis* Barker and Laughlin 1911 specimens that matured in lab-infected vole (*Microtus* spp. Schrank 1798) hosts had uterine coils that overlapped the intestinal ceca and a larger overall body size, while specimens that matured in lab-infected muskrats (*Ondatra zibethicus* Linnaeus 1766) had uterine coils that were restricted in width between the intestinal ceca and were generally smaller than the specimens from voles. Thus, if only considering morphology, two *Quinqueserialis* species may be recognized that were each host specific to one host species instead of one parasite species using two hosts. As a result of this misassignment, parasite species diversity would be overestimated, and host specificity would be underestimated. In contrast to comparing specimens from lab exposures, it can be more difficult to determine whether field-collected parasites are exhibiting phenotypic plasticity or if they are distinct species. For example, an echinostome trematode species with high morphological variation infecting rodent and canid hosts was hypothesized to be several species. However, genetic sequencing revealed low divergence between specimens at the cytochrome c oxidase gene region, suggesting that all the specimens belonged to a single species (Hildebrand et al, 2015). This example illustrates that genetic data can be used to make the boundary between intra- and inter-specific morphological variation clearer and demonstrates the importance of integrating several sources of information for species delineation.

The use of genetic data in investigations of parasites has increased over the past decades (Blasco-Costa et al, 2016). While DNA barcoding provides a relatively fast and efficient way of identifying parasite species, there are some caveats to using this method alone. Genetic parasite identification relies on matching sequences to those in public databases, limiting our ability to identify parasites that are not well studied. In addition, it assumes that the GenBank specimens were accurately identified, which is not always the case (e.g. Detwiler et al, 2010). Further, using solely genetic data may not indicate whether variation in many aspects of parasite life history, such as host specificity, morphology, and development, is occurring. Consequently, there has been a rising interest in integrative taxonomy, an approach to species delineation that combines genetic, morphological and ecological data (Dayrat, 2005).

An integrative approach to parasite identification is not only essential for species delineation, but also for inferring parasite life cycles. Due to the small size of parasite larvae and adults, and the lack of distinguishing morphological features, it is challenging to link parasite life cycle stages without experimental studies and genetic data (Blasco-Costa et al, 2016). In the absence of DNA sequences, the only way to link larval stages to adults was through a series of experimental infections (Gonchar and Galaktionov, 2017). Due to the complexity of trematode life cycles, involving many hosts, this method may not be feasible nor economical for many trematode species. Further, obtaining institutional animal care ethics approval may not be possible depending on the host species used by the parasite of interest. Even if DNA sequencing is used, parasite stages from all hosts in the life cycle need to be sampled, which can be challenging depending upon who those hosts are. These challenges may help to explain why many studies focus either on collecting larvae from snails (e.g. Gordy et al, 2016) or adults from vertebrate hosts (e.g. McKenzie and Welch, 1979). This type of approach means that many

parasite life cycles remain unknown or their accuracy is unverified. For example, if there are cryptic species present, then it could be that the description of the nominal species life cycle is inaccurate. Thus, a holistic approach that includes sampling both the intermediate and final hosts and integrating morphological and genetic data is essential in understanding the evolutionary ecology and diversity of parasites.

The genus *Quinqueserialis* Harwood 1939 is an appealing model for implementing an integrative study of species diversity and life history. This group is small and thus it is tractable to thoroughly investigate aspects of each species' life history. Parasites in this genus require two hosts to complete their life cycle: a freshwater gastropod intermediate host and mammalian final hosts such as muskrats and voles (Herber, 1942). It is relatively easy to collect all the hosts in the life cycle at a single location, especially with the help of fur trappers (McKenzie and Welch, 1979; Detwiler et al, 2012). Further, this group of parasites has a large geographic distribution extending from the Canadian Arctic to the southern United States making is possible to study host-parasite interactions in several locations throughout the range.

Five species of *Quinqueserialis* have been described worldwide. The first species, *Quinqueserialis wolgaensis* Skvortsov 1935, was described from the European water vole (*Arvicola terrestris* Linnaeus 1758) in the Volga river valley, Russia (Skvortsov, 1935). Later, this species was synonymized with *Quinqueserialis hassalli* McIntosh and McIntosh 1934 (a species described in North America) due to similarities in morphology and final host use (both use vole species) (Rausch, 1952B; Smith, 1954). However, later studies would find that *Q. hassalli* was not a valid species, which returned species level status to *Q. wolgaensis* (Kinsella, 1971). Further, *Q. wolgaensis* was confirmed as morphologically different from another North American species, *Quinqueserialis quinqueserialis*. With the introduction of muskrats from

North America into Eurasia and the ecological overlap between voles and muskrats, it was suggested that the parasites from voles in Russia could be the same species as those inhabiting muskrats in North America (Kinsella, 1969). However, *Q. wolgaensis* had a smaller overall body size, small oral sucker, and shorter metraterm than other *Quinqueserialis* spp. and remained a valid species (Kinsella, 1971).

The other four species were described in North America, though only two are still valid (Barker and Laughlin, 1911; McIntosh and McIntosh, 1934; Rausch, 1952A; Kinsella, 1971). The uncertainty surrounding species diversity in North America revolves around morphological descriptions from few specimens and limited geographic sampling. One valid species is *Q. quinqueserialis*, which was morphologically described from a single specimen from a muskrat and was not deposited into a museum (Barker and Laughlin, 1911). Because there is no holotype for *Q. quinqueserialis*, the validity of the congeners, *Q. hassalli* and *Quinqueserialis zibethicai* Gupta 1962, were questioned. The species *Q. hassalli* was morphologically described from three specimens, two of which were *Q. quinqueserialis* vouchers deposited in museums (McIntosh and McIntosh, 1934). These two museum specimens were reassigned to *Q. hassalli* because they were collected from a different final host, the meadow vole (*Microtus pennsylvanicus* Ord 1815), and varied morphologically from the *Q. quinqueserialis* description (McIntosh and McIntosh, 1934). Comparative morphological studies determined that the width of the uterine coils in adult worms was a distinguishing feature between *Q. quinqueserialis* and *Q. hassalli* (Smith, 1954). Later, experimental infections determined that this trait was subject to host-induced phenotypic plasticity and was therefore not reliable in distinguishing between the two species (Kinsella, 1971). Because of this morphological variation, Kinsella (1971) concluded that *Q. hassalli* was not a distinct species and synonymized it with *Q. quinqueserialis*.

Another valid species found in North America, *Q. floridensis* Rausch 1952, was morphologically described from specimens collected from round-tailed muskrats (*Neofiber alleni* True 1884) (Rausch, 1952A). Comparative morphological studies showed that this species was distinct from the other *Quinqueserialis* spp. due to the nature of the vitelline follicles and overall smaller body size (Smith, 1954; Kinsella, 1971). In addition to *Q. hassalli*, another described species is no longer recognized as valid. The species *Q. zibethicai* was morphologically described from a single specimen found in the duodenum of a muskrat (Gupta, 1962). Again, the type specimen used for description was not deposited in a museum (Kinsella, 1969). Further, despite many surveys of muskrat helminths across North America, no other specimens belonging to this species have been found (Kinsella, 1969). Comparisons to other *Quinqueserialis* species suggested that *Q. zibethicai* could be an erroneous description of a *Notocotylus* spp. Diesing 1839 specimen (Kinsella, 1969). Muskrats act as a definitive host for the species *Notocotylus filamentis* Barker 1915, which has also been found in the duodenum (Barker, 1915). However, because there is no *Q. zibethicai* type specimen available for further investigation, and more specimens have not been subsequently identified, Kinsella (1971) proposed that *Q. zibethicai* be considered a *species inquirenda.*

All of these species descriptions focused on the adult stages found within the mammalian hosts, so the life cycles and range of snail hosts used by larvae remains unclear. Only *Q. quinqueserialis* has had intermediate hosts identified, the freshwater snails *Gyraulus parvus* Say 1817 in North America, and *Gyraulus stroemi* Westerlund 1881 in Russia (Herber, 1942; Zhaltsanova and Beliakova, 1986). However, *G. stroemi* was confirmed as an intermediate host by experimentally infecting laboratory mice with metacercariae sourced from the naturally infected field snails and morphologically identifying adults recovered from the lab-infected mice

(Zhaltsanova and Beliakova, 1986). Because both *Q. wolgaensis* and *Q. quinqueserialis* occur in Russia, genetic identification of larvae from infected snails would be required to confirm the role of *G. stroemi* in either *Q.* spp. life cycle. Similarly, in North America, surveys of snail hosts are needed to complete life cycles and to better understand intermediate host specificity. Laboratory experiments using *Q. quinqueserialis* found that specimens reached sexual maturity in 12 rodent hosts (Kinsella, 1971). Whether *Q. floridensis* can infect mammals other than round-tailed muskrats is not known. Thus, the host specificity at each point in the life cycle for all of the North American species of *Quinqueserialis* remains unclear. Identifying both intermediate and final host specificity is critical for understanding transmission dynamics of each species. By accurately identifying parasite species and life cycles, we will have a better understanding of the basic biology of parasites and this will then make studying their diversification possible.

Here, we used integrative taxonomy to characterize species diversity of *Quinqueserialis* parasites in North America. Due to the low interspecific morphological variation between *Quinqueserialis* species and the high occurrence of crypsis reported among trematodes, we predicted that cryptic *Quinqueserialis* species may be detected. After accurately identifying *Quinqueserialis* spp., we confirmed whether descriptions of host specificity and life cycles needed revision. Due to low sampling effort and limited geographic extent of past surveys (i.e. none in Canada), we predicted that novel hosts, especially intermediate host snails, would be identified.

1.2 Methodology

1.2.1 Specimen collection

Hosts were field-collected at six locations throughout five states and provinces in North America: Inuvik, Northwest Territories and Churchill and Winnipeg, Manitoba, Canada, and

Mankato, Minnesota, Blacksburg, Virginia, and Florence, Alabama, USA (Table S1.1**)**. Muskrat carcasses were salvaged from licensed trappers at each location, excluding Churchill, from 2015-2018. Carcasses were frozen at -20 C until necropsy. Voles were either live-trapped or snap-trapped at the same locations where muskrats were collected (Table S1.1). Upon recovery, voles (*Microtus* spp.) were euthanized in the field via anesthetic overdose followed by cervical dislocation (Animal Use Protocol AC11347, Appendix 2). Voles were sorted into individual bags and transported to the lab for necropsy. The intestinal tract, from the duodenum to colon, of each host was sectioned, separated in petri dishes, and opened longitudinally. The contents of each section of intestinal tract were searched systematically for *Quinqueserialis* spp. adults under a stereomicroscope. Live parasites were heat killed and relaxed in 70 C distilled water and preserved in 80% EtOH for molecular and morphological analysis. Adult worms were initially identified as *Quinqueserialis* spp. by the presence of 5 rows of ventral papillae (Barker and Laughlin, 1911).

Freshwater snails were hand-collected at three locations where muskrats and voles were obtained: Inuvik, NT; Winnipeg, MB; and Mankato, MN (Table S1.1). A total of six species of snails were collected throughout the locations: *Gyraulus circumstriatus* Tryon 1866, *Promenetus exacuous* Say 1821, *Gyraulus crista* Linnaeus 1758, *Planorbula* sp. Say 1821, *Helisoma* sp. Swainson 1840, and *Lymnaea elodes* Say 1821. Snail species were identified as described by Gagnon and Detwiler (Manuscript in review, Appendix 3). In the lab, snails were assessed for parasites by placing them in individual well plates filled with well water, which were then placed under lights to stimulate cercarial (larval swimming stage of trematodes) emergence. After three hr, snails were examined under a dissecting microscope for emerging cercariae (Figure 1.1). A subset of snails was then crushed to determine if there were latent infections. Monostome

morphotype cercariae were distinguished from other larval trematodes based on the absence of a ventral sucker and the presence of eye spots (Schell, 1985). Larvae (cercariae and rediae) were preserved in 80% EtOH for molecular analysis.

The holotype specimen for *Q. hassalli* and a paratype of *Q. floridensis* were loaned from the Smithsonian Institution and included in the morphological analyses. No genetic data could be obtained from these specimens as they were stained and permanently mounted. Fifteen additional specimens identified as *Quinqueserialis* spp. were also loaned from the Smithsonian Institution to assess intraspecific morphological variation (Table S1.3).

1.2.2 Morphological measurements

Prior to DNA extraction, two gravid adult worms were haphazardly selected from each infected host. A voucher image was captured of each worm using an Axio Cam ICcI digital camera connected to a Stereo Discovery V8 microscope and Axio Imager M2 compound microscope (Zeiss). These images were used to assess total body length and width, and oral sucker length and width (μm) so that the anterior section of the worm could be used in DNA extraction.

Once the tissue sample was retrieved, adult worms were stained with acetocarmine, dehydrated in ethanol, cleared in xylene and mounted in Canada Balsam to create hologenophore-type vouchers (Pleijel et al, 2008). Hologenophores are samples from which parts are removed for molecular study and the remaining organism is deposited as a voucher. An adult worm recovered from the same host as molecular voucher worms was also permanently mounted to create paragenophore-type vouchers (Pleijel et al, 2008). Paragenophores are collected from the same host as the sample used in molecular studies, thereby it is assumed that they come from the same population.

For each specimen, we attempted to measure a total of 11 morphological traits included in *Quinqueserialis* spp. descriptions. However, 4 of these morphological traits were not included in the analyses because measurements could not be obtained from each specimen because they were not clearly visible without staining and were removed with the anterior end of the worm for DNA extraction. These features included: metraterm length and width, Mehlis gland length and width, cirrus length, and cirrus sac length and width. The 13 measurements of seven morphological traits included in the analyses were: width of uterine coils, length of the right and left rows of vitelline follicles, width and length of the ovary, and width and length of the two testes (μm) (Figure 1.2). Larval parasites were live-mounted to capture voucher images. All morphological characters were measured using ZEN Microscope and Imaging Software (Zeiss).

1.2.3 Molecular sequencing

After a voucher image of the whole worm was captured, a 1 mm^2 piece of the anterior end of the body was removed for DNA extraction. Entire larvae collected from the same snail host as imaged larvae were used in DNA extractions. Because trematode larvae reproduce asexually inside snail hosts, we assumed all collected larvae represented a single haplotype (Esch et al, 2002). 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 MilliQ water.

We attempted to amplify approximately 1000 base pairs (bp) of the 28S ribosomal RNA gene and 500 bp of the cytochrome c oxidase subunit I (CO1) mitochondrial gene region for each individual. The 28S RNA gene region was targeted because it is highly conserved and commonly utilized to investigate interspecific variation among trematodes (Blasco-Costa et al, 2016). 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 10x buffer, 1.5 µM of $MgCl_2$, 0.5 µM of each primer, 0.5 µM of dNTP and 0.05/µL units Taq polymerase. The CO1 amplifications were carried out in 25 µL reactions containing 5 µL of extracted DNA, 2.5 µM of 10x buffer, 3.5 µM of $MgCl_2$, 0.5 µM of each primer, 0.5 µM of dNTP and 0.5/µL units Taq polymerase. The primers and thermocycling conditions used for each gene region are summarized in Table 1.1. If amplification at the 28S gene region was unsuccessful with the LSU-5 forward primer, then samples were amplified with the 300F primer instead, resulting in a smaller gene fragment (Table 1.1). Products were visualized with a 2% agarose gels and products with single bright bands were purified with a PCR clean up kit (MO BIO Laboratories. Inc) and sequenced in both directions at the Hospital for Sick Children, Toronto, ON.

Contigs were constructed and manually edited by eye using Sequencher 4.1 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. Genetic similarity was used to match life cycle stages. 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 cutoffs were used to assign specimens to species groups, and genetic distances between and among species was calculated with uncorrected p-distance in MEGA.

1.2.4 Multivariate analyses

Multivariate statistical analyses were performed on 13 morphological measurements on a total of 96 specimens (Table S1.2). Each variable was tested for normality with the Shapiro-Wilk test, and then log-transformed if not normally distributed ($P<0.05$). Principal component analyses (PCA) were performed to determine if specimens clustered according to nominal species (Table S1.3). These analyses included specimens that we field-collected and genetically identified. These analyses also included museum specimens that were morphologically identified by other researchers (Table S1.2**)**. Results from the PCA were used to assign specimens to groups that were then analyzed in a linear discriminant analysis (LDA). The LDA was used to determine what morphological characters discriminated each group. 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).

1.3 Results

Specimens identified as *Q. quinqueserialis* were field-collected from at least one host at the six sampling locations (Table S1.1). Prevalence of *Q. quinqueserialis* infection was 81% (83/102) in muskrats and 8% (9/117) 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.* Of the six snail species collected, three were infected with monostome-type cercariae: *Gyraulus circumstriatus*, *Gyraulus crista*, and *Promenetus exacuous*. The prevalence

of monostome infections was 1.6% (14/870) in *G. circumstriatus*, 7.4% (4/27) in *G. crista,* and 5.1% (5/98) in *P. exacuous*.

1.3.1 Morphological variation within the genus *Quinqueserialis*

Thirteen morphological measurements were analyzed for a total of 96 specimens identified as *Quinqueserialis* spp.: 27 museum specimens, 15 field-collected paragenophores, and 54 field-collected hologenophores (Table 1.2). Two specimens were excluded from the multivariate analyses due to missing morphological traits. 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 prior to analysis. The first and second principal components accounted for 82% of the total observed morphological variation (Table 1.3). The first principal component (PC1) accounted for 69% of the total variation (eigenvalue= 9.1). 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= 1.6). 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.

Museum specimens identified as *Q. hassalli* clustered with both museum and field-collected specimens identified as *Q. quinqueserialis* (Figure 1.3A). Notably, the type specimen used in the original *Q. hassalli* description fell within this group (Figure 1.3B). Among the specimens identified as *Q. quinqueserialis*, those from voles were generally larger than those from muskrats (Figure 1.4). The museum specimens identified as *Q. floridensis* formed a separate cluster along PC2 from those identified as *Q. quinqueserialis* and *Q. hassalli*. The *Q.*

floridensis specimens were the smaller than specimens in the other clusters and had wider uterine coils (Figure 1.3A). There was a third cluster consisting of museum specimens identified as *Q. hassalli* from voles and specimens from field-collected voles. This cluster separated from the *Q. floridensis* specimens along PC1 and from the *Q. quinqueserialis*/*Q. hassalli* cluster along PC2 (Figure 1.3A). Specimens within this cluster were larger than *Q. quinqueserialis* specimens, had wider uterine coils, but had relatively smaller oral suckers (Figure 1.3A). The three clusters depicted in Figure 1.4 were used to assign specimens to groups for the linear discriminant analysis (LDA). In the LDA, the three groups are subsequently referred to as: *Q. quinqueserialis*, *Q. floridensis* and *Quinqueserialis* n. sp.

The linear discriminant analysis run on 13 morphological characters separated the three species with 95% accuracy (Wilks Lambda= 0.2, χ^2= 71, $p < 0.001$). The first canonical function clearly discriminated *Q. quinqueserialis* specimens from the other two species, whereas the second canonical function discriminated *Q. floridensis* from *Q.* n. sp (Figure 1.5). The specimens of *Q. floridensis* and *Q.* n. sp. were all assigned to their *a priori* groups whereas four specimens of *Q. quinqueserialis* were misclassified as *Quinqueserialis* n. sp. (4.2% misclassification rate). These uncertainties are indicated by the higher dispersion and overlap among specimens of *Q. quinqueserialis* and *Q.* n. sp. (Figure 1.5). The discriminatory power of the model was associated with 5 variables: oral sucker length, oral sucker width, left testes width, left vitellaria length, and width of uterine coils.

1.3.2 Genetic identification of *Quinqueserialis* spp.

In total, 49 individual specimens were genetically identified. For the mitochondrial gene, we generated 54 CO1 sequences (each 511 bp), including four larval sequences from four snail hosts, 21 adult sequences from 11 vole hosts, and 29 adult sequences from 18 muskrat hosts.

Among these sequences, there were 28 unique haplotypes. For the nuclear gene region, we generated 78 partial 28S sequences (each 891 bp), including 15 larval sequences from 15 snail hosts, 19 adult sequences from nine vole hosts, and 43 adult sequences from 27 muskrat hosts. There were two unique 28S haplotypes.

The mean genetic distance at the CO1 gene region between *Q. quinqueserialis* and *Q.* n. sp. was 10% (over 452 bp), which is two times the percent 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.* n. sp. The mean genetic distance at the 28S gene region between *Q. quinqueserialis* and *Q.* n. sp. 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 either *Q. quinqueserialis* and *Q.* n. sp. specimens. Genetic comparisons of *Q. quinqueserialis* and *Q.* n. sp. to *Q. floridensis* specimens could not be completed as DNA could not be extracted from the museum specimens. Rediae recovered from *G. circumstriatus*, *G. crista*, and *P. exacuous* collected in Manitoba and the Northwest Territories were genetically identical at the 28S and CO1 gene regions to adult *Q. quinqueserialis* worms recovered from voles and muskrats in these regions.

1.4 Discussion

This study is the first to incorporate genetic and morphological data focused on characterizing species in the genus *Quinqueserialis.* Although we hypothesized that cryptic species may be found, both PCA and LDA analyses demonstrated the presence of three morphologically distinct parasite species in North America. Two of the three species were also genetically distinct, while the remaining species requires additional sampling as all known

specimens cannot be sequenced. Thus, in contrast to many other trematode groups, at present there is no evidence for crypsis in *Quinqueserialis* spp. Yet, our study confirmed that *Quinqueserialis* spp. diversity was underestimated because a new species was discovered. Importantly, if using morphology alone, this new species could be misclassified as *Q. quinqueserialis* and vice versa due to host-induced phenotypic plasticity. 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 life history 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 did not support the hypothesis of cryptic *Quinqueserialis* species. Instead, morphological analyses revealed three clusters representing separate parasite species. Given that the genetic analyses confirmed two of the three clusters, we are confident that there are three distinct species in our sampling. Cryptic species are hypothesized to occur more frequently among trematodes than any other helminth taxa (Pérez-Ponce 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 integrated molecular and morphological analyses. For *Quinqueserialis*, most studies use 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). If studies using genetics alone propose cryptic species complexes, parasites should be considered provisionally cryptic because morphological analyses may reveal diagnostic morphological differences that can be used in species descriptions (Pérez-Ponce de León and Nadler, 2010). 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 linear discriminant analysis. However, the width of the uterine coils has been shown to be subject to host-induced phenotypic plasticity (Figure 1.3, Kinsella, 1971). The intraspecific variation observed in this trait could explain the 4.2% misclassification rate of the LDA. Thus, while not cryptic, 4% of *Quinqueserialis* specimens collected could be misidentified, 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.

Host-induced phenotypic plasticity among *Q. quinqueserialis* resulted in specimens from vole hosts being larger and having uterine coils that overlapped with their intestinal ceca compared to specimens from muskrats. This type of plasticity was previously noted by Rausch (1952B) and Kinsella (1971) and led to an erroneous description of *Q. hassalli* (McIntosh and McIntosh, 1934; Kinsella, 1971). Not surprisingly, we found that the type specimen of *Q. hassalli* clustered with *Q. quinqueserialis* specimens in the PCA, supporting Kinsella's (1971) decision to synonymize the species with *Q. quinqueserialis*.

In addition to the historical misclassification, our analyses suggested that host-induced phenotypic plasticity could lead to misclassification of *Q. quinqueserialis* with the newly discovered *Quinqueserialis* n. sp. This misclassification is notable, as our study demonstrated that these two species co-occur at the same location and in the same host species, *Microtus pennsylvanicus* voles. Further, there were four museum specimens identified as *Q. quinqueserialis* from meadow voles in Minnesota and Virginia that clustered with the novel *Quinqueserialis* species. Without further sampling in the source locations of these museum specimens and subsequent genetic identification, we cannot confirm whether these specimens

belong to the novel species and have been misidentified as *Q. quinqueserialis*. However, if these specimens are genetically distinct then museum records would need to be updated.

Our study increases the number of 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 agree with Kinsella's conclusions, but also reveal a third novel species occurring in meadow voles in Churchill, MB. Not only is this species morphologically distinct, but it is genetically different from *Q. quinqueserialis* at both the nuclear 28S and mitochondrial CO1 gene regions. Thus, according to the criteria for the morphological and genetic species concepts, these two parasite groups could be designated as different species (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 (Table 1.2). 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. We did not obtain vouchers of the Eurasian species, *Q. wolgaensis*, thus the validity of this species remains unknown. Although, morphological comparisons between the North American *Quinqueserialis* species and *Q. wolgaensis* concluded that this species could be distinguished by its smaller oral sucker and wider body (Table 1.2, Kinsella, 1969). Future research should use the integrative taxonomic approach to confirm the validity of *Q. floridensis* and *Q. wolgaensis*.

In addition to improving estimates of species diversity in this group, we also improved our knowledge of their life cycles. We genetically linked rediae from three snail species to adult

stages of *Q. quinqueserialis* from two mammalian hosts, confirming the life cycle in two locations. The three snail species are new host records for *Q. quinqueserialis*: *G. circumstriatus*, *G. crista* and *P. exacuous* (Gagnon and Detwiler, Manuscript in review, Appendix 3). All three snails were used as intermediate hosts at both locations, Inuvik, NT and Winnipeg, MB. Based on our current results, there is no life cycle variation across geography, at least in the northern and central regions of the *Q. quinqueserialis* range (Gagnon and Detwiler, Manuscript in review, Appendix 3). Two intermediate snail hosts had been previously reported, one in the United States (*Gyraulus parvus*) and one in Russia (*Gyraulus stroemi*) (Herber, 1942; Zhaltsanova and Beliakova, 1986). Here, we report the first naturally infected snail hosts in Canada and increase the known host range to five snail hosts. At each life cycle stage, *Q. quinqueserialis* can successfully exploit more than one host species. We found *Q. quinqueserialis* infecting three mammalian final hosts: muskrats, meadow voles, and a tundra vole. In addition, other vole species such as the montane vole (*Microtus montanus* Peale 1848), prairie vole (*Microtus ochrogaster* Wagner 1842), and the singing vole (*Microtus miurus* Osgood 1901), are suitable hosts for *Q. quinqueserialis* (Rausch and Tiner, 1949; Kinsella, 1969; Haukisalmi et al, 1995). This generalist strategy is advantageous because it facilitates colonization of hosts and new locations and increases the chances of avoiding local extinction (Adamson and Caira, 1994). The success of this strategy is demonstrated in *Q. quinqueserialis*, with its widespread distribution and lack of divergent cryptic lineages.

1.5 Conclusions

Many researchers assume trematode species are cryptic without using in depth morphological analyses to confirm this hypothesis. Using an integrative taxonomic approach, we tested the cryptic species hypothesis and found no evidence for crypsis in the genus

Quinqueserialis. However, similar to our prediction, *Quinqueserialis* diversity was underestimated, but for a different reason. We found a novel species in a location that had not previously been sampled. Including this undescribed species, we confirmed the presence of at least three species of *Quinqueserialis* in North America, including two which were nominal. We produced novel genetic and morphological vouchers that will be publicly available and aid in future studies. Our broad geographic sampling and snail surveying led to the discovery of three novel intermediate host records for *Q. quinqueserialis*, improving our understanding of parasite host specificity and life cycles. This research is significant because knowledge of life cycles and host use are essential to understanding the spread of parasitic disease. Further, because we accurately assessed parasite diversity, we can now investigate questions regarding parasite evolutionary ecology.

Literature cited

Adamson, M.L. and Caira, J.N. 1994. Evolutionary factors influencing the nature of parasite specificity. Parasitology **109**: S85-S95.

Abdi, H. and Williams, L.J. 2010. Principal component analysis. WIREs Computational Statistics **2**: 433-459.

Baker, R.J. and Bradley, R.D. 2006. Speciation in mammals and the genetic species concept. Journal of Mammalogy **87**: 643-662.

Barker, F.D. and Laughlin, J.W. 1911. A new species of trematode from the muskrat, *Fiber zibethicus*. Transactions of the American Microscopical Society **30**: 261-274.

Barker, F.D. 1915. Parasites of the American muskrat, *Fiber zibethicus*. Journal of Parasitology **4**: 187-197.

Blasco-Costa, I., Balbuena, J.A., Raga, J.A., Kostadinova, A., and Olson, P.D. 2010. Molecules and morphology reveal cryptic variation among digeneans infecting sympatric mullets in the Mediterranean. Parasitology **137**: 287-302.

Blasco-Costa, I., Cutmore, S.C., Miller, T.L., and Nolan, M.J. 2016. Molecular approaches to trematode systematics: "best practice" and implications for future study. Systematic Parasitology **93**: 295-306.

Blouin, M.S. 2002. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. International Journal for Parasitology **32**: 527-531.

Dayrat, B. 2005. Towards integrative taxonomy. Biological Journal of the Linnean Society **85**: 407-415.

Detwiler, J.T., Bos, D.H., and Minchella, D.J. 2010. Revealing the secret lives of cryptic species: examining the phylogenetic relationships of echinostome parasites in North America. Molecular Phylogenetic and Evolution **55**: 611-620.

Detwiler, J.T., Zajac, A.M., Minchella, D.J., and Belden, L.K. 2012. Revealing cryptic parasite diversity in a definitive host: echinostomes in muskrats. Journal of Parasitology **98**: 1148-1155.

Esch, G.W., Barger, M.A., and Fellis, K.J. 2002. The transmission of digenetic trematodes: style, elegance, complexity. Integrative and Comparative Biology **42**: 304-312.

Gonchar, A. and Galaktionov, K. V. 2017. Life cycle and biology of *Tristriata anatis* (Digenea: Notocotylidae): morphological and molecular approaches. Parasitology Research **116**: 45-59.

Gordy, M.A., Kish, L., Tarrabain, M., and Hanington, P. C. 2016. A comprehensive survey of larval digenean trematodes and their snail hosts in central Alberta, Canada. Parasitology Research **115**: 3867: 3880.

Gupta, S.P. 1962. On a new trematode, *Quinqueserialis zibethicai* sp. nov. of the subfamily Notocotylinae from the duodenum of muskrat (*Ondatra zibethicus*) from Canada. Indian Journal of Helminthology **14**: 139-144.

Haukisalmi, V., H. Henttonen, and G.O. Batzli. 1995. Helminth parasitism in the voles *Microtus oeconomus* and *M. miurus* on the North Slope of Alaska: host specificity and the effects of host sex, age and breeding status. Annales zoologici Fennici **32**: 193–201.

Herber, E.C. 1942. Life history studies on two trematodes of the subfamily Notocotylinae. Journal of Parasitology **28**: 179-196.

Hildebrand, J., Adamczyk, M., Laskowski, Z., and Zaleśny, G. 2015. Host-dependent morphology of *Isthmiophora melis* (Schrank, 1788) Luhe, 1909 (Digenea, Echinostomatinae)—morphological variation vs. molecular stability. Parasites & Vectors **8**: 481.

Kinsella, J.M. 1969. Intraspecific variation of *Quinqueserialis quinqueserialis* (Barker and Laughlin 1911) in rodent hosts. PhD Dissertation, University of Montana.

Kinsella, J.M. 1971. Growth, development, and intraspecific variation of *Quinqueserialis quinqueserialis* (Trematoda: Notocotylidae) in rodent hosts. Journal of Parasitology **57**: 62-70.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution **33**: 1870-1874.

Leung, T.L.F., Keeney, D.B., and Poulin, R. 2009. Cryptic species complexes in manipulative echinostomatid trematodes: when two become six. Parasitology **136**: 241-252.

McIntosh, A. and McIntosh, G. 1934. A new trematode, *Notocotylus hassalli*, n. sp. (Notocotylidae), from a meadow mouse. Proceedings of the Helminthological Society of Washington **1**: 36-37.

McKenzie, C.E., and Welch, H.E. 1979. Parasite fauna of the muskrat, *Ondatra zibethica* (Linnaeus, 1766), in Manitoba, Canada. Canadian Journal of Zoology **57**: 640-646.

Moszczynska, A., Locke, S.A., McLaughlin, D., Marcogliese, D.J., and Crease, T.J. 2009. Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. Molecular Ecology Resources **9**: S75-S82.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., and Wagner, H. 2019. Vegan: Community ecology package. R package version 2.5-4.

Pérez-Ponce de León, G. and Nadler, S.A. 2010. What we don't recognize can hurt us: a plea for awareness about cryptic species. The Journal of Parasitology **96**: 453-464.

Pérez-Ponce de León, G. and Poulin, R. 2017. An updated look at the uneven distribution of cryptic diversity among parasitic helminths. Journal of Helminthology **92**: 197-202.

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P., and Thollesson, M. 2008. Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. Molecular Phylogenetics and Evolution **48**: 369-371.

Poulin, R. 2011. Uneven distribution of cryptic diversity among higher taxa of parasitic worms. Biology Letters **7**: 241-244.

Poulin, R. and Leung, T.L.F. 2010. Taxonomic resolution in parasite community studies: are things getting worse? Parasitology **137**: 1967-1973.

Price, T.D., Qvarnström, A., and Irwin, D.E. 2003. The role of phenotypic plasticity in driving genetic evolution. Proceedings of the Royal Society Series B **270**: 1433-1440.

Rausch, R. and Tiner, J.D. 1949. Studies on the parasitic helminths of the North Central States. II. Helminths of voles (*Microtus* spp.) Preliminary Report. The American Midland Naturalist **41**: 665-694.

Rausch, R. 1952A. Helminths from the round-tailed muskrat, *Neofiber alleni nigrescens* Howell, with descriptions of two new species. Journal of Parasitology **38**: 151-156.

Rausch, R. 1952B. Studies on the helminth fauna of Alaska. XI. Helminth parasites of microtine rodents—taxonomic considerations. Journal of Parasitology **38:** 415-444.

Schell, S. C. 1985. Handbook of trematodes of North America, North of Mexico. University Press Idaho, Moscow, Id, USA, P. 263.

Skvortsov, A.A. 1935. On a study of the helminth fauna of the water rat, *Arvicola terrestris*. (In Russian). Vestnik Microbiologii Epidemiologii i Parazitologii. **13**: 317-326.

Smith, C.F.1954. Studies on *Quinqueserialis hassalli* and taxonomic considerations of the species of *Quinqueserialis* (Trematoda: Notocotylidae). Journal of Parasitology **40**: 209-215.

Stunkard, H.W. 1957. Intraspecific variation in parasitic flatworms. Systematic Biology **6**: 7-18.

Vilas, R., Criscione, C.D., and Blouin, M.S. 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology **131**: 1-8.

Venables, W.N. and Ripley, B.D. 2002. Modern applied statistics with S. 4th Ed. Springer, NY, USA.

Zhaltsanova, D.S., and I. V. Beliakova. 1986. Intermediate host of the trematode *Quinqueserialis quinqueserialis* (Trematoda, Notocotylidae) in the USSR and the morphology of its parthenitae and larvae. (In Russian). Parazitologiia **20**: 323–326.

Table 1.1: Primers and thermocycling conditions for PCR with *Quinqueserialis* spp.

Conditions used to amplify the partial 28S rRNA gene region and partial cytochrome c oxidase subunit I (CO1) mtDNA gene.

Fragment	Forward (F)/ Reverse (R)	Primer Name	Sequence (5'-3')	Reference	Thermocycler profile
28S	F	LSU-5	TAG GTC GAC CCG CTG AAY TTA AGC A	Olson et al, 2003	94˚C—3 min 94˚C—1 min 56˚C—45 sec 72˚C—2 min (X 35) 72˚C—7 min
	R	1500R	GCT AGG GAA ACT TCG	Olson et al, 2003	
	F	300F	CAA GTA CCG TGA GGG AAA GTT G	Olson et al, 2003	95 ˚C—3 min 94 ˚C—45 sec 56 ˚C—30 sec 72 ˚C—2 min (X 40) 72 ˚C—7 min
	R	1500R	GCT AGG GAA ACT TCG	Olson et al, 2003	
CO1	F	DICE 1F	TTW CNT TRG ATC ATA AG	Moszczynska et al, 2009	95 ˚C—2 min 95 ˚C—30 sec 50 ˚C—30 sec 72 ˚C—1 min (X 35) 72 ˚C—10 min
	R	DICE 11R	GCW GWA CHA AAT TTH CGA TC	Van Steenkiste et al, 2015	

Table 1.2: Comparative morphological data for four *Quinqueserialis* species.
Measurements of 13 morphological variables of three species of *Quinqueserialis* found in North America: *Q. quinqueserialis, Q. floridensis, Quinqueserialis* n. sp. and the Eurasian species, *Q. wolgaensis*. Measurements of *Q. wolgaensis* were obtained from Kinsella (1969).

Species	***Q. quinqueserialis* (N=83)**			***Q. floridensis* (N=5)**			***Quinqueserialis* n sp (N=8)**			***Q. wolgaensis* (N=5)**	
Morphological features	**Mean**	**±SE**	**Range**	**Mean**	**±SE**	**Range**	**Mean**	**±SE**	**Range**	**Mean**	**Range**
Total length	3615	78	1922-5232	1817	192	1354-2299	3614	196	2897-4386	3077	2842-3391
Total width	1251	35	601-1967	1018	92	729-1236	1739	111	1142-2036	2095	1976-2210
OS length	352	8	182-508	169	19	132-239	287	32	164-474	NA	NA
OS width	387	8	229-585	171	16	141-222	331	21	233-442	290	280-300
Left Testes length	470	15	180-823	225	41	106-305	596	42	425-778	454	370-500
Left Testes width	294	9	154-499	177	24	91-220	472	36	346-635	NA	NA
Right Testes length	483	16	187-880	260	42	121-338	640	50	431-858	NA	NA
Right testes width	302	10	134-521	179	31	99-237	461	37	297-598	NA	NA
Ovary length	320	10	123-576	179	29	108-237	383	41	299-635	278	240-300
Ovary width	230	8	107-394	173	26	107-239	297	23	217-376	NA	NA
Width of Uterine Coil	647	18	271-1072	774	86	557-962	1219	89	707-1488	1071	1014-1170
Left Vitellaria Length	899	23	499-1452	236	38	123-318	1112	76	789-1415	NA	NA
Right Vitellaria Length	892	23	488-1377	322	59	120-428	1024	82	580-1300	NA	NA

Table 1.3: Principal component analysis variance and loadings.
Principal component analysis of morphological measurements of 96 *Quinqueserialis* spp. specimens. Variance and loadings of the first two principal components conducted on a covariance matrix of 13 morphological characters.

	PC1	**PC2**
Variance (%)	69	12
Total length	-1.38	0.69
Total width	-1.39	-0.46
Oral sucker length	-1.20	0.97
Oral sucker width	-1.15	0.92
Left testes length	-1.50	-0.1
Left testes width	-1.46	-0.45
Right testes length	-1.51	-0.16
Right testes width	-1.47	-0.52
Ovary length	-1.41	0.01
Ovary width	-1.45	-0.37
Width of uterine coils	-1.14	-0.95
Left vitellaria length	-1.39	0.34
Right vitellaria length	-1.36	0.32

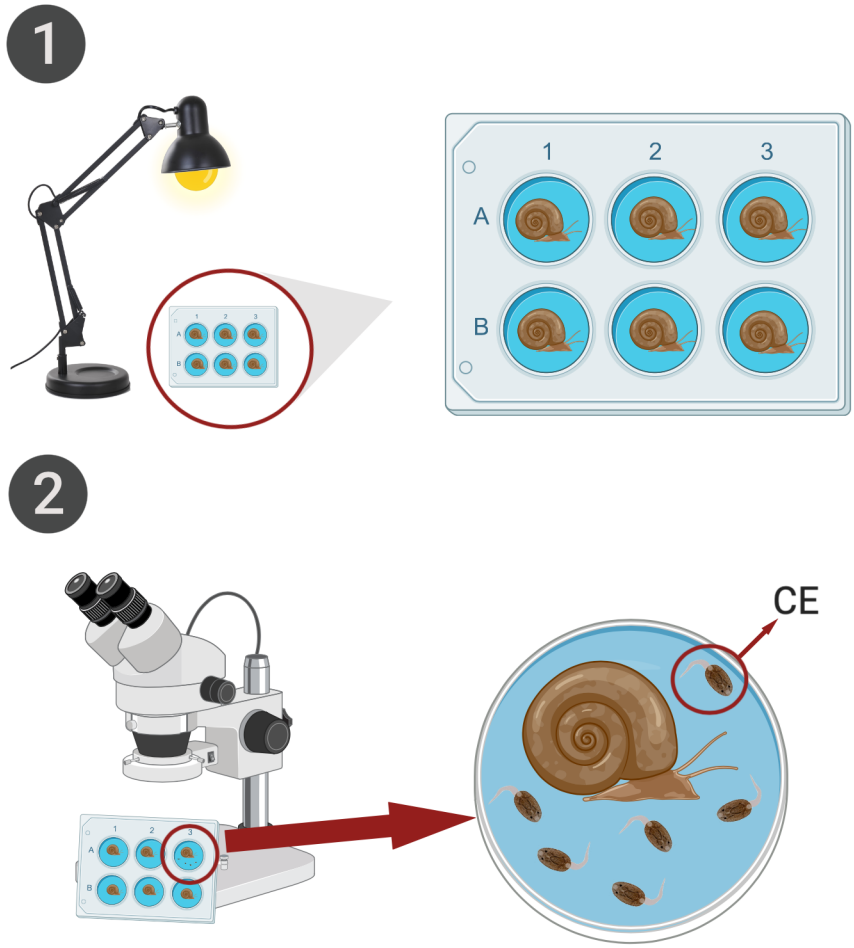

Figure 1.1: Method of stimulating cercarial emergence from intermediate host snails.
1) Snails are placed in 6-well cell culturing plates filled with well water. The plates are placed under incandescent lights to provide light and heat stimulation. 2) After a minimum of 3 hr, each well is examined for the presence of cercariae (CE) under a stereomicroscope. Created with BioRender.com.

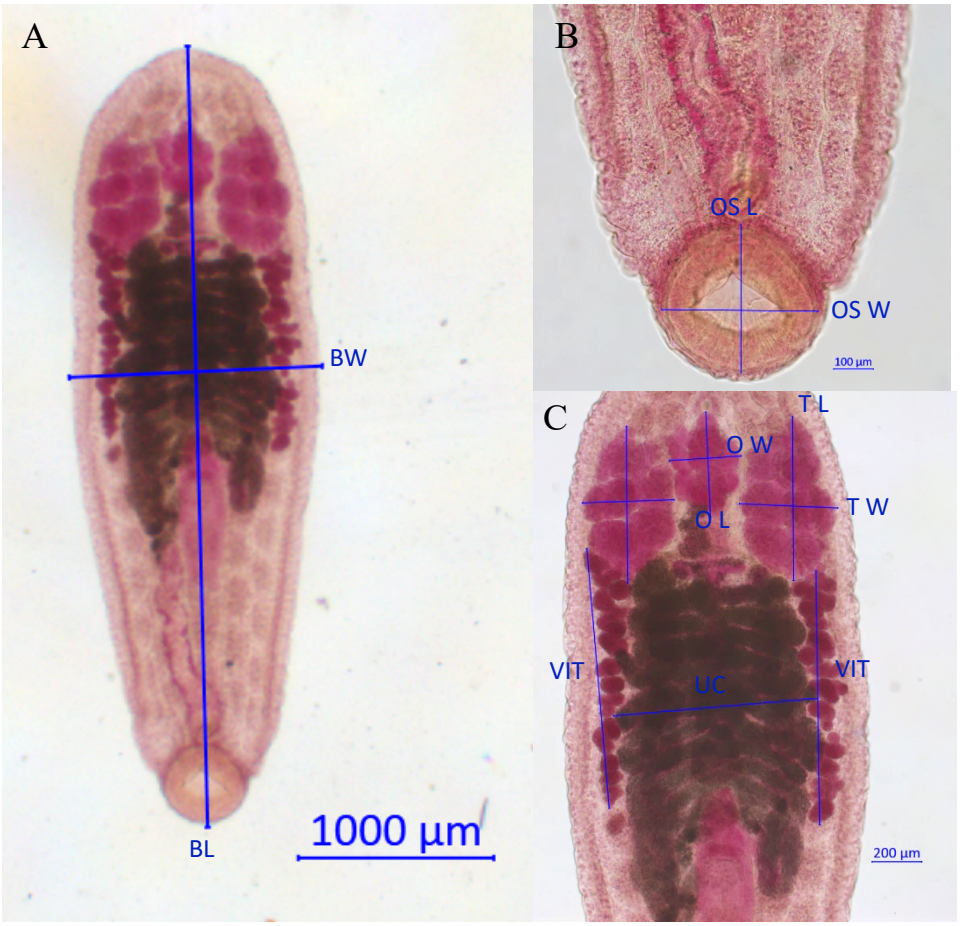

Figure 1.2 Morphological traits measured on 96 adult *Quinqueserialis* specimens.
A) Full body view with body length (BL) and body width (BW) demarcated, B) Oral sucker (OS) length (L) and width (W), and C) Measurements of reproductive organs including length of vitellaria (VIT), width of uterine coils (UC), left and right testes (T) length and width, and ovary (O) length and width.

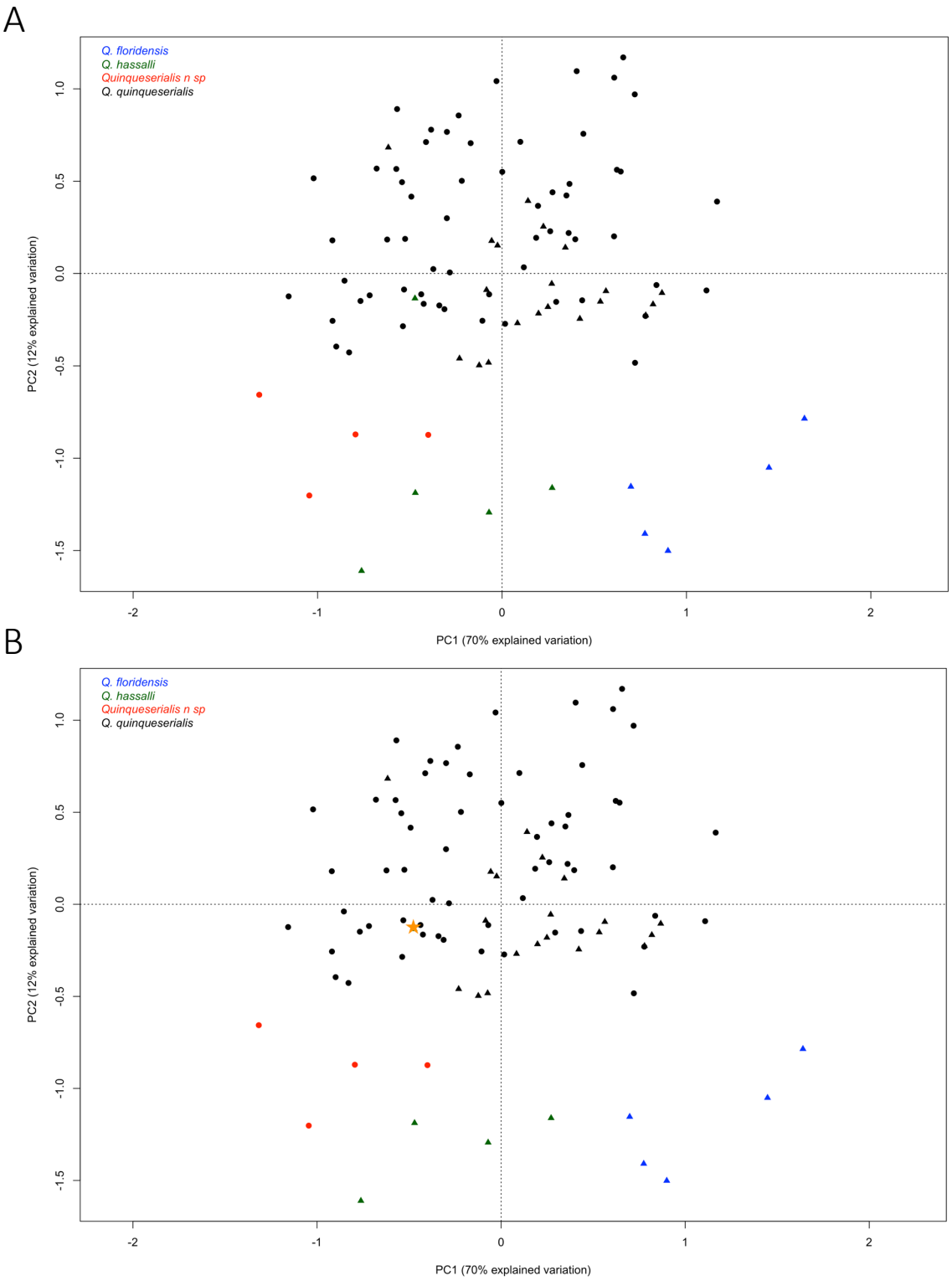

Figure 1.3: Principal component analysis of *Quinqueserialis* spp. specimens. Colours correspond to species: *Quinqueserialis floridensis* (blue), *Quinqueserialis hassalli* (green), *Quinqueserialis* n. sp. (red), and *Quinqueserialis quinqueserialis* (black). Specimens indicated by circles were field collected and museum specimens are indicated by triangles. A) PCA of 13 morphological measurements of 96 *Quinqueserialis* spp. specimens. B) PCA with the *Q. hassalli* type specimen indicated by the orange star.

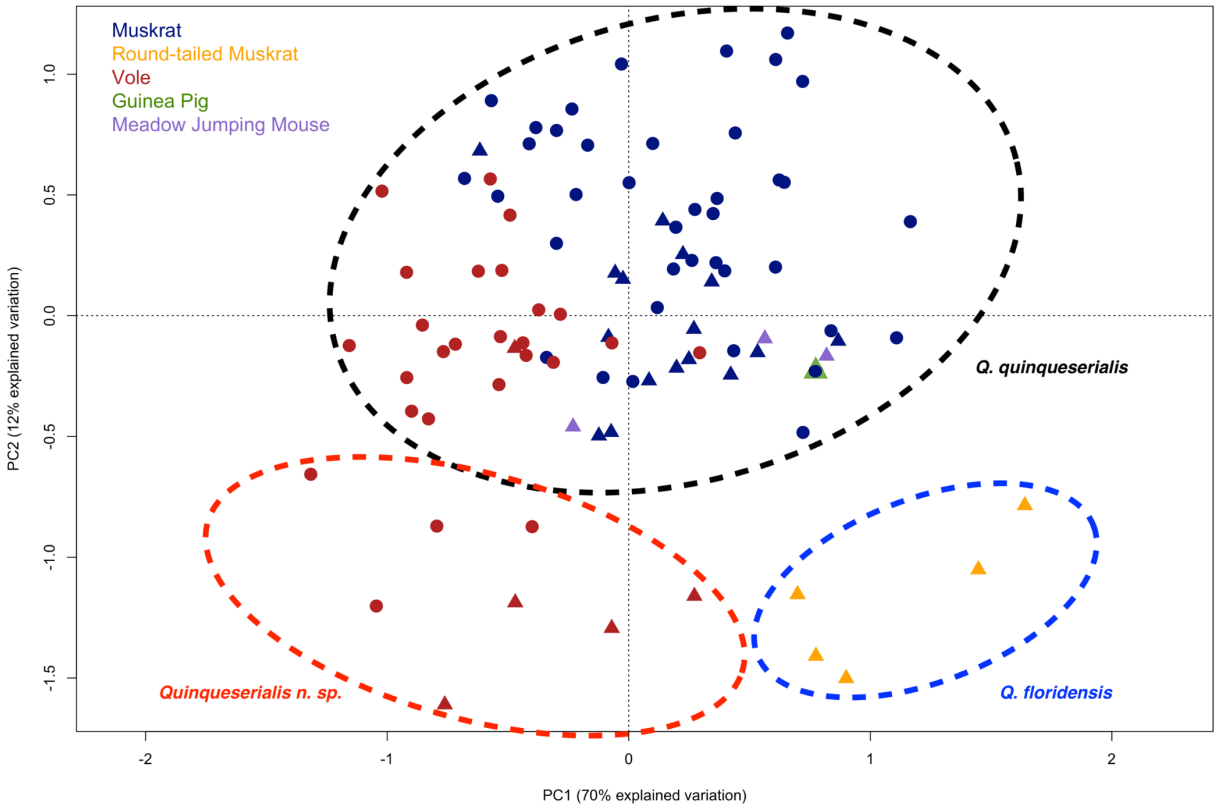

Figure 1.4: Principal component analysis of *Quinqueserialis* spp. from five host species. PCA of 13 morphological measurements of 96 *Quinqueserialis* spp. specimens. Specimens decrease in body size from left to right along PC1. Colours correspond to hosts: muskrats (navy), voles (red), round-tailed muskrat (yellow), meadow jumping mouse (purple), and guinea pig (green; point enlarged so that it is not obscured by other point). Ellipses indicate species: *Quinqueserialis floridensis* (blue), *Quinqueserialis* n. sp. (red), and *Quinqueserialis quinqueserialis* (black). Specimens indicated by circles were field collected and museum specimens are indicated by triangles.

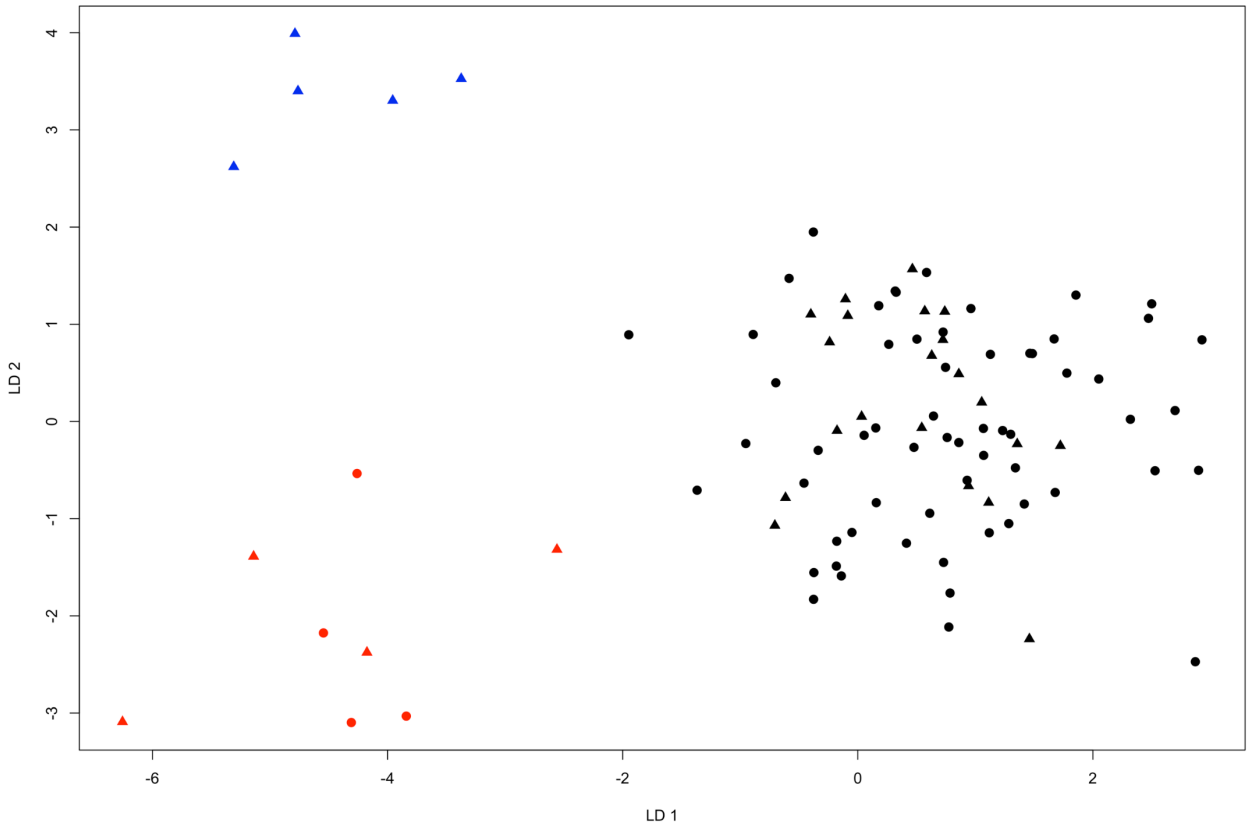

Figure 1.5: Linear discriminant analysis of three *Quinqueserialis* species.
LDA of 13 morphological measurements of 96 specimens assigned to three taxa: *Quinqueserialis quinqueserialis* (black), *Quinqueserialis floridensis* (blue), and *Quinqueserialis* n. sp (red). Includes field-collected (circles) and museum specimens (triangles).

Chapter 2: Investigating the influence of host specificity and geography on trematode speciation.

2.1 Introduction

Diversification among parasitic helminths has been extensive, with an estimated 75,000 species exploiting vertebrate hosts (Poulin and Morand, 2000). Understanding the factors that led to their diversification is of broad interest because parasites are indicators of recent and historical ecological associations (Price, 1977; Brooks and Hoberg, 2000). Price (1977) identified several factors that may affect radiation among parasites: host specificity, host population structure, geographic distribution, and mobility of hosts. Yet, determining their influence on parasite speciation is complex, as several of these factors may be acting simultaneously to lead to patterns of extant species diversity (Nadler, 1995). For example, host specificity and geographic distribution can act as isolating mechanisms that drive the speciation of parasites. They could act together to increase isolation and diversification among parasites, but diversification could also occur even if just one of these factors was promoting isolation (Hoberg and Brooks, 2008).

Host specificity is a fundamental life history characteristic of parasites because, in the most general sense, it represents the range of host species in which a parasite can infect, grow, and reproduce (Lymbery, 1989; Poulin et al, 2011). Host use varies widely among parasite species ranging from specialism (using a single host species) to generalism (using several host species) (Poulin and Keeney, 2007). The continuum in host specificity reflects the breadth of a parasite's ecological niche and is the product of ecological, evolutionary, physiological and immunological interactions with hosts. If parasites specialize in host species, then parasite populations could diverge (Poulin, 2007). For instance, the trematode *Macvicaria crassigula* Linton 1910 infects two fish species *Diplodus vulgaris* Geoffroy Saint-Hilaire 1817 and

Diplodus sargus Linnaeus 1758, but a cryptic sister species, *Macvicaria* sp., was discovered in a different fish *Diplodus annularis* Linnaeus 1758 (Jousson et al, 2000). In this case, genetic analysis showed that a more highly, host specific parasite lineage diverged from a more generalist lineage. There was no evidence of coevolution in the *Macvicaria* and *Diplodus* phylogenies, suggesting that the cryptic species likely evolved by host-switching driven by the feeding habits of the host fish (Jousson et al, 2000).

The designation of a parasite as a specialist or generalist is complicated by the fact that host specificity may vary among the different stages in a parasite's life cycle. Trematode life cycles include several types of larval stages that must infect at least two hosts to complete their development. Further, each life stage may have its own pattern of host use compared to the other stages. Among trematode parasites, the first intermediate host stage is thought to be more host specific than the second intermediate host and definitive host stages (Adamson and Caira, 1994; Lockyer et al, 2004). For instance, trematode species usually infect a single species, genus or family of mollusk (Noble et al, 1989; Adema and Loker, 1997). Higher specificity for the first intermediate hosts suggests that parasites could speciate with changes in these host populations, but also due to parasite-parasite interactions (Jousson et al, 2000). For example, two sibling species of *Echinoparyphium* Dietz 1909 trematodes infect the same definitive host but use different first intermediate host species. These separate transmission routes may eliminate competition between two closely related parasites for host resources (McCarthy, 1990). However, isolation with the definitive host may also promote speciation. The two *Echinoparyphium* species mentioned above inhabited separate sections of the definitive host's gastrointestinal tract, which could reduce resource competition and promote reproductive isolation (McCarthy, 1990). In this case, even though both parasites utilize the same host species,

they could be considered specialists because they evolved a preference for different sites, or microhabitats within the same host species (Adamson and Caira, 1994). Selection for site specificity within a host could lead to divergence within a parasite species that uses the same host species (Adamson and Caira, 1994). Over time, maintenance of this microhabitat specialization could isolate populations within a parasite species and lead to closely related species that diverged in sympatry in either the first intermediate or definitive host (McCoy, 2003).

Host specificity is closely linked to geography, as the spatial structure of host and parasite populations depends on the geographic distribution of hosts (Thompson, 1999). Host specificity can vary over a parasite's geographic range because parasites are exposed to communities of potential host species that vary in composition and density from one locality to the next (Poulin et al, 2011). Further, the level of vagility of the host across its range directly affects the amount of gene flow between parasite populations (Price, 1977). Highly mobile hosts, such as migratory birds, may dampen genetic structure and processes that would lead to speciation (Price, 1977). For example, larval *Diplostomum pseudospathaceum* Niewiadomska 1984 trematode parasites from snails collected from lakes separated by up to 300 km showed low genetic differentiation, likely due to gene flow facilitated by dispersal from the avian definitive host (Louhi et al, 2010). Conversely, low host vagility and geographic isolation can lead to adaptive divergence and speciation in affected parasite populations (Lively, 2018). Structured host populations interacting with variable parasite communities can form geographic mosaics of parasite speciation (Lively, 1999). In these mosaics, parasites may continually adapt to infect local host genotypes creating a coevolutionary hotspot. For example, cross infections with intermediate host snails exposed to trematode eggs from an allopatric population had a low

infection frequency compared to trematode eggs from the same population as the snails (King et al, 2011). Hosts and their distribution across geography influence the structure of parasite populations, which can lead to divergence and speciation.

Parasite geographic distributions can be an indicator of whether vicariance events occurred in a group. Vicariance events could involve the separation of host and parasite populations by the rise of a geographic barrier, leading to isolation and speciation (Hoberg and Brooks, 2008). However, vicariance events can also be associated with parasite range expansions and host switching, making it difficult to trace which factor influenced speciation (Paterson et al, 2003). For instance, lungworm species in the genus *Varestrongylus* Bhalerao 1932 in Eurasia and the Nearctic are hypothesized to have diversified through host colonization and range expansion, often in relation to shifts in climate and habitat (Verocai et al, 2018). However, range expansion and host colonization can also lead to parasite extinction. While there are many factors and events that can influence parasite evolution, determining which ones have occurred and resulted in speciation is complex. The simplest model proposes that parasites cospeciate with their hosts, but there are several alternatives models to co-speciation including that parasites speciate independently of their hosts, can fail to colonize new host lineages (i.e. "missing the boat"), or simply do not speciate (Page, 2003). Currently, there is no overwhelming support for one of these processes being the main mode of parasite speciation. Thus, elucidating how parasite species evolved requires investigation into their contemporary distributions in both hosts and geographic space.

To explore the factors that have influenced trematode speciation, we chose the genus *Quinqueserialis* Harwood 1939 (Trematoda). First, this system is relatively tractable as there are a limited number of described parasite species. In North America, there are three valid species,

though only two are described (Barker and Laughlin, 1911; Rausch 1952A; Gagnon, Chapter 1). This genus has a large geographic distribution extending from the Canadian Arctic to the southern United States making it possible to collect hosts in several locations throughout the range (See Chapter 1; Rausch, 1952B; Detwiler et al, 2012). Parasites in this group infect mammalian definitive hosts: meadow and tundra voles (*Microtus pennsylvanicus* Ord 1815 and *M. oeconomus* Pallas 1776*,* respectively) and muskrats (*Ondatra zibethicus* Linnaeus 1766) in the case of *Q. quinqueserialis* Barker and Laughlin 1911, meadow voles in the case of *Quinqueserialis* n. sp., and the round-tailed muskrat (*Neofiber alleni* True 1884) in the case of *Q. floridensis* Rausch 1952 (See Chapter 1; Barker and Laughlin, 1911; Rausch, 1952A). All the hosts in the life cycle have limited dispersal capabilities eliminating the confounding issue of parasites being dispersed by mobile hosts such as migrating birds. If locations are separated by a distance that exceeds the dispersal capabilities of the hosts, then these locations may represent independent replicates in which to study host-parasite interactions and parasite speciation.

Here, we collected parasites from all or some hosts in the life cycle (snails, voles, and muskrats) from six new sites within their predicted geographic range that extended from the north to the south (Northwest Territories, Canada to Alabama, USA). We combined these field collections with museum specimens originating from mammalian hosts and other geographic locations from throughout North America. Morphological and genetic analyses were integrated to estimate parasite species diversity and validate the geographic range and host specificity of each parasite species. Due to the limited vagility of the definitive hosts, we predicted that if geography had a strong influence on parasite speciation, then sister *Quinqueserialis* species will not share sympatric distributions. If host specificity has had more of an influence on speciation, then sister *Quinqueserialis* species will not infect the same subset of hosts.

2.2 Methodology

2.2.1 Specimen collection

Muskrats and voles were field collected at six locations representing the northern to southern extent of *Quinqueserialis* spp. range in North America: Inuvik, Northwest Territories; Churchill, Manitoba; Southern Manitoba; Minnesota, Virginia, and Alabama, USA (Table S1.1). Muskrats and voles were collected and necropsied as described in Section 1.2.1. Recovered *Quinqueserialis* specimens from infected hosts were treated and preserved as described in Section 1.2.1. Infected snails were field collected at two of the locations: Inuvik, NT and Winnipeg, MB. Snails were collected and processed as described in Section 1.2.1. Larval *Quinqueserialis* parasites were preserved as described in Section 1.2.1. Seventeen specimens identified as *Quinqueserialis* spp. representing eight additional locations (Illinois, Iowa, Maryland, Montana, Michigan, New York, Washington, and Wyoming) and two additional definitive hosts (meadow jumping mouse and guinea pig) were loaned from the Smithsonian Institute.

2.2.2. Morphological measurements

Voucher images of field-collected specimens were captured as described in Section 1.2.2. Field-collected specimens were stained with acetocarmine, dehydrated in ethanol, cleared in xylene and mounted in Canada Balsam to create permanent mounts. Permanent mounts and museum specimens were imaged, and 13 morphological characters were measured as described in section 1.2.2.

2.2.3 Molecular sequencing

DNA was extracted from haphazardly selected *Quinqueserialis* specimens (1-3 gravid worms per host individual) collected from naturally infected muskrats and voles, respectively.

Before field-collected specimens were stained and mounted, a 1 mm^2 section of the anterior end of the worms was removed to be used in DNA extraction. DNA was extracted from entire larvae from infected snail hosts as described in section 1.2.3. One of two methods were used to extract DNA, chelex beads or Qiagen DNeasy Blood & Tissue kit, as described in section 1.2.3. Extracted DNA was stored at -20 C until polymerase chain reaction (PCR) could be performed. We attempted to sequence each parasite individual at a mitochondrial (cytochrome c oxidase subunit 1, CO1) and a nuclear (28S rRNA) gene regions. These gene regions were amplified and sequenced as described in section 1.2.3.

Once sequences were aligned, we determined haplotype similarity using DNAsp v5 (Librado and Rozas, 2009). To visualize genetic variation within species and to demonstrate gene flow between different geographic populations, we constructed haplotype networks of *Quinqueserialis quinqueserialis* and *Quinqueserialis* n sp. using TCS1.21 (Clement et al, 2000). Genetic distances between individuals and between species was calculated with uncorrected p-distance in MEGA 7.0 (Kumar et al, 2016).

2.2.4 Data analyses

The 13 morphological characters were tested for normality using the Shapiro-Wilkes test, and any character that was not normally distributed ($P>0.05$) was log-transformed. A principal component analysis (PCA) was performed on field-collected, genetically identified specimens and on museum specimens to determine if specimens clustered according to geographic location and host use. Specimens were labeled by geographic location prior to analysis.

Latitudinal clines of genetic variation were assessed based on the concept of isolation-by-distance (Wright, 1943). Specimens sequenced at the CO1 gene were labeled by sampling location and a mean genetic distance by group matrix (p-distances) was generated in MEGA. A

pairwise geographic distance matrix was generated using the latitude and longitude coordinates of each location using the Geographic Distance Matrix Generator (Ersts, 2012). To test the correlation between genetic and geographic matrices, a Mantel test (1000 permutations) was performed. All analyses were conducted with the "vegan" package in Rstudio 1.1.463 (Oksanen et al, 2019).

2.3 Results

As detailed in Section 1.3, both morphological and genetic sequences analyses indicated the presence of three putative species of *Quinqueserialis* in North America. *Quinqueserialis quinqueserialis* was morphologically and genetically distinct from *Q.* n. sp. Both of these parasite species were morphologically distinct from *Q. floridensis*.

2.3.1 Host specificity among *Quinqueserialis* species

Among the three species, only one of the *Quinqueserialis* species was isolated by host species: *Q. floridensis*. Specimens identified as *Q. floridensis* clustered morphologically according to their host species, the round-tailed muskrat (Figure 2.1). No genetic data was obtained from the *Q. floridensis* museum specimens because DNA cannot be obtained from specimens after the staining process.

In contrast, *Q. quinqueserialis* was found in four mammalian host species: muskrats (morphology and genetics), voles (morphology and genetics), meadow jumping mice (morphology only), and guinea pig (morphology only) (Figure 2.1). Individuals of *Q. quinqueserialis* formed one cluster despite originating from different host species (Figure 2.1). Yet, there was some subdivision within this cluster with larger worms being found in voles and smaller worms found in muskrats (Section 1.3). Although there was some host-induced morphological variation, network analysis with genetic sequences indicated no association with

host species (Figure 2.2). Three species of snails were infected with *Q. quinqueserialis*: *Gyraulus circumstriatus* Tryon 1866, *Gyraulus crista* Linnaeus 1758, and *Promenetus exacuous* Say 1821. At the 28S gene region, *Q. quinqueserialis* from muskrats, voles, and snails were identical and represented by a single haplotype (810 base pairs (bp)). At the CO1 gene region, *Q. quinqueserialis* from muskrats, voles and snails exhibited genetic variation and were represented by 27 haplotypes (452 bp, Figure 2.2).

Similar to *Q. quinqueserialis*, individuals of *Q.* n. sp. formed one cluster according to host. In this case, it was only recovered from a single host species, the meadow vole (*Microtus pennsylvanicus*) (Figure 2.1). All *Q.* n. sp. specimens were genetically identical at both the 28S (810 bp) and CO1 (452 bp) genes. However, *Q.* n. sp. is not isolated within voles, as *Q. quinqueserialis* specimens also occurred in vole hosts and even at the same location (Churchill, Manitoba).

2.3.2 Influence of geography

In addition to being isolated by host species, *Q. floridensis* was also isolated by geography. This parasite species only occurred in the southernmost extent of the *Quinqueserialis* spp. range in Florida, USA (Figure 2.3A). *Quinqueserialis* n. sp. was only found in one site, Churchill, MB, and these specimens formed a single cluster (Figure 2.3B). However, four *Q. quinqueserialis* museum specimens from Minnesota and Virginia clustered along with *Q.* n. sp. Without genetic data we cannot confirm what species these specimens belong to, and thus whether *Q.* n. sp. occurs in these locations. In addition to using the same host species (vole), *Q.* n. sp. also co-occurred geographically with *Q. quinqueserialis*. Notably, Churchill, MB is the only site where two parasite species co-occurred. In contrast, museum and field specimens identified as *Q. quinqueserialis* were distributed across Canada and the United States and there

was no clustering by location (Figure 2.3A). Genetically identified *Q. quinqueserialis* did not cluster morphologically according to geographic location (Figure 2.3B). At the 28S gene region, specimens identified as *Q. quinqueserialis* did not vary genetically by geographic location. At the CO1 gene region, specimens identified as *Q. quinqueserialis* varied genetically and showed some structuring according to geographic location (Figure 2.2). Specimens from the northern region (Inuvik, NT and Churchill, MB) of the *Q. quinqueserialis* range were more similar to each other than to specimens from the southern region (VA and AL) of the range, and vice versa. Specimens from the central regions (MN and MB) represented intermediate haplotypes (Figure 2.2). The most frequent haplotype was recovered from Inuvik, NT, Churchill, MB and southern Manitoba and was therefore inferred to be the ancestral haplotype (Clement et al, 2000). The Mantel test showed a significant relationship between genetic p-distance and geographic distance, indicating that genetic similarity of *Q. quinqueserialis* populations declines with increasing geographic distance from the north to south (Figure 2.4, $r^2= 0.30$ $P= 0.05$).

2.4 Discussion

We used a system in which parasite species were accurately delimited with integrative taxonomy to determine the relative influences of host specificity and geography on parasite speciation. We found *Quinqueserialis* parasites in six locations including areas in which it had never been reported. By combining our samples with museum specimens, we increased the number of host species and sampling within North America. Based on extensive sampling of hosts and localities from throughout the range of the parasite genus in North America, we found that the influences of host specificity and geography on the speciation of *Quinqueserialis* differed among the taxa. Only one species, *Q. floridensis*, was isolated by geography and host specificity suggesting that both factors have contributed to the divergence of this species. In

contrast, neither of the other species, *Q. quinqueserialis* and *Q.* n. sp. were isolated by geography or definitive host specificity as they co-occurred in the same host species and site with each other. Thus, it remains unclear how *Q.* n. sp. diverged, especially because despite extensive geographic and host sampling, it has only been recovered from one site in North America. Relative to the other two parasite species, *Q. quinqueserialis* has a large host and geographic range. However, mtDNA variation within *Q. quinqueserialis* exhibited some spatial structuring according to geographic region. This result agrees with the prediction of parasite isolation by geography because all the hosts in the life cycle have limited mobility and dispersal.

We found evidence of speciation due to geographic and host isolation in only one of the three *Quinqueserialis* species examined. The PCA (13 measurements of 7 morphological traits) clustered *Q. floridensis* specimens away from the two other *Quinqueserialis* species by both its host, the round-tailed muskrat, and by geography. Because all the *Q. floridensis* specimens were permanently mounted museum specimens, we could not make genetic comparisons to the other parasite species. New collections and storage of specimens in ethanol will enable this work in the future. However, round-tailed muskrats are rare and protected in Florida, making sampling more difficult (Lefebvre and Tilmant, 1992). Nonetheless, our results suggest that host specificity and geography have influenced the speciation of *Q. floridensis*. However, it remains unclear if either factor has had more of an influence on divergence. We hypothesize that *Q. floridensis* speciated through host-switching. The geographic range of hosts used by *Q. quinqueserialis*, muskrats and meadow voles, do not extend into Florida (Cassola, 2016A & B). It is possible that a host-switching event from muskrats to the round-tailed muskrat, in combination with geographic isolation thereafter, led to divergence and speciation. Speciation following host-switching has been observed in several host-parasite systems. For instance, radiation of circumarctic

microphallid trematodes is believed to have been caused in part by host switching from gulls to waders and again to sea-ducks (Galaktionov et al, 2012). Muskrats, voles, and round-tailed muskrats all belong to the subfamily Arvicolinae Gray 1821 (Buzan et al, 2008). The phylogenetic similarities among these host species may suggest that for *Quinqueserialis* spp., the host physiology and immunology may be similar enough to increase the likelihood of establishment after host-switching. Alternatively, *Q. floridensis* could be the result of co-speciation with their hosts. If the common ancestor of *Quinqueserialis* species infected an arvicoline common ancestor, the parasites may have co-speciated with their hosts (Hafner and Nadler, 1998). Genetic data from all extant *Quinqueserialis* species and phylogenetic analyses of both the parasites and the hosts would be required to test either hypothesis.

Similar to *Q. floridensis*, *Q.* n. sp. appears to have a restricted geographic and host distribution. *Quinqueserialis* n. sp. occurred only in one geographic location and was found in a single host species. However, four museum specimens from Minnesota and Virginia clustered with *Q.* n. sp. While we did set traps for voles in Minnesota and Virginia, no voles or only uninfected voles were captured, respectively. Therefore, it is possible that the novel species occurs in other geographic locations and was not sampled. Even so, this parasite species is not isolated in geography or host specificity because it co-occurs with *Q. quinqueserialis* in voles in the same location. Churchill, MB, was the only location where two species of *Quinqueserialis* were found simultaneously. There are two hypotheses that explain how *Q.* n. sp. and *Q. quinqueserialis* could have diverged in sympatry. First, host specificity at the intermediate host stage could result in speciation (Lockyer et al, 2004). *Quinqueserialis quinqueserialis* can infect up to four snail intermediate hosts, three of which were found infected in two locations (Gagnon and Detwiler, Manuscript in review, Appendix 3). Whether *Q. quinqueserialis* snail host

specificity varies in other parts of its geographic range requires further sampling. The intermediate host of *Quinqueserialis* n. sp. is not known, but if this species infects a different snail species it could have resulted in divergence and speciation. Surveys of snail species to find potential *Quinqueserialis* n. sp. intermediate hosts are required to test this hypothesis.

Second, parasites can be resource specialists, which could result in strict site specificity within the same host (Adamson and Caira, 1994). While *Quinqueserialis* n. sp. and *Q. quinqueserialis* both exploited meadow voles, they were found in different individuals making it unclear as to whether they can co-infect the same individual host. The *Q. quinqueserialis* specimens are specific to the cecum of muskrats and voles, whereas *Q.* n. sp. was recovered from the small intestine of its vole hosts. If both species can exploit the same host concurrently, then within-host site specificity could have played a role in speciation (McCarthy, 1990). Further investigation into host use and site specificity by *Quinqueserialis* n. sp. is required to disentangle its evolutionary history. Vole hosts would need to be live captured, freshly euthanized and immediately necropsied to observe microhabitat specificity. All recovered specimens from a host would need to be preserved individually and sequenced to determine genetic differentiation.

Because both *Q. quinqueserialis* and *Q,* n. sp. use vole definitive hosts, hybridization between diverged populations of *Q. quinqueserialis* could have led to subsequent divergence and speciation (*Q.* n. sp.) (Detwiler and Criscione, 2010). Speciation through hybridization may occur because of the creation of novel phenotypes arising from genetic variation as a result of transfer of adaptive traits from the parental populations/species (Detwiler and Criscione, 2010). These novel phenotypes may promote reproductive isolation, eventually leading to divergence. Hybridization has been observed in nature in several trematode systems including *Fasciola hepatica/gigantica* populations (Agatsuma et al, 2000). A common method of identifying

putative hybrids is nuclear-mitochondrial gene discordance, where sequences from one gene may match one parental species while the other gene matches the other parent (Detwiler and Criscione, 2010). In the case of *Quinqueserialis* n. sp., both the nuclear 28S gene and the CO1 mitochondrial gene were distinct from *Quinqueserialis quinqueserialis*. Thus, at this point there is no evidence for hybridization among divergent *Q. quinqueserialis* populations. However, further geographic sampling, and additional sequencing of a less conserved nuclear gene or whole genome sequencing would provide more evidence to support this conclusion.

It is possible that *Quinqueserialis* n. sp. and *Q. quinqueserialis* diverged in allopatry and presently occur in sympatry following colonization by one of the host species. However, it is difficult to determine whether congeneric parasite species that occur in sympatry also diverged in sympatry (McCoy, 2003). This idea is consistent with the taxon pulse hypothesis (TPH), where a barrier could have initially separated the two species and has since disappeared (Hoberg and Brooks, 2008). Understanding the phylogeography of parasite host species can inform the evolutionary history of parasite species. The arvicolid rodents first appeared around five million years ago and were Holarctic in distribution (Chaline et al, 1999). The genus *Microtus* is proposed to have originated in Asia and expanded and radiated in Europe and North America approximately one million years ago (Barbosa et al, 2018). The ancestor of the *Ondatra* lineage is believed to have originated between five and two million years ago in Oregon (Chaline et al, 1999). We hypothesize that vole and muskrat populations during this time were infected with *Q. quinqueserialis*, but a population of voles may have been isolated within glacial refugia during the last glacial maximum, leading to the divergence of its *Quinqueserialis* population. The area of Churchill, MB was covered by the Laurentian ice sheet from 115,000 to 10,000 years ago (Dyke, 2004). Voles and muskrats may have colonized the area, along with the two distinct

species of *Quinqueserialis,* following the retreat of the Laurentian ice sheet at the end of the last glacial maximum. A complete phylogeny of the genus would be required to test this hypothesis. In addition to sampling the North American species, this work would need to include the Eurasian species *Q. wolgaensis* Skvortsov 1935. This parasite uses the vole *Arvicola terrestris* Linnaeus 1758 and its inclusion in a phylogenetic analysis of the genus would indicate which *Quinqueserialis* species is basal. Then more specific hypotheses about the evolutionary divergence of *Quinqueserialis* n. sp. could be tested.

In contrast to the other two North American species, *Q. quinqueserialis* had a broad geographic and host distribution. This parasite species was found across Canada and the United States and despite the prediction of morphological and genetic structure due to limited host dispersal, *Q. quinqueserialis* showed no morphological and genetic clustering according to host and geographic range. However, only specimens from muskrats were obtained from the southern extent of the *Q. quinqueserialis* range, thus there are gaps in the knowledge of host specificity in this region. This parasite species did not cluster morphologically according to geographic location or host species although specimens from voles were generally larger than those from muskrats (Figure 2.1). There was genetic structure at the CO1 gene within the species that corresponded to geographic location (Figure 2.2). Genetic similarity between *Q. quinqueserialis* populations declined with increasing geographic distance (Figure 2.4). However, the intraspecific genetic variation (0.76%, 452 base pairs) was much less than the 5% proposed as a species-level cutoff among helminths (Section 1.3.2; Vilas et al, 2005).

Despite the broad geographic range of *Q. quinqueserialis,* there is relatively low genetic structure which is indicative of gene flow occurring between populations. The ability to infect multiple host species at both stages in the life cycle may increase the amount of gene flow

occurring between populations (Lejeunesse and Forbes, 2002). This may be why *Q. quinqueserialis* parasites are widespread throughout the continent. On the other hand, this low genetic variation may be a relic of historical geographic distribution (Brooks and Hoberg, 2000). There was a significant relationship between genetic similarity and geographic distance that indicates a trend of increasing genetic divergence between geographically disparate populations (Figure 2.4). More contemporary gene flow between populations would first need to be assessed with more variable markers like microsatellite or single nucleotide polymorphisms (SNPs) to determine whether populations are diverging.

2.5 Conclusions

After accurately identifying species diversity of *Quinqueserialis* parasites in North America, we assessed how host specificity and geography may have influenced speciation in this group. In the case of *Q. floridensis*, we found support for both our hypotheses, host and geographic isolation, because this parasite species infected a unique host species and did not share sympatric distributions with *Q. quinqueserialis* and *Q.* n. sp. However, for the two other species, we found that neither host specificity nor geography clearly explained the patterns of speciation suggested by the current dataset. Sampling of potential intermediate hosts of *Quinqueserialis* n. sp. are required to further inform how host specificity may have influenced speciation. Future explorations of the processes underlying speciation among *Quinqueserialis* parasites would benefit from a molecular phylogeny that includes all *Quinqueserialis* species (North American and Eurasian).

Literature Cited

Adamson, M.L. and Caira, J.N. 1994. Evolutionary factors influencing the nature of parasite specificity. Parasitology **109**: S85-S95.

Adema, C.M. and Loker, E.S. 1997. Specificity and immunobiology of larval digenean snail associations. *In* Advances in trematode biology. B. Fried and T.K. Graczyk (eds.). CRC Press, Boca Raton, Fl, USA. P. 230-253.

Agatsuma, T.; Arakawa, Y.; Iwagami, M.; Honzako, Y.; Cahyaningsih, U.; Kang, S.Y.; Hong, S.J. 2000. Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica.* Parasitology International **49**: 231-238.

Barbosa, S., Paupério, J., Pavlova, S.V., Alves, P.C., and Searle, J.B. 2018. The *Microtus* voles: resolving the phylogeny of one of the most speciose mammalian genera using genomics. Molecular Phylogenetics and Evolution **125**: 85-92.

Barker, F.D. and Laughlin, J.W. 1911. A new species of trematode from the muskrat, *Fiber zibethicus*. Transactions of the American Microscopical Society **30**: 261-274.

Buzan, E.V., Krystufek, B., Hänfling, B., Hutchinson, W.F. 2008. Mitochondrial phylogeny of Arvicolinae using comprehensive taxonomic sampling yields new insights. Biological Journal of the Linnean Society **94**: 825–835.

Brooks, D.R. and Hoberg, E.P. 2000. Triage for the biosphere: the need and rationale for taxonomic inventories and phylogenetic study of parasites. Comparative Parasitology **67**: 1-25.

Cassola, F. 2016A. *Ondatra zibethicus*. The IUCN Red List of Threatened Species 2016: e.T15324A22344525. http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T15324A22344525. en. Accessed on 12 July 2019.

Cassola, F. 2016B. *Microtus pennsylvanicus* (errata version published in 2017). The IUCN Red List of Threatened Species 2016: e. T13452A115114123. http://dx.doi.org/10.2305/IUCN.UK. 2016-3. RLTS.T13452A22347596.en_Accessed on 12 July 2019.

Chaline, J., Brunet-Lacomte, P., Montuire, S., Viriot, L. and Courant, F. 1999. Anatomy of the arvicoline radiation (Rodentia): paleogeographical, paleoecolgical history and evolutionary data. Annales Zoologici Fennici **36:** 239-267.

Clement, M., Posada, D., and Crandall, K.A. 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology **9**: 1657–1661.

Detwiler, J.T. and Criscione, C.D. 2010. An infectious topic in reticulate evolution: introgression and hybridization in animal parasites. Genes **1**: 102-123.

Detwiler, J.T., Zajac, A.M., Minchella, D.J., and Belden, L.K. 2012. Revealing cryptic parasite diversity in a definitive host: echinostomes in muskrats. Journal of Parasitology **98**: 1148-1155.

Dyke, A.S. 2004. An outline of North American deglaciation with emphasis on central and northern Canada. Developments in Quaternary Sciences **2:** 373-424.

Ersts, P.J. 2012. Geographic Distance Matrix Generator. 1.2.3. American Museum of Natural History, Center for Biodiversity and Conservation. Release date: 2016 6 20. URL: http://biodiversityinformatics.amnh.org/open_source/gdmg

Galaktionov, K., Blasco-Costa, I., and Olson, P.D. 2012. Life cycles, molecular phylogeny, and historical biogeography of the '*pygmaeus'* microphallids (Digenea: Microphallidae): widespread parasites of marine and coastal birds in the Holarctic. Parasitology **139**: 1346-1360.

Hafner, M.S. and Nadler, S.A. 1988. Phylogenetic trees support the coevolution of parasites and their hosts. Nature **332**: 258-259.

Hoberg, E.P. and Brooks, D.R. 2008. A macroevolutionary mosaic: episodic host-switching, geographical colonization and diversification in complex host-parasite systems. Journal of Biogeography **35**: 1533-1550.

Jousson, O., Bartoli, P., and Pawloski, J. 2000. Cryptic speciation among intestinal parasites (Trematoda: Digenea) infecting sympatric host fishes (Sparidae). Journal of Evolutionary Biology **13**: 778-785.

King, K.C., Delph, L.F., Jokela, J., and Lively, C.M. Coevolutionary hotspots and coldspots for host sex and parasite local adaptation in snail-trematode interaction. Oikos **120**: 1335-1340.

Kumar, S., Stecher, G., and Tamura, K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution **33**: 1870-1874.

Lajeunesse, M.J. and Forbes, M.R. 2002. Host range and local parasite adaptation. Proceedings of the Royal Society of London Biological Sciences **269**: 703-710.

Lefebvre, L.W. and Tilmant, J.T. 1992. Round-tailed muskrats. *In* Rare and Endangered Biota of Florida, Vol. 1, Mammals. S.R. Humphrey and R.E. Ashton (eds.). University Press of Florida, Gainesville, Fl, USA. P. 276-286.

Librado, P., and Rozas, J.. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics **25**: 1451–1452.

Lively, C.M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. American Naturalist **153**: S34-S47.

Lively, C.M. 2018. Habitat heterogeneity, host population structure, and parasite local adaptation. Journal of Heredity **109**: 29-37.

Lockyer, A.E., Jones, C.S., Noble, L.R., and Rollinson, D. 2004. Trematodes and snails: an intimate association. Canadian Journal of Zoology **82**: 251-269.

Louhi, K.R., Karvonen, A., Rellstab, C., and Jokela, J. 2010. Is the population genetic structure of complex life cycles parasites determined by the geographic range of the most motile host? Infection, Genetics, and Evolution **10**: 1271-1277.

Lymbery, A.J. 1989. Host specificity, host range and host preference. Parasitology Today **5**: 298.

McCarthy, A.M. 1990. Speciation of echinostomes: evidence for the existence of two sympatric sibling species in the complex *Echinoparyphium recurvatum* (von Linstow 1873) (Digenea: Echinostomatidae). Parasitology **101**: 35-42.

McCoy, K. 2003. Sympatric speciation in parasites—what is sympatry? Trends in Parasitology **19**: 400-404.

Nadler, S.A. 1995. Microevolution and the genetic structure of parasite populations. Journal of Parasitology **81**: 395-403.

Noble, E.R., Noble, G.A., Schad, G.A., and MacInnes, A.J. 1989. Parasitology: the biology of animal parasites. Lea and Febiger, Philadelphia, Pa, USA. Pp. 574.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., and Wagner, H. 2019. Vegan: Community ecology package. R package version 2.5-4.

Page, R.D.M. 2003. Introduction. *In* Tangled Trees: Phylogeny, cospeciation, coevolution. R.D.M. Page (ed.). The University of Chicago Press, Chicago, Il, USA. P. 1-16.

Paterson, A.M., Palma, R.L., and Gray, R.D. 2003. Drowning on arrival, missing the boat, and *X*-events: how likely are sorting events? *In* Tangled Trees: Phylogeny, cospeciation, and coevolution. R.D.M. Page (ed.). The University of Chicago Press, Chicago, Il, USA. P. 287-300.

Poulin, R. 2007. Are there general laws in parasite ecology? Parasitology **134**: 736-776.

Poulin, R. and Morand, S. 2000. The diversity of parasites. Quarterly Review of Biology **75**: 277-293.

Poulin, R. and Keeney, D.B. 2007. Host specificity under molecular and experimental scrutiny. Trends in Parasitology **24**: 24-28.

Poulin, R., Krasnov, B.R., and Mouillot, D. 2011. Host specificity in phylogenetic and geographic space. Trends in Parasitology **27**: 355-361.

Price, P.W. 1977. General concepts on the evolutionary biology of parasites. Evolution **31**: 405-420.

Rausch, R. 1952A. Helminths from the round-tailed muskrat, *Neofiber alleni nigrescens* Howell, with descriptions of two new species. Journal of Parasitology **38**: 151-156.

Rausch, R. 1952B. Studies on the helminth fauna of Alaska. XI. Helminth parasites of microtine rodents—taxonomic considerations. Journal of Parasitology **38:** 415-444.

Thompson, J.N. 1999. Specific hypotheses on the geographic mosaic of coevolution. American Naturalist **153**: S1-S14.

Verocai, G.G., Kutz, S.J., and Hoberg, E.P. 2018. Historical biogeography among species of *Varestrongylus* lungworms (Nematoda: Protostrongylidae) in ungulates: episodic expansion and host colonization linking Eurasia and North America. Parasitology Research **117**: 2125-2137.

Vilas, R., Criscione, C.D., and Blouin, M.S. 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. Parasitology **131**: 1-8.

Wright, S. 1943. Isolation by distance. Genetics **28**: 114-138.

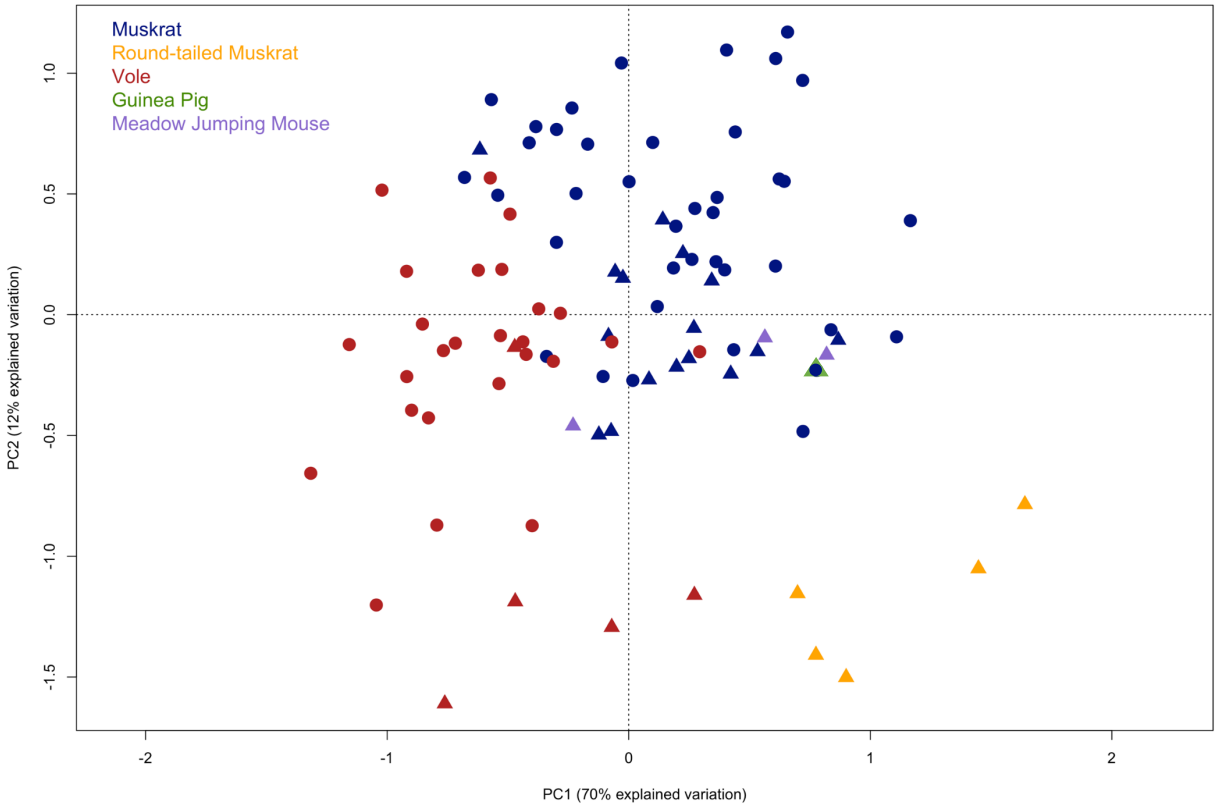

Figure 2.1 Principal component analysis of *Quinqueserialis* spp. specimens according to host use.
PCA of 13 morphological measurements of 96 *Quinqueserialis* spp. specimens. Colour corresponds to host species: Muskrat (*Ondatra zibethicus*) in navy, round-tailed muskrat (*Neofiber alleni*) in orange, voles (*Microtus pennsylvanicus* and *M. oeconomus*) in red, guinea pig (*Cavia porcellus*) in green enlarged point (as to not be obscured by other point), and meadow jumping mouse (*Zapus hudsonius*) in purple. Specimens indicated by circles were field collected and museum specimens are indicated by triangles.

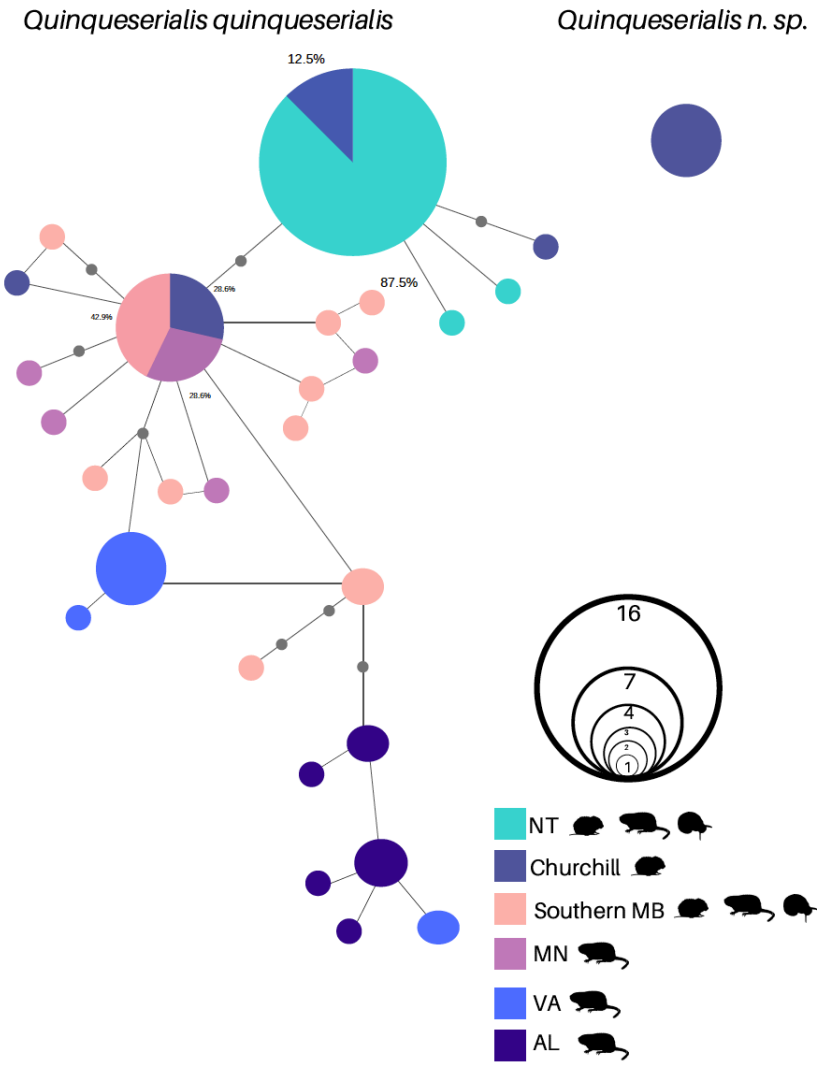

Figure 2.2: Statistical parsimony network for cytochrome c oxidase 1 mtDNA haplotypes. Haplotype network of the cytochrome c oxidase gene for 60 *Quinqueserialis* spp. individuals represented by 28 haplotypes. Each connection is a single mutational step with grey circles representing inferred haplotypes. Observed haplotypes are shown as circles with the geographic location (s) indicated by colour. Size of the circles correspond to the frequency of the haplotype. Hosts of *Quinqueserialis* spp. sampled indicated in the legend.

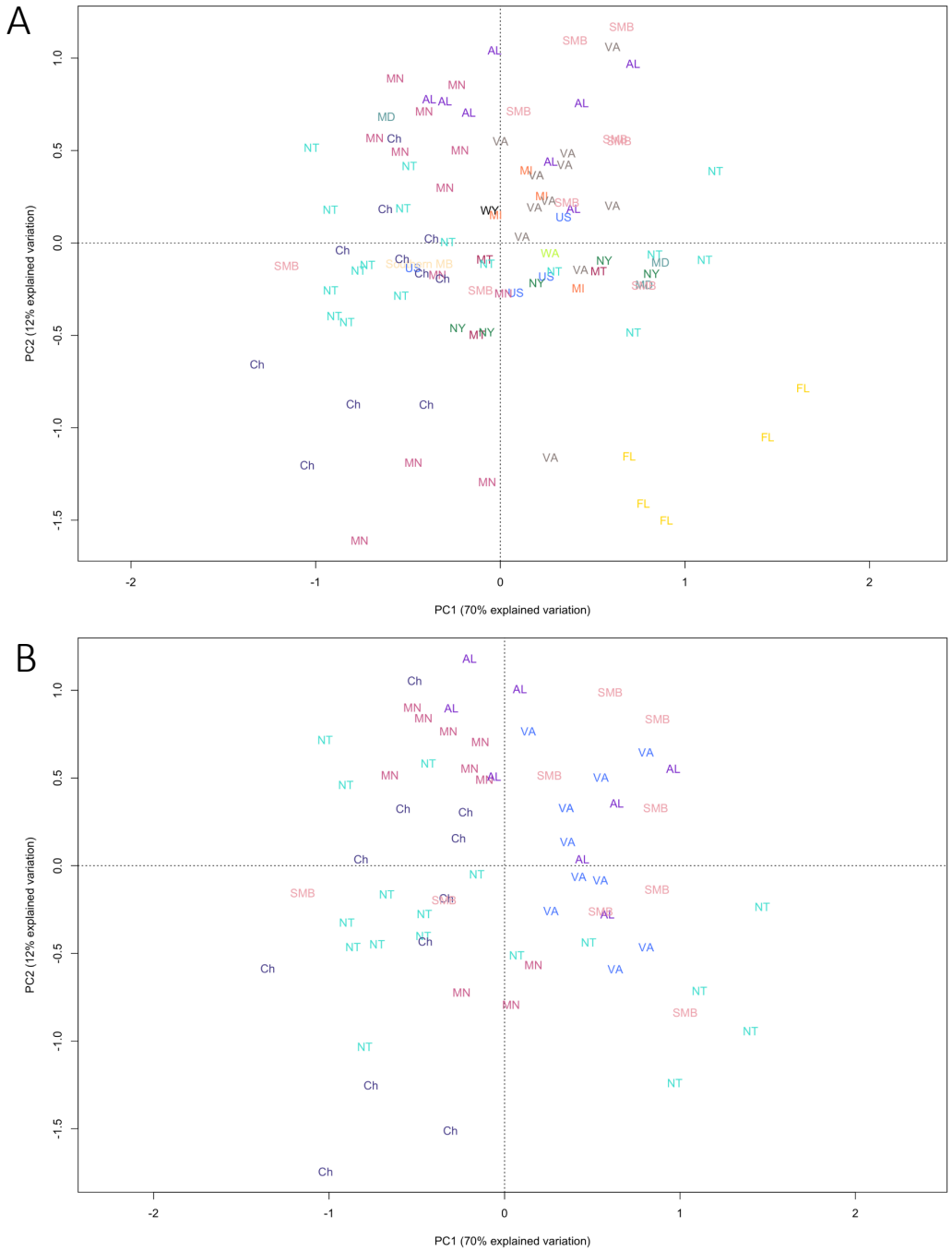

Figure 2.3 Principal component analysis of *Quinqueserialis* spp. specimens by geographic location.

A) PCA of 13 morphological measurements of 96 *Quinqueserialis* spp. specimens. Acronyms and colours correspond to geographic location: Alabama (AL), Churchill, MB (Ch), Florida (FL), Maryland (MD), Michigan (MI), Minnesota (MN), Montana (MT), Northwest Territories (NT), New York (NY), Southern Manitoba (SMB), Virginia (VA), Washington (WA), and Wyoming (WY). B) PCA of 13 morphological measurements of 66 field-collected (50 of which sequenced at the cytochrome c oxidase subunit 1 gene) *Quinqueserialis* spp. specimens. Acronyms and colours correspond to geographic location.

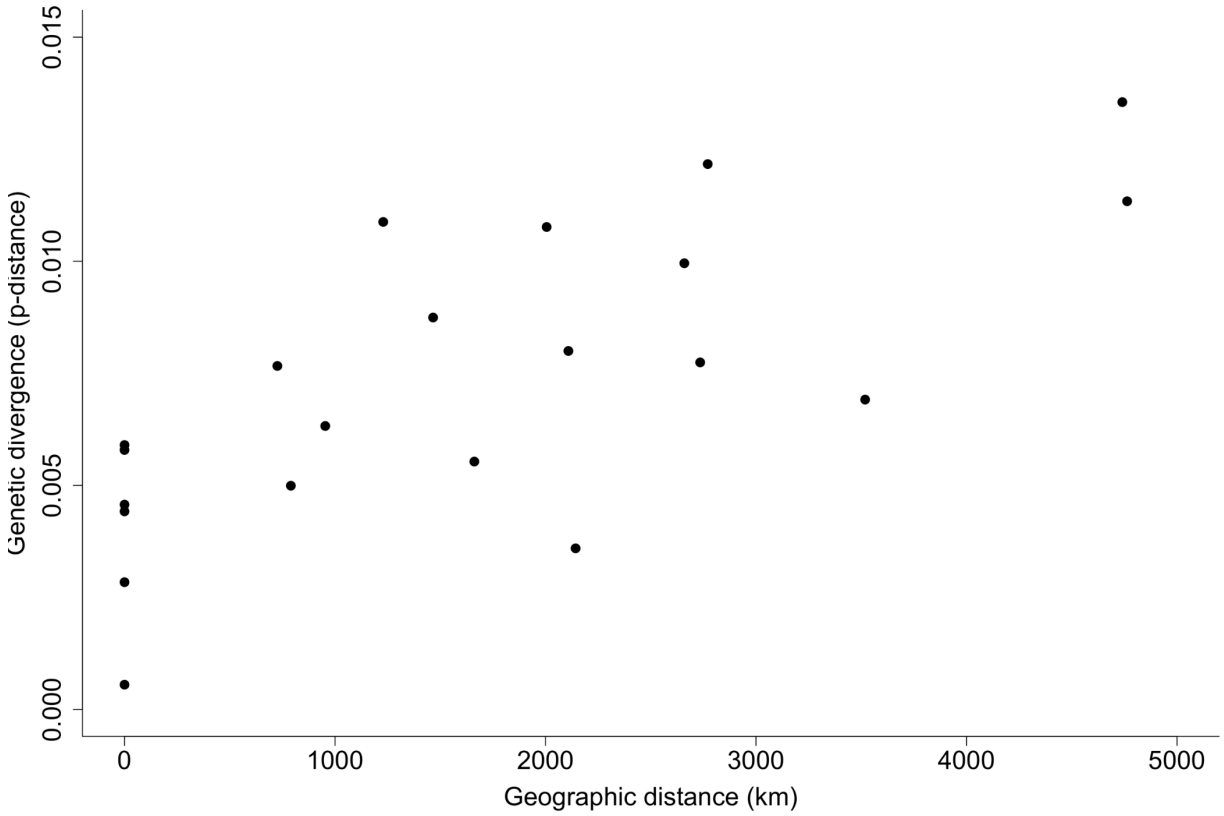

Figure 2.4: Geographic cline of genetic divergence among *Quinqueserialis quinqueserialis* populations.
Isolation by distance of *Q. quinqueserialis* populations from the six sampling locations (Northwest Territories, Churchill, Southern MB, Minnesota, Virginia, and Alabama). Genetic divergence corresponds to pairwise p-distance (number of base pair differences between sequences) plotted against geographic distance (km) between locations.

Thesis Conclusion

Using an integrative taxonomic approach, we assessed diversity of *Quinqueserialis* Harwood 1939 species in North America. Chapter 1 demonstrated that integrating data from morphological analyses and gene sequencing can accurately delimit parasite species. In contrast to our prediction that cryptic species would be found, we found three morphologically distinct clusters and confirmed that two of them were also genetically distinct based on differences at a nuclear and mitochondrial gene. Therefore, in accordance with the morphological and genetic species concepts, we concluded that there are at least three species of *Quinqueserialis* in North America. One of these species, *Q.* n. sp. is an undescribed demonstrating that broader geographic sampling, especially in the northern parts of parasites' range, can lead to discoveries of new parasite species. Additionally, we filled gaps in our knowledge of parasite life cycles by demonstrating that one species, *Q. quinqueserialis* Barker and Laughlin 1911 can use three additional snail species as intermediate hosts. Larvae from three snail species were genetically linked to *Q. quinqueserialis* adults, confirming the life cycle in Inuvik, NT and Winnipeg, MB. The three snail host species were previously undocumented as *Q. quinqueserialis* hosts, demonstrating there is still much to learn about parasite host specificity and life cycles. Because we accurately assessed species diversity and host use, we could then address questions regarding the evolutionary ecology of *Quinqueserialis*. In Chapter 2, we investigated how host specificity and geography have influenced speciation in this group. Using multivariate analyses to determine if *Quinqueserialis* spp. specimens clustered according to host and/or location, we demonstrated that the factors influencing speciation can vary between taxa. Only *Q. floridensis* Rausch 1952 was isolated by host and by geography being limited to one definitive host species and the southeastern US. In contrast, *Q. quinqueserialis* was found in four definitive host species

and distributed throughout Canada and the United States. This parasite species exhibited host-induced phenotypic plasticity and spatial genetic structure of isolation-by-distance from the northern to southern parts of its range. For the newly discovered species, *Q.* n. sp., the factors that led to its divergence remain unclear, as this species co-occurred with *Q. quinqueserialis* in both its host species, the meadow vole, and its location, Churchill. A complete *Quinqueserialis* spp. phylogeny with species from outside North America is required to disentangle how these species arose. The only other known species of *Quinqueserialis* occurs in Russia and there are no publicly available sequences.

Overall, the 49 novel DNA sequences generated in this study will help to identify either larval or adult *Quinqueserialis* spp. specimens in future studies. Additionally, morphological vouchers produced in this study will be vital to further investigations into *Quinqueserialis* taxonomy and ecology. Further, the sequence and morphological vouchers from this study will be key to solving the life cycle of *Quinqueserialis* n. sp. which remains unknown. We demonstrated that using an integrative and holistic approach is essential to understanding parasite ecology and evolution. Parasites represent an extremely diverse group covering many taxa, and yet there is still much to learn about their basic life history and evolution. Knowledge of parasite biogeography, life cycles, and host use is important in understanding disease ecology and interactions with other organisms in the ecosystem[1]. Thus, parasites represent a fundamental component of the environment, and we must continue to learn and understand their biology.

[1] Price, P.W. Westoby, M., Rice, B., Astatt, P.R. Fritz, R.S., Thompson, J.N. and Mobley, K. 1986. Parasite mediation in ecological interactions. Annual Review of Ecology and Systematics **17**: 487-505.

Appendix 1

Table S1.1: Prevalence of *Quinqueserialis* spp. infection in field-collected hosts.
Infection prevalence in mammalian final hosts and monostome-morphotype cercariae in snail intermediate hosts from 6 sampling locations.

Location	GPS Coordinates	Host Species (N)	Parasite Species	Prevalence % (Range)
Inuvik, NT	68.2986, -133.6018	*Ondatra zibethicus* (10)	*Q. quinqueserialis*	100 (228-1006)
		Microtus pennsylvanicus (8)		38 (1-7)
		Microtus oeconomus (1)		100 (12)
		Myodes rutilus (45)		0
		Gyraulus circumstriatus (179)		1.1 (NA)
		Promenetus exacuous (5)		0
		Lymnaea elodes (14)		0
		Valvata sp. (6)		0
	68.6232, -135.3692	*Ondatra zibethicus* (12)	*Q. quinqueserialis*	100 (69-669)
		Microtus pennsylvanicus (3)		67 (1-44)
		Gyraulus circumstriatus (107)		0
		Promenetus exacuous (40)		10 (NA)
		Planorbula sp. (53)		0
Churchill, MB	NA	*Microtus pennsylvanicus* (34)	*Q. quinqueserialis*	5.9 (3-14)
			Q. n. sp.	5.9 (1-4)
Southern MB	49.1291, -97.283	*Ondatra zibethicus* (25)	*Q. quinqueserialis*	68 (2-170)
		Microtus pennsylvanicus (10)		10 (NA)
		Myodes gapperi (12)		0
		Zapus hudsonius (1)		0
		Gyraulus circumstriatus (432)		1.4 (NA)
		Promenetus excacuous (4)		25 (NA)
		Helisoma trivolvis (100)		0
		Lymnaea elodes (133)		0
	50.3135, -96.7082	*Ondatra zibethicus* (31)	*Q. quinqueserialis*	64.5 (1-131)

		Gyraulus circumstriatus (54)		1.9 (NA)
		Promenetus exacuous (43)		2.3 (NA)
		Gyraulus crista (1)		0
		Planorbula sp. (6)		0
		Helisoma trivolvis (117)		0
		Lymnaea elodes (70)		0
	50.1881, -97.1167	*Ondatra zibethicus* (17)	*Q. quinqueserialis*	100 (34-211)
		Gyraulus circumstriatus (69)		7.3 (NA)
		Gyraulus crista (18)		16 (NA)
		Promenetus exacuous (6)		0
		Lymnaea stagnalis		0
Minnesota*	43.8492, -93.8578	*Gyraulus* sp. (5)	*Q. quinqueserialis*	0
		Gyraulus crista (8)		0
Virginia*	37.2011, -80.5652	*Microtus pennsylvanicus* (3)		0
Alabama	34.3489, -86.2873	*Ondatra zibethicus* (7)	*Q. quinqueserialis*	100 (13-291)

*Adult *Q. quinqueserialis* specimens from muskrats (*Ondatra zibethicus*) were donated from collections by Dr. L. Belden and Dr. R. Sorensen from Virginia and Minnesota, respectively. No prevalence data available.

Table S1.2: Final hosts of 96 specimens included in the Principal Component Analysis.

Species	Number of specimens	Number of specimens from host species	Replicate specimens from individual host?
Quinqueserialis quinqueserialis	87	Muskrats (56) Voles (25) Guinea Pig (1) Meadow Jumping Mouse (3)	1-3 per host.
Quinqueserialis floridensis	5	Round-tailed muskrat (5)	2-3 per host.
Quinqueserialis n. sp.	4	Voles (2)	1-3 per host.

Table S1.3: Catalog numbers of *Quinqueserialis* spp. museum specimens.
Catalog number and accompanying data of 19 museum lots identified as *Quinqueserialis* spp. loaned from the Invertebrate Zoology Collection at the Smithsonian National Museum of Natural History.

Catalog Number	Species Identification	Host Identification	Location	Collector
USNM 1319422	*Notocotylus hassalli**	*Arvicola riparius*	US	Hassall, A.
USNM 1343043	*Notocotylus quinqueserialis*	Guinea Pig	Beltsville, Maryland	Krull, W.H.
USNM 1350530	*Notocotylus quinqueserialis*	*Ondatra zibethicus*	Baker Lake, Washington	Metcalf, H.E.
USNM 1355028	*Notocotylus quinqueserialis*	*Ondatra zibethicus*	Blackwater	Dozier
USNM 1390832	*Notocotylus quinqueserialis*	*Ondatra zibethicus*	Onieda Lake, New York	Mueller, J.F.
USNM 1350529	*Notocotylus quinqueserialis*	*Ondatra zibethicus*	US	La Rue, G.R.
USNM 1346536	*Quinqueserialis floridensis*	*Neofiber alleni*	Putnam County, Florida	Quay, W.B.
USNM 1327181	*Quinqueserialis hassalli**	*Microtus pennsylvanicus*	East Falls Church, Virginia	Chapin, E.A.
USNM 1341119	*Quinqueserialis hassalli**	*Microtus pennsylvanicus*	Cass Lake, Minnesota	Dikmans, G.
USNM 1319419‡	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Baltimore, Maryland	Hassall, A.
USNM 1330294	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Eastern Shore, Maryland	Price, E.W.
USNM 1338717	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Dixbono, Michigan	Ameel, D.J.
USNM 1341145	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Miles City, Montana	Wehr, E.E.
USNM 1353411	*Quinqueserialis quinqueserialis*†	*Charitonetta albeola*	Mendenhall Flats, Alaska	Williams, R.B.
USNM 1370253‡	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Williamson County, Illinois	Jilek, R.
USNM 1385033	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Wyoming	McIntosh, A.
USNM 1353615	*Quinqueserialis* sp.*	*Zapus hudsonius*	Tompkins County, New York	Whitaker, J.
USNM 1395179	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Michigan	Wu, K.
USNM 1365347	*Quinqueserialis quinqueserialis*	*Ondatra zibethicus*	Bog Lake, Michigan	Unknown

* Species name needs to be changed to *Q. quinqueserialis*.
‡ Accurate measurements could not be obtained from this specimen and so it was not included in the analyses.
† Specimen only had three rows of ventral papillae and is likely a misidentified *Notocotylus* sp. parasite. For this reason, it was not included in the analyses.

Appendix 2

Small mammal trapping protocol

Field trapping and euthanasia protocol was approved by the University of Manitoba Fort Garry Campus Animal Care Committee (AC11347). Territorial/State scientific collection permits were obtained prior to trapping voles in Manitoba, Inuvik, NT and Blacksburg, VA.

In Manitoba and Virginia, Sherman style live-traps were used to capture voles at the same marshes where infected muskrats were trapped and donated by licensed local trappers (Abbreviated Protocol for Minimal Animal Involvement). Traps were set along a linear transect or along the banks of bodies of water. Each trap was insulated with cotton balls and baited with a mixture of peanut butter and oats. The GPS coordinates of each trap were recorded, and each trap location was demarcated with fluorescent flagging tape. Traps were set at sunset and checked at sunrise the following morning. Bite-proof leather gloves were worn by each person handling recovered voles. Each vole was euthanized on site via anesthetic overdose followed by cervical dislocation. We placed voles in a 1L glass jar with cotton soaked with 1 mL isoflurane (University of Montana, Standard Operating Procedures, 2017, https://www.umt.edu/research/LAR/sops/SOPopendropisoflurane.php). The soaked cotton was placed in an open 15 mL conical tube so that the liquid isoflurane was not making contact with the vole's skin. All persons who handled isoflurane wore nitrile gloves. Proper ventilation was ensured as the voles were euthanized in the field. Voles were left in the sealed jar until they were unresponsive to stimuli, or for 5 min to ensure overdose. Voles were then removed from the jar and euthanasia was verified via cervical dislocation.

In Northwest Territories, snap traps were used to mortally capture voles (NWTWCC 2018_014). Snap traps were set along a linear transect or along the banks of bodies of water.

Traps were baited with a mixture of peanut butter and oats. The GPS coordinates of each trap were recorded, and each trap location was demarcated with fluorescent flagging tape. Traps were set in the evening and recovered the following morning. As the voles were deceased upon recovery, nitrile gloves were worn to handle voles.

Deceased voles were placed in Ziploc bags, labelled appropriately, and into a cooler with ice for transport. Voles were necropsied immediately in the laboratory or stored at 4 C until time of necropsy. Post necropsy, the remains of the voles were incinerated (University of Manitoba), or disposed of following the regulations of the host institution.

Appendix 3

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Consistent intermediate host use across a broad geographic range by a trematode parasite (Notocotylidae, *Quinqueserialis quinqueserialis*)

D. K. Gagnon and J. T. Detwiler

Department of Biological Sciences, University of Manitoba, Winnipeg, Canada, R3T 2N2

Correspondence should be sent to: Jillian.Detwiler@umanitoba.ca

ABSTRACT

Knowledge of helminth life cycles is essential to understanding their host specificity, geographic distribution, and transmission. Many helminth life cycle descriptions are based on field collections in a limited part of the parasite's range. However, it is important to determine whether helminth life cycles and host specificity remain consistent across their geographic range so that we may better understand their life history and epidemiology. Here, we investigated whether the life cycle of a widespread trematode, *Quinqueserialis quinqueserialis* (Trematoda, Notocotylidae) varies across its geographic range. Four species of planorbid snails; *Gyraulus* sp., *Gyraulus crista*, *Promenetus exacuous* and *Planorbula* sp., were collected at 5 locations in Canada (3 in Manitoba, 2 in Northwest Territories). Snails and parasite larvae were morphologically and genetically identified to species. Total prevalence of *Q. quinqueserialis* infections in snail hosts at the 5 locations was 2.1% (N=1016). Three species of snails were infected with *Q. quinqueserialis* rediae: *Gyraulus circumstriatus*, *G. crista*, and *P. exacuous*. These 3 species of snails were infected in both the central locations (Manitoba) and northern extant (Northwest Territories) of this parasite's geographic range, and thus no life cycle variation due to geography was noted. This is the first report of snails naturally infected with *Q.*

quinqueserialis in Canada. Further, these novel host records demonstrate that this trematode species is not as host specific for first intermediate host species as previously described.

KEY WORDS

Life cycle variation, Host survey, New host records, Host specificity, Geographic range

Understanding the life cycles of helminth parasites is essential to establishing their host specificity, geographic range, and transmission. However, most helminth life cycles have not been solved or if they have, they have been revised as more life history studies of the parasite have been completed (Bolek et al, 2016). One reason for these revisions is that many life cycle descriptions are based on field collections in one part of the entire range of the parasite. Some parasites have large geographic ranges that can span entire continents. For such parasites, it is important to determine whether their life cycle and host specificity is similar across that range.

One example of a parasite with an established life cycle and a large geographic range is *Quinqueserialis quinqueserialis* (Trematoda, Digenea, Notocotylidae). This life cycle was "solved" when the adult stage was linked to the larval stage by Herber (1942). Initially, *Q. quinqueserialis* was described from adults collected from muskrats (*Ondatra zibethicus*) and meadow voles (*Microtus pennsylvanicus*) in Nebraska and Alaska (Barker and Laughlin, 1911; Rausch, 1952). This trematode species has been reported in muskrats across the United States and Canada, with the northern limit of its range above the arctic circle and the southern limit of its range reaching the southeastern United States (i.e. Rausch, 1952; Detwiler et al, 2012). Decades later, the life cycle was completed in North America after larvae were found in the freshwater snail *Gyraulus parvus* (Say) collected in Michigan (Herber, 1942). Then, over 40 years later in Russia, another snail species, *Gyraulus stroemi* (Westerlund) was identified as the intermediate host of *Q. quinqueserialis* through field surveys and experimental infections using

field-infected snails and laboratory mice (Zhaltsanova and Beliakova, 1986). However, a second *Quinqueserialis* species, *Q. wolgaensis*, that infects water voles (*Arvicola terrestris*) also occurs throughout Russia (Skvortsov, 1935). Thus, without genetic identification of rediae from infected *G. stroemi* in Russia, it is unclear whether this snail species acts as intermediate host to either *Q. quinqueserialis* or *Q. wolgaensis*. The report of a potential novel host in a different part of the parasite's range suggests that as first intermediate host specificity is examined across a wide geographic range, additional hosts may be found. Yet, most subsequent reports of *Q. quinqueserialis* have been of the adult stage identified from the definitive hosts (e.g. McKenzie and Welch, 1979; Rigby and Threlfall, 1981; Haukisalmi et al, 1995; Zabiega, 1996; Seegers et al, 1997; Detwiler et al, 2012). Thus, knowledge of the intermediate host range is limited. For example, there are no reports of *Q. quinqueserialis* in naturally-infected snails in Canada.

To complete the life cycle of *Q. quinqueserialis* and test the hypothesis that life cycle variation will occur in different parts of the parasite's geographic range, we surveyed 4 species of pulmonated snails in the family Planorbidae. Snails were collected at 5 locations (3 in Manitoba and 2 in Northwest Territories, Canada) in June-August 2018 and examined for the presence of *Q. quinqueserialis*. The locations included a pond adjoining a river (Marais River, MB: 49°07.75'N, 097°16.98'W); a road-side ditch abutting a wetland (Libau, MB: 50°18.81'N, 096°42.49'W); a marsh (Oak Hammock Marsh, MB: 50°11.287'N, 097°07.004'W); a creek (Onion Lake Creek, NWT: 68°17.95'N, 133°35.81'W): and a channel (Leland Channel, NWT: 68°37.39'N, 135°22.15'W).

Over a 2 hr period, snails were haphazardly collected by hand, placed in buckets containing water from the collection location, and transported back to the laboratory. Snails were placed in 6-well cell culture plates filled with well water and exposed to light for a minimum of

3 hr to promote emergence of cercariae (Detwiler and Minchella, 2009). If cercariae emerged from the snail, the morphotypes were identified using the larval trematode key by Schell (1985). Live cercariae with a monostome morphotype were imaged with an Axio Imager M2 compound microscope (Zeiss). Due to equipment limitations at the field station in Northwest Territories, no images of live cercariae were obtained from this location. Snails infected with the monostome morphotype were crushed and rediae and cercariae were preserved in 80% ethanol. Snails that did not shed cercariae were also crushed and examined for latent infections. Snail species were initially identified using shell morphology characteristics following descriptions by Clarke (1981). Snail foot tissue was preserved in 80% ethanol.

Rediae collected from the same snail host as imaged cercariae were used in DNA extractions. Tissue samples were soaked in MilliQ water to remove the ethanol 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. A 1000 base pair (bp) fragment of the *28S* ribosomal RNA gene region was amplified using the forward primer 300F (5' CAA GTA CCG TGA GGG AAA GTT G 3') and the reverse primer 1500-R (5' GCT AGG GAA ACT TCG 3') (Olson et al, 2003). The *28S* gene region was targeted to compare our sequences to an isolate of *Q. quinqueserialis* deposited in GenBank (JQ670848.1; Detwiler et al, 2012). Amplification was carried out in a 25 µl reaction solution containing 2 µL of extracted DNA, 2.5 mM 10x buffer, 1.5 mM $MgCl_2$, 0.5 mM of dNTPs and each primer, and 0.05 unit/µL of Taq polymerase. The amplification had an initial denaturation at 95 C for 3 min followed by 40 cycles of denaturation (94 C, 45 sec), annealing (56 C, 30 sec), and extension (72 C, 2 min), with a final extension phase at 72 C for 7 min.

To confirm the morphological species identification of field-collected snails, we performed DNA barcoding with the cytochrome c oxidase subunit I (*CO1*). DNA was extracted from snails using the E.Z.N.A. Mollusc DNA Kit according to the manufacturer's instructions (Omega Bio-Tek, cat. No. D3373-02). The Folmer region (~650 bp) of *CO1* was targeted using primers LCO1490 (5'GGTCAACAAATCARAAAGATATTGG 3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA 3') (Folmer et al 1994). Amplification was carried out in 25 µL reaction volumes with 50 ng of DNA, 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, and 0.05 unit/µl Taq polymerase. The amplification was completed with a thermocycler profile following that of Gordy et al (2016).

Polymerase chain reaction (PCR) products were visualized with 2% agarose gels and products with single bright bands were purified with a PCR clean up kit (MO BIO Laboratories Inc., Carlsbad, California). Purified products were sequenced in both directions at the Hospital for Sick Children, Toronto, Ontario, Canada using an ABI 3730XL instrument. Contigs were constructed and manually edited by eye in Sequencher (Sequencher v. 4.1, 2009) and alignments were generated using ClustalW in MEGA 7.0 (Kumar et al, 2016). Genetic distances between all individuals was calculated with p-distance in MEGA.

Total prevalence of monostome infections in planorbid snails collected at the 5 locations was 2.1% (N=1016) with a mean prevalence (±SE) of 2.8% (±1.5) per location (Table 1). A total of 14 sequences were obtained from 14 individual rediae that were each recovered from an infected snail: 3 from Northwest Territories and 11 from Manitoba. All of these *28S* sequences were identical to each other but were 0.4% different across 907 bp compared to a sequence from an adult *Q. quinqueserialis* collected from a muskrat in Virginia (JQ670848.1). One sequence

representing the larval *Q. quinqueserialis* collected in Northwest Territories and Manitoba was deposited in GenBank (MK251989.1).

Among the 5 locations, 4 species of planorbid snails were collected, however monostome infections occurred in 3 species: *Gyraulus circumstriatus* (Tryon), *Gyraulus crista* (Linnaeus), and *Promenetus exacuous* (Say) (Table 1). Using shell morphology, we identified the snails as *G.* sp, *G. crista,* and *P. exacuous*. Initially, we did not identify *Gyraulus* to species because of the phenotypic plasticity observed in key shell characteristics such as shell opacity and striae (Clarke, 1981). Further, *G. parvu*s and *G. circumstriatus* can be confused based on morphological identification alone (Clarke, 1981). BLAST searches of genetic sequences from morphologically identified samples of *G. crista* and *P. exacuous* had high percent identity to other GenBank samples identified as the same species, respectively. A genetic sequence from *G. crista* collected from Oak Hammock Marsh, MB was 97% identical over 655 bp to *G. crista* (KC495836.1). Two genetic sequences from *P. exacuous* from Libau, MB and Leland Channel, NWT were 100% identical over 604 bp to *P. exacuous* (MG421197.1, MG421621.1, MG421977.1, MG422064.1, MG422315.1, MG422776.1, MG423081.1, MG423194.1, MG423308.1, MG423392.1, MG423429.1, MG423538.1). Three genetic sequences from *G.* sp collected from Marais River and Oak Hammock Marsh, MB; Onion Creek, NWT were 99 % identical over 670 bp to *G. circumstriatus* (MF544182.1, MF544479.1, MF545123.1, MG421037.1, MG421138.1, MG421420.1, MG421423.1, MG421962.1, MG421983.1, MG422619.1, MG422627.1, MG422893.1, MG422911.1, MG423191.1, MG423252.1, MG423237.1, MG423312.1). One significant contribution of this study is to have deposited genetic vouchers for the snail hosts. Without these vouchers, it is difficult to determine whether the snails in the previous life cycle studies were correctly identified (i.e. Herber, 1942,

Zhaltsanova and Beliakova, 1986). Future studies can use our genetic sequences of hosts to further evaluate life cycle variation in *Q. quinqueserialis*.

Our study confirms that *Q. quinqueserialis* has a wide geographic range in Canada that spans from the Northwest Territories to Manitoba (~2816-2989 km). On one hand, our results suggest that *Q. quinqueserialis* is not as specialized for gastropod hosts as previously described. By using at least 4 host species instead of a single snail host, it could be considered more of a generalist parasite (Poulin et al, 2011). On the other hand, the same set of host species was used by the parasite at the northern extant and in central locations (Northwest Territories-Manitoba) suggesting consistency in host specificity across a broad geographic range (Poulin et al, 2011). Further, these 3 host species belong to the same family Planorbidae. Thus, despite using a relatively high number of compatible intermediate hosts, *Q. quinqueserialis* parasites may be considered more restricted in the sense that host specificity does not vary according to geography and host phylogeny.

Prevalence of *Q. quinqueserialis* was relatively low in each snail host (Table 1). However, *Q. quinqueserialis* is successful in infecting definitive hosts typically with prevalence of infection over 80%, with individuals hosting a mean of 100 worms (e.g. McKenzie and Welch, 1979; Rigby and Threlfall, 1981). The discrepancy between first intermediate host and definitive host prevalence demonstrates transmission success despite low infection numbers among snail hosts. Exploiting more than one intermediate host species could increase the likelihood of transmission to the definitive host. *Quinqueserialis quinqueserialis* parasites do not use a mobile second intermediate host, but instead cercariae disperse and encyst on aquatic vegetation (Herber, 1942). Thus, exploiting several intermediate host species within the same

habitat, in addition to the metacercarial stage persisting in the environment, could increase transmission success.

Life cycle and host range information is critical for understanding the epidemiology of wildlife disease. Here, we demonstrate the importance of host surveys across a broad geographic range in determining the host specificity and infection dynamics of trematode parasites. In contrast to our hypothesis, we found no evidence for life cycle variation across the geographic range of the trematode *Quinqueserialis quinqueserialis*. Instead, we found that the same set of gastropod host species are first intermediate hosts for this parasite. Importantly, all of these hosts were not previously known to transmit this parasite.

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LITERATURE CITED

Barker, F. D., and J. W. Laughlin. 1911. A new species of trematode from the muskrat, *Fiber zibethicus*. Transactions of the American Microscopical Society 30: 261-274.

Bolek, M.G., Stigge, H.A. and Gustafson, K.D. 2016. The iron wheel of parasite life cycles: Then and now! *In* A Century of Parasitology: Discoveries, ideas and lessons learned by scientists who published in The Journal of Parasitology, 1914-2014. J. Janovy Jr and G.W. Esch (eds.). John Wiley & Sons, Ltd, Chichester, UK, p. 131-147.

Clarke, A. H. 1981. The freshwater molluscs of Canada. National Museum of Natural Sciences, Ottawa, Canada, 446 p.

Detwiler, J. T., and D. J. Minchella. 2009. Intermediate host availability masks the strength of experimentally-derived colonization patterns in echinostome trematodes. International Journal for Parasitology 39: 585-590.

Detwiler, J. T., A. M. Zajac, D. J. Minchella, and L. K. Belden. 2012. Revealing cryptic parasite diversity in a definitive host: Echinostomes in muskrats. Journal of Parasitology 98: 1148-1155.

Folmer, O. M., Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294-299.

Gordy, M.A., L. Kish, M. Tarrabain, and P. C. Hannington. 2016. A comprehensive survey of larval digenean trematodes and their snail hosts in central Alberta, Canada. Parasitology Research 115: 3867-3880.

Haukisalmi, V., H. Henttonen, and G.O. Batzli. 1995. Helminth parasitism in the voles *Microtus oeconomus* and *M. miurus* on the North Slope of Alaska: host specificity and the effects of host sex, age and breeding status. Annales zoologici Fennici 32: 193–201.

Herber, E. C. 1942. Life history studies on two trematodes of the subfamily Notocotylidae. Journal of Parasitology 28: 179-196.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870-1874.

McKenzie, C. E., and H. E. Welch. 1979. Parasite fauna of the muskrat, *Ondatra zibethica* (Linnaeus, 1766) in Manitoba, Canada. Canadian Journal of Zoology 57: 640-646.

Olson, P. D., T. H. Cribb, V. V. Tkach, R. A. Bray, and D. T. J. Littlewood. 2003. Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). International Journal for Parasitology 33: 733-755.

Poulin, R., B.R. Krasnov, and D. Mouillot. 2011. Host specificity in phylogenetic and geographic space. Trends in Parasitology 27: 355-361.

Rausch, R. 1952. Studies on the helminth fauna of Alaska. XI. Helminth parasites of microtine rodents—taxonomic considerations. Journal of Parasitology **38:** 415-444.

Rigby, M.D. and W. Threlfall. 1981. Helminth parasites of the muskrat (*Ondatra zibethicus* (L.)) in Newfoundland. Canadian Journal of Zoology 59: 2172-2176.

Seegers, G., S. Baumeister, S. Kuschfeldt, K. Pohlmeyer, and M. Stoye. 1997. Nematode and trematode fauna of the muskrat (*Ondatra Zibethicus* LINK, 1795) in Lower Saxony. Deutsche Tierarztliche Wochenschrift 104: 503–504.

Sequencher® version 4.1. 2009. Sequence analysis software, Gene Codes Corporation, Ann Arbor, Michigan.

Schell, S. C. 1985. Handbook of trematodes of North America, North of Mexico. University Press Idaho, Moscow, Idaho, 263 p.

Skvortsov, A.A. 1935. On a study of the helminth fauna of the water rat, *Arvicola terrestris*. (In Russian). Vestnik Microbiologii Epidemiologii i Parazitologii. 13: 317-326.

Zabiega, M.H. 1996. Helminths of mink, *Mustela vison*, and muskrats, *Ondatra zibethicus*, in Southern Illinois. Journal of the Helminthological Society of Washington 63: 246–250.

Zhaltsanova, D.S., and I. V. Beliakova. 1986. Intermediate host of the trematode *Quinqueserialis quinqueserialis* (Trematoda, Notocotylidae) in the USSR and the morphology of its parthenitae and larvae. (In Russian). Parazitologiia 20: 323–326.