When you come to a fork in the road, take it.

- Yogi Berra

University of Alberta

A molecular assessment of range expansion of the northern or virile crayfish (*Orconectes virilis*), crayfish-based community co-structure, and phylogeny of crayfish-affiliated symbionts

by

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To my parents, Judy and Jim, and my sister, Caitlin,
for your unconditional love and support through
all twists and turns in my path to the present.

Abstract

Geographical limits of a species' range are determined in part by the environmental tolerances of that species, and also by its past and current ability to colonize new areas. Range shifts are a common occurrence in the evolutionary history of almost all taxa; however, anthropogenically-mediated activities have facilitated rapid changes in the distribution of many species directly (via introduction) or indirectly (via alteration in habitat or environment). Identification of the pathways underlying range expansion is critical if we are to understand the ecological and evolutionary potential of a species facing changing abiotic and biotic factors.

The northern crayfish, *Orconectes virilis* (Hagen), has rapidly expanded its western range edge along several rivers in the prairies of North America. Although the spatial extent and timing of spread appear to indicate that the species has responded to a large-scale change in environmental conditions, several lines of evidence suggested that human-mediated introduction may also have played a role in range expansion of the species. The species is currently contiguously distributed along rivers across the Interior Plains, but is also found in several lakes and waterways disjunct from the core range. In this thesis I use phylogeographic analysis to identify two genetically distinct *O. virilis* haplogroups in the region. One haplogroup is spatially and genetically consistent with patterns expected from gradual post-glacial expansion; however, the second is suggestive of frequent and widespread introduction. I developed microsatellite markers that allowed me to assess fine-scale intra-river genetic patterns associated with spread of *O. virilis*. I found that genetic patterns were not consistent across rivers, and that recent range expansion has resulted from a combination of natural spread and human-mediated introduction.

Two groups of crayfish-associated symbionts were found on *O. virilis* in the Interior Plains, branchiobdellidan worms and entocytherid ostracods. The distributions of these organisms suggest that factors affecting range limits differ among host and symbionts. Little is known of the ecology and evolution of these

symbionts, not only in the Prairies, but also across their global distribution. My examination of phylogenetic patterns of North American branchiobdellidans provides the best supported evolutionary hypothesis of the order to date.

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Chapter 1. The evolution of a thesis

1.1 Main text

Geographic limits to species' ranges vary as individuals and populations respond to fluctuations in several factors that affect persistence and survival (e.g., environment, competition, predator-prey interactions). Although range shifts occur commonly throughout the evolutionary history of almost all species (Hewitt 1996, 2000), anthropogenically-mediated activities have facilitated recent and rapid changes in the distribution of many taxa, both directly (via spread of propagules) and indirectly (via modifications of habitat or other environmental characteristics) (Chen et al. 2011; Parmesan and Yohe 2003; Wilson et al. 2009). The ability to distinguish between 'natural,' or unassisted, spread and spread associated with human-mediated introduction is not always simple, particularly when patterns of spread are not closely monitored and post-expansion distribution appears to be contiguous relative to core areas within range of the species. However, these mechanisms of expansion may differ drastically in their ecological and evolutionary impacts, and in the way shifts in ranges are managed.

Numerous studies have demonstrated the utility of molecular genetic methods for examining patterns of spread in both native and introduced organisms. Our current understanding of genetic patterns of unassisted spread is largely based on observations of post-glacial expansion (e.g., Bernatchez and Wilson 1998; Excoffier et al. 2009; Hewitt 1996, 2004). A few recent studies have examined the consequences of rapid unassisted (e.g., climate-driven) range expansions (Banks et al. 2010; Dawson et al. 2010; Garroway et al. 2011), highlighting important differences in establishment of genetic structure over short temporal scales. Additional information of genetic patterns of rapid range expansion is provided by recent studies of spread following introduction of invasive species (e.g., Bronnenhuber et al. 2011; Darling and Folino-Rorem 2009; Ramakrishnan et al. 2009; Watts et al. 2010), although such patterns are largely influenced by characteristics of the initial founding event(s).

The northern or virile crayfish, *Orconectes virilis* (Hagen, 1870), is one of the most widespread crayfish species in North America, with a core distribution – defined as the largely contiguous area within the overall range supporting the highest densities of individuals – ranging west to east from Alberta and Montana to Maine and New Brunswick, and north to south from the northern Prairie Provinces to Texas (McAlpine et al. 1999; Taylor et al. 2005; Williams et al. 2011a). Over half of its core range is found in areas of northern North America that were covered by the Laurentide ice sheet during the last glacial maximum of the Wisconsinan ~ 21,000 ya. As such the species has demonstrated an ability to successfully take advantage of colonization opportunities. In addition, the wide distribution of *O. virilis* suggests that the species has broad environmental tolerance levels which may in turn facilitate the spread and establishment of the species.

In recent decades *O. virilis* has greatly increased its distribution. Much of this expansion has been human-mediated, resulting in disjunct populations far beyond the limits of the core range. Although some of these introductions were intentional (e.g., to supplement food stocks for sport fishes, Johnson 1986; Sheldon 1989; for culinary purposes, Skurdal et al. 1999), most have been undocumented releases likely via several different pathways (e.g., bait release, escape or release from private or institutional aquaria, escape from areas of pond weed management; Larson and Olden 2008; Lodge et al. 2000; DiStefano et al. 2009). The northern crayfish has also exhibited apparent recent and substantial extension to its core range, particularly along its western range edge in the northern Prairies. However, prior to my research the history and distribution of *O. virilis* in this region was not well known. Therefore, except for scattered anecdotal sightings or reports of the species by fisherpersons or biologists, there was little context, historical or contemporary, available to clearly understand the timing and extent of recent expansion or the potential mechanisms underlying the spread of *O. virilis*.

An initial objective of my doctoral research was to document the current distribution of the northern crayfish across the Prairies region and to use these

data, in combination with anecdotal observations and limited historical reports (e.g., Faxon 1885; Hagen 1870; Rawson and Moore 1944), to develop hypotheses about the mode(s) and potential source(s) for the recent westward expansion of *O. virilis* (Chapter 2; Williams et al. 2011a). A second objective was to use molecular markers to characterize genetic structure and diversity of *O. virilis* and test theoretical expectations of the aforementioned hypotheses. To understand this apparent recent range expansion of *O. virilis* in the western Prairies, both historical and contemporary processes affecting genetic patterns at regional and local scales must be taken into account. Phylogeographic methods can be used to assess if regional patterns of genetic structure and diversity are consistent with post-glacial colonization, and can also be used to identify potential source regions of introductions (Chapter 3). However, sequence-based phylogeographic analyses are often limited in scale of inference, particularly in younger systems colonized by expansion from a single glacial refugium, where genetic diversity and structure is expected to be low (Bernatchez and Wilson 1998; Hewitt 2004). Highly variable molecular markers, such as microsatellites, are frequently used in studies examining relationships between and among populations and/or individuals. Consequently, I developed a novel suite of microsatellite markers for *O. virilis* (Chapter 4; Williams et al. 2010) and used them to assess genetic patterns within rivers where recent spread had been documented relative to rivers in which *O. virilis* are known to have been historically established (Chapter 5).

In 2002, Dr. Heather Proctor (University of Alberta) discovered unidentified branchiobdellidans, or crayfish worms, on *O. virilis* collected in the North Saskatchewan River in Edmonton, Alberta. Branchiobdellidans were previously unknown from the Prairie Provinces (Gelder et al. 2002), but are common ectosymbionts of crayfishes across the Holarctic (Gelder 1999). The presence of these worms on *O. virilis* in a recently colonized reach of the North Saskatchewan River suggested that the crayfish and branchiobdellidans had concurrently expanded their ranges, and thus likely had a shared colonization history. As such, there was an opportunity to use the branchiobdellidans as

proxies to examine *O. virilis* history, particularly with respect to recent movements.

Several studies have demonstrated the utility of symbionts in elucidating host ecology and evolution (review in Nieberding and Olivieri 2007). The main criterion for such an approach is a common history between (or among) host and symbiont(s), driven largely by the nature of the host-symbiont interaction and transmission dynamics (e.g., Charleston and Perkins 2006; Whiteman and Parker 2005). Branchiobdellidans have the potential to be useful as good proxies for examining host history, particularly in the Prairies. Northern crayfish populations in this region are relatively young due to the impacts of the Wisconsinan glaciations. Consequently, resolution of molecular markers for *O. virilis*, including microsatellites, might be too coarse to reflect the fine-scale movements of the crayfish. Although the life cycle of all branchiobdellidan species remain unknown, my personal observations suggest that time from cocoon deposition to sexual maturity is between one and two months. In contrast, northern crayfish reach maturity between one and two years of age, typically reproduce once per year in northern latitudes, and have an average life span of three years (Sawchyn 1986; Weagle and Ozburn 1972). Thus, the life cycle of branchiobdellidans is much faster than that of *O. virilis*, and might facilitate finer genetic structuring of the worms relative to the crayfish. The obligate nature of the branchiobdellidan-crayfish symbiosis is reproductive, whereby cocoons must be deposited directly on the carapace of a living crayfish (Young 1966). Branchiobdellidans can survive for several months off a host on the substrate, but appear to not enter the water column or to move about in search for additional hosts (Young 1966). As such, the worms are transmitted primarily through direct contact among host individuals, both vertically from female parent to her offspring and horizontally during social interactions between unrelated individuals. In the Prairie region, host-specificity is high due to the presence of only a single host species.

Therefore, an additional objective of my research was to use branchiobdellidans as another marker to help assess colonization patterns of *O. virilis*. Site-level branchiobdellidan diversity can be high, with a record of eight

species representing five genera, and at least six of these species on a single individual crayfish (Gelder 1996). Thus, species composition could potentially be used as a rough marker to trace host origins if host-branchiobdellidan communities differ among rivers or regions. However, a survey of *O. virilis* across the Prairies resulted in the identification of only two species, *Cambarincola vitreus* Ellis, 1919 and *Cambarincola chirocephalus* Ellis, 1919, with the latter restricted to the eastern and southern limits of the sampled area (Chapter 6; Williams et al. 2009). Further, the distribution of branchiobdellidans was not entirely coincident with that of *O. virilis*, although *Cambarincola vitreus* is distributed throughout the North Saskatchewan and Battle Rivers, representing two of the three major expansion fronts of interest. To examine individual- and population-level genetic patterns for the worm, and hence the potential to use their genetic patterns to interpret invasion routes of the crayfish, I set out to develop a suite of microsatellite markers for *C. vitreus*. Despite the high number of repeat-bearing clones I was able to obtain from the species (over 800), I was left with only one viable microsatellite marker. I therefore began examining variation in several sequences for population and regional variation within *C. vitreus* with a modified objective of assessing co-phylogeographic structure of the worm and host crayfish. I first screened the 658 base pair barcoding region of cytochrome *c* oxidase subunit I (COI) in *C. vitreus* collected widely across the central Interior Plains and surrounding regions (i.e., Alberta, Saskatchewan, Manitoba, North Dakota, and Minnesota). Surprisingly I found only two haplotypes, differing by a single base pair, across all sampled *C. vitreus*, in contrast to 39 COI (barcoding region) haplotypes found in *O. virilis* spanning the same geographic area. Screening of 16S rDNA and internal transcribed spacer I (ITS1) gave similar results, although in both cases no variation was observed.

It appeared that despite the theoretical potential for the utility of branchiobdellidans as proxies for host history, the practical reality was that my methods had failed to achieve the results necessary to examine host structure via the symbiont. However, I had concurrently screened a handful of additional branchiobdellidan species from various locations across North America and found

very different patterns of genetic diversity and structure, despite limited intra-specific spatial sampling. Thus, the overall lack of genetic structure in *C. vitreus* appears to be species, or region, specific. Very little is known about genetic relationships within, or even among, branchiobdellidan species. In addition to observing differing patterns of diversity in various branchiobdellidan species, my sequences contradicted several results from a previous attempt at reconstructing a molecular phylogeny for the order Branchiobdellida (Gelder and Siddall 2001). Continued sampling of branchiobdellidan species and the optimization and sequencing of five partial or full genes now provides a better understanding of phylogenetic relationships among branchiobdellidan species (Chapter 7; Williams et al. In review).

Approximately three and half years into my research I stumbled across entocytherid ostracods, representing a second group of obligate crayfish symbionts on *O. virilis* I had collected in the Prairie Provinces. These entocytherids can be, and were, easily overlooked or mistaken for grains of sand on the host. The species diversity and distribution of entocytherid ostracods in Canada is not well known. By re-examining preserved *O. virilis* in my collections, I was able to identify the ostracod – *Thermastrocythere riojai* (Hoff, 1943) – and establish the species' range across the northern prairies region (Chapter 8; Williams et al. 2011b). Similar to the branchiobdellidans, the distribution of *T. riojai* is more restricted than that of *O. virilis*, and also differs from those of *C. vitreus* and *C. chirocephalus*. Although the discovery of *T. riojai* was too late in the study to assess their genetic diversity and structure, the spatial aspects of *O. virilis*-based community structure provide novel insight. For example, entocytherids found on *O. virilis* in Swift Current Creek in southwestern Saskatchewan suggest human-mediated introduction as the next closest site where the species was present is over 100 km away and downstream of a major dam.

The following seven chapters detail data collected and analysed over the course of my doctoral research. Chapters that have been published or are in review are presented in the corresponding journal manuscript format.

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Chapter 2. Range extension of the northern crayfish, *Orconectes virilis* (Decapoda, Cambaridae), in the western Prairie Provinces of Canada[1]

2.1 ABSTRACT

The northern crayfish, *Orconectes virilis* (Hagen, 1870), is the northernmost-ranging crayfish species in North America. However, little is known of the distribution of *O. virilis* in the western Prairie Provinces of Canada, which comprise the northwestern portion of the species' contiguous range. We combined extensive sampling data with confirmed reports of crayfish to describe a recent westward range expansion and the current distribution of *O. virilis* in Saskatchewan and Alberta. The reports suggest that the vast majority of colonization of the western Prairie Provinces may have resulted from natural movement, although anthropogenic introduction may be, in part, responsible for the described expansion of *O. virilis*. The description of potential movement patterns and current range has implications for the continued monitoring and management of the species across the region.

2.2 INTRODUCTION

The widespread northern or virile crayfish, *Orconectes virilis* (Hagen, 1870), has the northernmost range of any crayfish species in North America (Hamr, 2002). Although it is the only species of crayfish known to occur in Saskatchewan and Alberta, Canada, little is known about the details of the distribution of *O. virilis* in these provinces, as evidenced by question marks in the species range map of Crocker & Barr (1968: 95), and more recently that of Hamr (2002: 586).

The earliest records of *O. virilis* in the Prairie Provinces were provided by Hagen (1870) and restated in the works of Faxon (1885), Harris (1903), and Huntsman (1915). These records were limited to collections made from Lake Winnipeg, the Red River, and Saskatchewan River in Manitoba during the

[1] A version of this chapter has been published. Williams, B.W., H.C. Proctor, and T. Clayton. 2011. Crustaceana 84:451-460. Formatting follows this journal's guidelines.

Assiniboine and Saskatchewan Exploring Expedition of 1858. The first published records of *O. virilis* in Saskatchewan were provided by Rawson & Moore (1944), who found the species to be common in lakes in the Qu'Appelle River drainage in east-central Saskatchewan, as well as the Saskatchewan River drainage and Lake Winnipegosis drainage in northeastern Saskatchewan. In addition, Rawson & Moore (1944) reported the presence of *O. virilis* in Cold Lake on the Saskatchewan-Alberta border in the Beaver River drainage, a distribution later extended westward along the Beaver and Amisk Rivers of Alberta (Aiken 1968a). Unfortunately, Rawson & Moore (1944) appears to have been missed in subsequent descriptions of the range of *O. virilis* (Crocker & Barr, 1968; Aiken, 1968a). Although Crocker & Barr (1968) and Aiken (1968a) described the range of the species reaching into Saskatchewan, they inaccurately referenced the early works of Faxon (1885), Harris (1903), and Huntsman (1915), which had provided records no further west than western Manitoba.

Despite its presence in the Beaver River system of northern Alberta (Aiken, 1968a), *O. virilis* appears to have been historically absent from much of the western Prairie Provinces. Although their survey sites were restricted to lakes, Rawson & Moore (1944) noted that crayfish were not found in any of the sampled areas in the central or western part of Saskatchewan. Aiken (1967, 1968a) failed to detect crayfish outside of the Beaver River drainage during an extensive survey across Alberta in 1964 (fig. 2-1). A macroinvertebrate study in the Oldman and South Saskatchewan Rivers of southern Alberta during 1975-77 (Culp & Davies, 1982) also failed to detect the presence of *O. virilis*. Similarly, crayfish were not encountered during intensive invertebrate collections in the Battle River in Alberta in the late 1980s (A.-M. Anderson, Alberta Environment, pers. comm.).

In the early 1990s, anglers began reporting crayfish catches and sightings (e.g., live individuals, moulted carapaces, and dissociated chelae) in areas of Alberta outside of the Beaver River drainage. As a means of assessing the apparent movement of crayfish into Alberta, one author (TC) compiled written and verbal reports from a variety of sources including anglers, commercial fisherman, fishing guides, and biologists. We use these data, in combination with

extensive field sampling, to describe the current distribution of *O. virilis* in the Prairie Provinces of Alberta and Saskatchewan.

2.3 MATERIAL AND METHODS

Between June 2007 and August 2009 we conducted an extensive crayfish collection program across Alberta and Saskatchewan as part of a population genetics study of *O. virilis*. Sampling dates ranged from May to October throughout the study, when rivers and lakes were predominantly ice-free. Crayfish were collected by hand, kick-netting, or in baited Gee minnow traps (Wildlife Supply Company, Buffalo, NY, USA) deployed for up to 24 hours. Entrance holes of the minnow traps were enlarged to approximately 6 cm in diameter to enable larger crayfish to enter. Additional specimens were provided by collectors in Saskatchewan, Canada, and Montana and North Dakota, USA. Crayfish were identified to species using Hobbs (1972) and individually preserved in containers using 95% ethanol. The distribution of *O. virilis* was plotted in relation to modified sub-drainages, which are a combination of upper, central, and lower sub-drainages along a given named river (Natural Resources Canada 2008-2009).

2.4 RESULTS AND DISCUSSION

We collected crayfish from 103 sites. Confirmed reports of crayfish catches or sightings added five sites beyond the area sampled in Alberta (fig. 2-1, table 2-1). All obtained specimens were identified as *O. virilis*. Because a focus of the study was to confirm the presence of crayfish in Alberta, we surveyed few sites at which no crayfish were collected. However, of particular note was a failure to detect crayfish in the Red Deer River system of Alberta (sub-drainage G, fig. 2-1; survey data not shown). Our collections, in conjunction with confirmed incidence reports, document both the current distribution and a large range expansion of *O. virilis* into the northwestern portion of its contiguous range.

With the exception of works by Aiken (1967, 1968a, 1968b, 1969a, 1969b), crayfish in the Prairie Provinces have generally not been well-studied. Thus, the dearth of previous records in areas in which we found *O. virilis* might be a result

of inadequate targeted sampling rather than true absence. However, descriptions of presence or absence of crayfish by Rawson & Moore (1944) suggest that *O. virilis* may not have been present in much of western Saskatchewan. Similarly, results of the targeted survey by Aiken (1967, 1968a) suggest that *O. virilis* was likely absent from Alberta prior to 1964, with the exception of the Beaver River drainage. The first reports of crayfish observations in Alberta outside of the Beaver River drainage occurred in the early 1990s (Clayton, unpubl.), indicating a recent and rapid westward range expansion of *O. virilis*.

Although the mechanisms behind the spread of *O. virilis* into the western Prairie Provinces are currently unknown, the probable continuous distribution along rivers, including many that flow into Montana and North Dakota (e.g., Milk River, Frenchman River) suggest that the vast majority of colonization of the western Prairie Provinces may have resulted from natural movement. In contrast, the westernmost collections from McLeod Lake (site 1), Beyette Lake (site 2), East Pit Lake (site 15), and Nose Creek (site 47) (fig. 2-1, table 2-1) are isolated populations that range approximately 60 to 160 km from the nearest known river population. The disjunct distribution of these isolated populations may be a result of historic non-human-mediated movement (e.g., remnants of a previous range expansion followed by range contraction). However, McLeod Lake is heavily used for recreational purposes (i.e., swimming, boating, fishing), and it is unlikely that the presence of *O. virilis* would have gone unnoticed for decades prior to the first report in the late-1990s. In addition, East Pit Lake is a reclaimed coal mine with no in- or out-flow, and no viable routes of natural colonization. These two situations suggest at least some portion of the westward spread of *O. virilis* is due to anthropogenically-mediated movement.

The northern crayfish has been widely introduced outside of its native range, namely into western and eastern North America as well as Europe (e.g., McAlpine et al., 2007; Ahern et al., 2008; Larson et al., 2010). The reason for its initial introduction into East Coast areas of the U.S. and Europe were largely for culinary purposes (Faxon, 1885; Schwartz et al., 1963; Souty-Grosset et al., 2006). Due to the absence of a tradition of crayfish consumption in Canada

(Huntsman, 1915), it is unlikely that a culinary rationale is responsible for introduction of crayfish in Alberta and Saskatchewan. Introductions can also occur through the escape of crayfish from aquaculture and ornamental ponds, and deliberate release from pet aquaria and educational institutions (Lodge et al., 2000; Larson and Olden, 2008). Live bait release is frequently implicated in crayfish introductions (Lodge et al., 2000; DiStefano et al., 2009), and may best explain the spatio-temporal pattern reports of crayfish in southern Alberta ranging from the mid 1990s to the present. Initial reports of crayfish in southern Alberta, from 1993 on, came from lakes and reservoirs that are popular fishing areas, particularly for pike (Esocidae: *Esox lucius* Linnaeus, 1758); subsequent reports were predominantly from rivers downstream of those lakes and reservoirs (Clayton, unpubl.). This is in contrast to spatio-temporal patterns reported from northern Alberta. The earliest observations of *O. virilis* in northern Alberta outside the Beaver River drainage were in 1992 and 1993 from locations in the Battle and North Saskatchewan Rivers close to the Alberta-Saskatchewan border (Alberta Sustainable Resource Development [ASRD], unpubl.); subsequent reports from the Battle and North Saskatchewan Rivers appeared in areas to the west of the initial observations (ASRD, unpubl.), suggesting upstream expansion, either naturally or via human assistance.

Because of the relatively recent arrival of *O. virilis* into many waterways and waterbodies of Alberta, it is too early to forecast what effect the species will have on newly occupied areas. Although introduction of crayfish into novel habitats has the potential to dramatically affect native ecosystem diversity and function (e.g., Chambers et al., 1990; Dorn & Wojdak, 2004; Dorn & Mittlebach, 2004; Phillips et al., 2009), the spread of *O. virilis* may provide yet unknown ecological benefits. Further spread of *O. virilis* in the western Prairie Provinces may be limited by its inability to tolerate environmental conditions such as prolonged low temperatures (Aiken, 1969a) or high water velocity (Maude & Williams, 1983) which would be encountered further west along the glacial-fed rivers of Alberta.

Our collections of *O. virilis* from the Churchill River (site 12, fig. 2-1) have established the northernmost location for any crayfish species in North America

to date. Prior to this study, the northernmost record was found in the Amisk River, Beaver River drainage, Alberta, in an area disjunct from rest of the species' range (Aiken, 1968a). It is not surprising that the distribution of *O. virilis* is now known to continue east and north into the Churchill River, which is the mainstem of the Amisk and Beaver Rivers. The present report likely does not define the northern limit of *O. virilis*, which will undoubtedly be found further north and east within the Churchill River drainage area. Despite increasing our knowledge of the distribution of *O. virilis*, the absolute limits of the distribution in many parts of Canada remain largely unknown.

2.5 ACKNOWLEDGEMENTS

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Table 2-1

Locations of collections and confirmed reports of *Orconectes virilis* (Hagen, 1870) in Alberta and Saskatchewan, Canada, and adjacent areas of Montana and North Dakota, USA. Collection sites are geographically grouped by sub-drainage and site number, corresponding to fig. 2-1. Waterbody name and coordinates are provided for each site.

Modified sub-drainage	Site #	Waterbody	Location
Athabasca River (A)	1	McLeod Lake	54°17'36.04"N 115°39'04.86"W
	2	Beyette Lake	54°35'32.69"N 114°11'54.74"W
Beaver River (B)	3	Amisk Lake	54°36'14.12"N 112°38'49.74"W
	4	Amisk River	54°27'40.11"N 111°46'19.68"W
	5	Beaver River	54°23'21.00"N 110°45'18.00"W
	6	Beaver River	54°21'19.00"N 110°12'59.00"W
	7	Beaver River	54°15'33.40"N 109°13'01.07"W
	8	Beaver River	54°17'44.00"N 108°36'08.00"W
	9	Beaver River	54°17'44.00"N 108°18'08.00"W
	10	Beaver River	54°30'35.57"N 107°52'05.29"W
	11	Beaver River	55°09'10.21"N 107°35'52.60"W
Churchill River (C)	12	Churchill River	55°43'57.69"N 106°33'54.00"W
	13	Churchill River	55°38'35.97"N 104°44'00.61"W
	14	Churchill River	55°25'03.59"N 104°33'38.01"W
North Saskatchewan River (D)	15	East Pit Lake	53°35'03.45"N 114°27'49.97"W
	16	N. Saskatchewan River	53°22'12.40"N 113°45'01.99"W
	17	Lacombe Lake	53°38'13.36"N 113°39'12.53"W

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Modified sub-drainage	Site #	Waterbody	Location
	18	N. Saskatchewan River	53°30'06.14"N 113°33'38.42"W
	19	Bearspaw Lake	53°26'35.54"N 113°30'18.11"W
	20	N. Saskatchewan River	53°53'18.00"N 112°58'28.00"W
	21	N. Saskatchewan River	53°54'21.00"N 111°57'46.00"W
	22	N. Saskatchewan River	53°45'19.00"N 111°12'56.00"W
	23	N. Saskatchewan River	53°39'34.00"N 110°20'12.00"W
	25	N. Saskatchewan River	53°31'23.00"N 109°37'04.43"W
	26	N. Saskatchewan River	53°23'46.64"N 109°17'36.33"W
	27	N. Saskatchewan River	52°44'36.15"N 108°17'02.03"W
	28	N. Saskatchewan River	52°29'25.94"N 107°41'54.69"W
	29	Eagle Creek	52°13'56.93"N 107°22'46.69"W
	30	N. Saskatchewan River	52°38'44.14"N 106°50'31.19"W
	31	N. Saskatchewan River	52°56'45.35"N 106°26'07.46"W
	32	N. Saskatchewan River	53°14'42.33"N 105°25'59.96"W
Battle River (E)	33	Battle River	52°27'57.23"N 112°06'26.76"W
	34	Battle River	52°24'32.00"N 111°48'36.00"W
	35	Battle River	52°47'09.00"N 111°08'40.00"W
	36	Battle River	52°53'34.00"N 111°00'26.00"W
	37	Battle River	52°55'04.00"N 110°20'03.00"W
	38	Battle River	53°02'44.57"N 109°36'01.89"W
	39	Battle River	52°54'23.88"N 108°56'56.33"W
	40	Battle River	52°43'00.61"N 108°18'36.37"W

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Modified sub-drainage	Site #	Waterbody	Location
Saskatchewan River (F)	41	Saskatchewan River	53°14'38.00"N 105°04'19.18"W
	42	Saskatchewan River	53°14'12.61"N 104°27'51.06"W
	43	Torch River	53°32'20.25"N 104°04'08.91"W
	44	Carrot River	53°08'13.92"N 104°01'22.21"W
	45	Saskatchewan River	53°19'24.04"N 104°02'27.65"W
	46	Carrot River	53°21'56.36"N 103°15'49.75"W
Red Deer (G)	-	-	- -
Bow River (H)	47	Nose Creek	51°05'09.26"N 114°02'49.35"W
	48	Bow River	50°44'48.37"N 112°31'18.65"W
	49	Bow River	50°14'46.00"N 112°04'45.00"W
	50	Lake Newell Reservoir	50°22'43.27"N 111°54'38.02"W
South Saskatchewan River (I)	51	McGregor Lake	50°20'27.99"N 112°50'12.55"W
	52	Travers Reservoir	50°11'27.90"N 112°43'49.76"W
	53	Pothole Creek	49°24'34.21"N 112°51'54.85"W
	54	Henderson Lake	49°41'15.37"N 112°47'22.49"W
	55	Oldman River	49°51'23.00"N 112°37'29.00"W
	56	Oldman River	49°57'39.35"N 112°05'05.06"W
	57	S. Saskatchewan River	49°54'13.00"N 111°28'34.00"W
	58	S. Saskatchewan River	50°02 43.00"N 110°40'26.00"W
	59	S. Saskatchewan River	50°23'57.53"N 110°35'21.23"W
	60	S. Saskatchewan River	50°43'54.00"N 110°04'30.00"W
	61	S. Saskatchewan River	50°54'48.32"N 109°53'24.46"W

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Modified sub-drainage	Site #	Waterbody	Location
	62	S. Saskatchewan River	51°01'26.00"N 109°08'04.00"W
	63	S. Saskatchewan River	50°39'20.00"N 107°58'29.00"W
	64	Swift Current Creek	50°18'27.39"N 107°46'09.53"W
	65	S. Saskatchewan River	50°54'16.48"N 106°55'02.91"W
	66	S. Saskatchewan River	51°26'15.40"N 107°05'18.74"W
	67	S. Saskatchewan River	51°15'33.22"N 106°53'46.65"W
	68	S. Saskatchewan River	51°36'47.77"N 107°00'29.97"W
	69	S. Saskatchewan River	51°02'03.51"N 106°29'37.16"W
	70	S. Saskatchewan River	52°08'14.00"N 106°38'44.00"W
	71	S. Saskatchewan River	52°19'09.00"N 106°27'18.65"W
	72	S. Saskatchewan River	52°29'26.00"N 106°16'58.21"W
	73	S. Saskatchewan River	52°55'24.03"N 105°48'19.12"W
	74	S. Saskatchewan River	53°10'56.79"N 105°09'43.42"W
Qu'Appelle River (J)	75	Qu'Appelle River	50°59'06.00"N 106°24'56.00"W
	76	Moose Jaw Creek	50°23'38.11"N 105°29'48.61"W
	77	Last Mountain Lake	50°59'30.09"N 105°10'48.70"W
	78	Qu'Appelle River	50°38'30.48"N 104°55'41.52"W
	79	Wascana Creek	50°28'38.00"N 104°42'34.00"W
	80	Qu'Appelle River	50°39'41.77"N 103°36'11.01"W
	81	Qu'Appelle River	50°38'31.37"N 102°50'48.86"W
	82	Qu'Appelle River	50°48'15.81"N 104°34'50.06"W
	83	Qu'Appelle River	50°29'56.21"N 101°43'39.44"W

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Modified sub-drainage	Site #	Waterbody	Location
Assiniboine River (K)	84	Assiniboine River	52°05'08.43"N 102°48'49.81"W
	85	Assiniboine River	51°47'32.45"N 102°24'28.11"W
	86	Assiniboine River	51°31'57.17"N 101°52'37.36"W
Lake Winnipegosis (L)	87	Swan River	51°59'53.68"N 102°04'29.30"W
Missouri River (M)	88	Lake Frances	48°17'04.78"N 112°15'50.26"W
	89	Milk River	49°05'53.52"N 111°58'45.84"W
	90	Tiber Reservoir	48°20'32.36"N 111°09'46.30"W
	91	Fresno Reservoir	48°41'07.63"N 110°00'28.80"W
	92	Battle Creek	49°24'42.52"N 109°44'38.36"W
	93	Conglomerate Creek	49°30'23.66"N 109°02'50.49"W
	94	Frenchman River	49°20'05.93"N 108°25'00.03"W
	95	Frenchman River	49°15'00.00"N 107°43'00.00"W
	96	Nelson Reservoir	48°29 42.64"N 107°32'46.44"W
	97	Weatherall Creek	49°05'33.91"N 106°44'15.63"W
	98	Poplar River	49°01'46.36"N 105°53'37.73"W
Souris River (N)	99	Long Creek	49°03'44.85"N 103°29'52.41"W
	100	Rafferty Dam Reservoir	49°08'43.00"N 103°05'51.12"W
	101	Short Creek Dam	48°59'31.46"N 102°47'01.89"W
	102	Souris River	49°04'45.43"N 102°23'55.54"W
	103	Northgate Dam	48°55'22.37"N 102°16'16.87"W
	104	Moose Mountain Creek	49°15'40.18"N 102°14'18.76"W
	105	Moose Mountain Creek	49°33'28.36"N 102°15'17.70"W

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Modified sub-drainage	Site #	Waterbody	Location
	106	Antler River	49°11'34.44"N 101°42'39.46"W
	107	Pipestone Creek	50°02'39.35"N 101°40'36.77"W

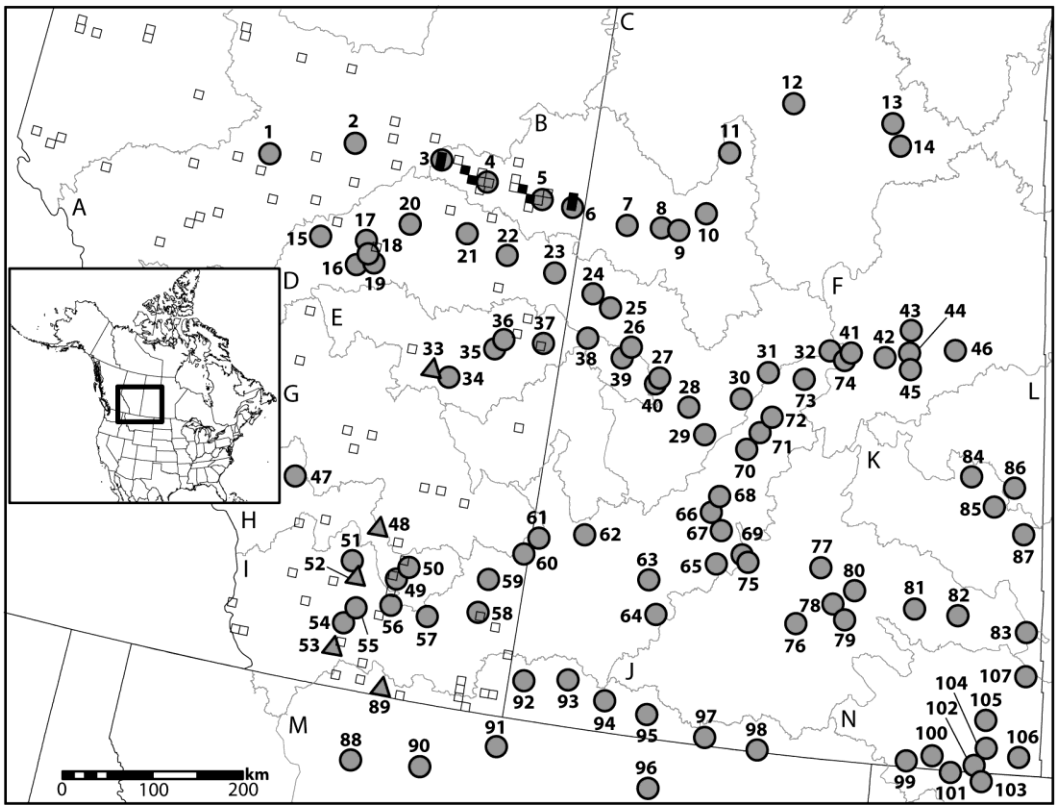

Fig. 2-1. Current distribution of the northern crayfish, *Orconectes virilis* (Hagen, 1870), in Alberta and Saskatchewan, Canada, and adjacent areas of Montana and North Dakota, USA, with respect to sub-drainage areas (labeled with upper-case letters; table 2-1). Sites at which we collected crayfish are indicated by numbered shaded circles; locations of confirmed reports of catches or sightings are shown by shaded triangles. Small squares indicate sites sampled by Aiken (1967), with black-filled and open squares indicating presence and absence of crayfish, respectively. Aiken's (1967) sites were plotted based on reported township-range locations.

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Chapter 3. Phylogeography of the virile crayfish *Orconectes virilis* (Hagen, 1870): patterns of post-glacial colonization and recent range expansion

3.1 Abstract

The northern crayfish, *Orconectes virilis*, is the northernmost-ranging crayfish species in North America. Its core distribution spans large regions of the Interior Plains and Laurentian Uplands that were covered by ice during the Wisconsinan glaciations. This distribution is indicative of widespread post-glacial colonization; however, *O. virilis* has undergone considerable recent range expansion along the western limits of its distribution that might be either continuation of post-glacial spread, or alternatively might be due to human-mediated introduction. The objectives of this chapter were to use a phylogeographic approach to determine if genetic structure of *O. virilis* across the Interior Plains is consistent with post-glacial colonization or if there is evidence of human-mediated introduction. I obtained a portion of cytochrome *c* oxidase I (COI) for 506 *O. virilis* individuals from 111 sites and nuclear glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for a subset of 211 individuals from 46 sites. I found three genetically distinct haplogroups in the Interior Plains. One haplogroup was found distributed throughout most of the prairies region and displayed patterns of genetic diversity and structure consistent with post-glacial expansion. Members of a second haplogroup were widely scattered around the eastern and western edges of the study area. This spatial distribution, in combination with patterns of genetic diversity and structure, suggested that these crayfish have been widely introduced. I observed regions of overlap between the first and second haplogroups, and patterns of hybridization and introgression. The third haplogroup was largely restricted to southern areas of the study area. Although represented by few individuals, observed patterns in genetic structure and high haplotype diversity in the third haplogroup is consistent with stable, glacially unsundered populations shown by several organisms near the Ozark region. Phylogeographic patterns across the study area reveal a complex evolutionary history of *O. virilis* and contemporary genetic changes as a result of human-mediated introduction.

3.2 Introduction

North American crayfishes are a diverse group of freshwater decapod crustaceans, representing more than 400 species and subspecies, or over 75% of the global crayfish taxonomic diversity (Taylor et al. 2007). Many of these taxa exhibit restricted ranges, likely reflective of high rates of speciation followed by limited dispersal. Concomitant with small range-size is a high risk of endangerment and extinction (Larson and Olden 2010). Indeed, almost 50% of North American crayfish species are considered threatened at some level as a result of habitat loss, water quality issues, and introduction of nonindigenous organisms (Lodge et al. 2000; Taylor et al. 2007). In contrast, frequent use of crayfishes in aquaculture, pond management, biological supply, the aquarium trade, and as bait has resulted in introduction and substantial range expansion of several species with substantial effects on native biodiversity and community structure (Lodge et al. 2000; Larson and Olden 2008). Despite high biodiversity and conservation issues, few studies have examined population-level genetic patterns of North American crayfishes.

The widespread northern or virile crayfish, *Orconectes virilis* (Hagen, 1870) (Cambaridae), is the northernmost ranging crayfish species in North America. Its core distribution spans large portions of the Interior Plains and Laurentian Uplands (Crocker and Barr 1968, Hamr 2002, Williams et al. 2011), much of which was covered by the Laurentide ice sheet during the Wisconsinan glaciations. As a result, populations across much of the core range are expected to be relatively young (< 15 000 years old), with colonization having tracked glacial retreat and having been affected by the drastic changes to drainage patterns associated with the post-glacial period (Pielou 1991). Genetic signatures of glacial events in North America are well documented for fishes (e.g., Bernatchez and Wilson 1998; Berendzen et al. 2008; Borden and Krebs 2009), and for some other freshwater animals (Cox and Hebert 2001; Dooh et al. 2006), which show varying levels of genetic structure and diversity as a function of taxon-specific demographic processes and responses to historical events. A previous analysis of *O. virilis* by Mathews et al. (2008) revealed clear genetic structure within the

species; however, restricted spatial sampling and uncertainty about endemicity of sampled populations limit the phylogeographic interpretations that can be made based on their data.

The northern crayfish has undergone considerable recent range expansion beyond the limits of its core range. It is considered nonindigenous in much of its contemporary range in the North American Atlantic region (Schwartz et al. 1963; Taylor et al. 1996; McAlpine et al. 2007), areas of the southeastern U.S. and Mexico, in several locations west of the continental divide (e.g., Sheldon 1989; Larson and Olden 2010), and in Europe (e.g., Souty-Grosset et al. 2006; Ahern et al. 2008). Although disjunct distributions and/or stocking records clearly indicate that many of these populations are indeed introduced (but see Crocker 1979), the mechanism of *O. virilis'* range expansion is not always evident. Changing environmental factors, both abiotic and biotic, have allowed some aquatic organisms to recently expand their range without direct human assistance (Gopurenko et al. 2003; Banks et al. 2010; Dawson et al 2010).

In several rivers of the central Interior Plains of U.S. and Canada, *O. virilis* has undergone apparent rapid expansion, achieving up to 250 km of river distance in less than 20 years (described in Williams et al. 2011). Crayfish catches and sightings within the period of expansion suggest different patterns of spread across drainages. Along rivers of the northern prairies region (northern Alberta and Saskatchewan), the virile crayfish was first observed in eastern localities; subsequent sightings to the west suggest upstream movement. In contrast, the first reports of *O. virilis* in the southern prairies (southern Alberta and Saskatchewan) were from western localities, primarily lakes and reservoirs that are popular fishing destinations. Subsequent observations came from eastern or downstream locales. This suggests human introduction followed by downstream movement. But overall, current distribution of the virile crayfish in the central Interior Plains is largely contiguous along waterways, with few cases of disjunct populations suggestive of introduction.

In this chapter I use a large dataset of mtDNA sequences supplemented with nuclear markers to analyse the phylogeographic structure of *O. virilis* and to

identify genetic patterns of expansion throughout the western portion of its core range. Specifically, I aim to determine what aspects of genetic structure are consistent with post-glacial colonization or if there is evidence of human-mediated introduction. As such, this chapter provides regional context and a broader background for the intra-river assessment of expansion mechanisms found in chapter 5.

3.3 Material and Methods

3.3.1 Sample collection and DNA extraction

Orconectes virilis specimens were collected between July 2006 and September 2010 from 107 sites across the central Interior Plains, western Canadian Shield, western Laurentian region, and scattered surrounding areas (Fig. 3-1; Table 3-2). This region represents a large portion of the species' core range. Crayfish were collected by hand or with baited Gee minnow traps and were placed directly into 95% ethanol. Total genomic DNA was extracted from mitochondrion-rich gill tissue using the DNeasy Blood and Tissue kit (QIAGEN, Inc., Valencia CA).

3.3.2 Sequence selection and amplification

I initially screened portions of two mitochondrial (16S rDNA and cytochrome oxidase I [COI]) and four nuclear genes (28S rDNA, Histone H3, internal transcribed spacer I [ITSI] and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) for 24 *O. virilis* individuals from sites widely scattered across the study area (see Table 3-1 for primers). Mitochondrial 16S has been suggested to be the most variable known gene in freshwater crayfishes (Crandall 1997; Fetzner and Crandall 2003; Buhay and Crandall 2005). However, I found that COI was more variable and provided better resolution than 16S, although general patterns of spatial genetic structure were similar. Histone H3 and 28S were invariant across the study area. Nuclear ITSI appeared to be hypervariable, but included repeat regions that prohibited sequencing of more than ~150 bp in either direction. However, with design of internal primers, the variability in this

region appears promising for future use as a marker for population-level analyses. The GAPDH gene showed variability at what appeared to be 4 single nucleotide polymorphism positions between two regions of the study area. As a result of my screening, I chose to use COI and GAPDH for this study.

I amplified a portion of COI for all crayfish and GAPDH for a subset of individuals. I performed amplification in 25 µL reactions consisting of 1x PCR reaction buffer (10 mM Tris pH 8.8, 0.1 % Triton X-100, 50mM KCl, 0.16 mg/ml BSA) 0.16 µM forward and reverse primers, 0.2 µM dNTPs, optimized volume of $MgCl_2$ (Table 3-1), 25-100 ng template DNA, and 0.3 U *Taq* DNA polymerase. The thermal profile for PCR amplification was 94°C for 1 minute, 3 cycles of 94°C for 30 seconds, annealing temperature (52°C COI; 58°C GAPDH) for 20 seconds, and 72°C for 5 seconds followed by 33 cycles of 94°C for 15 seconds, annealing temperature for 20 seconds, and 72°C for 1 second, with a final 72°C extension for 30 minutes. Amplified fragments were cleaned using ExoSAP-IT and subsequently sequenced and run on an ABI 3730 DNA Analyzer (Applied Biosystems).

I included 20 additional COI sequences from *O. virilis* specimens from south of the main study area that were previously published in Mathews et al. (2008), Filipova et al. (2010), and Taylor and Hardman (2002) (Genbank accession numbers, respectively: EU442725 – EU442742, FJ608578, AF474365). Twenty additional GAPDH sequences, also from these southern locales, were obtained from Mathews et al. (2008; Genbank accession numbers EU596264 – EU596284).

3.3.3 Phylogenetic analysis

Sequences were edited using SeqMan Pro (DNASTAR, Inc.) and aligned using ClustalW (Thompson et al. 1994). Allelic states in the nuclear GAPDH gene were called heterozygous when dual peaks were observed in both forward and reverse sequences. The COI haplotypes were identified in DnaSP v.5 (Librado and Rozas 2009). Akaike's information criterion (Akaike 1973) run in

jModelTest 0.1.1 (Guindon and Gascuel 2003; Posada 2008) indicated TPM1uf + Γ (Γ = 0.101) as the most appropriate substitution model for the COI dataset.

Maximum likelihood (ML) analysis of the COI dataset was carried out using RAxML v7.2.7 (Stamatakis 2006) via the CIPRES Science Gateway v. 3.1 (Miller et al. 2010). Statistical support for branching patterns was estimated concurrent with the tree search using RAxML rapid bootstrapping of 1000 replicates (Stamatakis et al. 2008). I also constructed a Neighbour-joining (NJ) tree in MEGA5 using the Tamura-Nei model of sequence evolution with Γ as identified above. Branch support was estimated using 1000 bootstrap replicates.

As intraspecific relationships are often not well-represented by bifurcating genealogies, I constructed a parsimony network for the COI dataset using TCS v 1.21 (Clement et al. 2000). I specified a connection limit of 20 mutational steps with gaps, resulting from differential sequence lengths between the present study and those incorporated from Mathews et al. (2008) and Filipova et al. (2010), considered as missing data. I calculated average Kimura 2-parameter (K2-P) distances within and among clades using MEGA5 (Tamura et al. 2011).

The most recent phylogenetic analyses of *O. virilis* have used *O. limosus* (Rafinesque, 1817) as an outgroup (Matthews et al. 2008; Filipova et al. 2010), despite a lack of morphological or molecular evidence to suggest a close relationship. In addition, Matthews et al. (2008) suggest that *Orconectes nais* (Faxon, 1885) and *Orconectes deanae* Reimer and Jester, 1975, previously supported by molecular data as close relatives to *O. virilis* (Taylor and Knouft 2006), might be members of a larger *O. virilis* complex. Consequently, I chose *Orconectes longidigitus* (Faxon, 1898) (GenBank accession no. AY701234, COI) and *Orconectes punctimanus* (Creaser, 1933) (GenBank accession no. AY701244, COI) as outgroup taxa based on concordance of phylogenetic relationships in Fetzner (1996) and Taylor and Knouft (2006).

3.3.4 Gene diversity and demographic analysis

I used DnaSP to calculate diversity estimates for the main mtDNA-based haplogroups identified in my phylogenetic analyses and for all locations with

sample sizes of two or more crayfish, including number of haplotypes (N_{hap}), number of polymorphic sites (N_p), haplotype diversity (h), nucleotide diversity (π), and mean number of paired differences (k). As I was unable to determine the distribution of haplotypes within sites sampled by Mathews et al. (2008), I could not calculate diversity estimates for haplogroup C (see Results), which was composed mostly of individuals from this previous study.

I assessed demographic history of our COI dataset by generating a mismatch distribution of pairwise differences (Slatkin and Hudson 1991) among haplotypes within each clade in Arlequin v.3.11 (Excoffier et al. 2005). I tested fit of our data to parameters estimated under both the demographic and spatial expansion models using the sum of square deviations between expected and observed mismatch over 1000 bootstrap iterations. To detect departures from equilibrium that would result from population expansions or bottlenecks, I also estimated several neutrality test statistics including Fu and Li's D^* and F^* (Fu and Li 1993) in DnaSP, and Tajima's D (Tajima 1989a, 1989b) and Fu's Fs (Fu 1997), in Arlequin.

3.4 Results

3.4.1 Phylogenetic analysis

The mitochondrial dataset was composed of 506 aligned partial COI sequences. Alignment was unambiguous, with no indels or frameshift mutations. Total alignment, based on the 484 new sequences from the current study, was 583 bp. Sequences from Filipova et al. (2010) and Mathews et al. (2008) differed in length, representing 520 bp and 538 bp, respectively, of the total alignment. Nonoverlapping segments of sequence were considered missing data in all phylogenetic analyses. I identified 47 COI haplotypes. Although the sequence from Filipova et al. (2010) was identical to my sequence h1 across the entire 520 bp overlap, it was considered a 48th haplotype (h34) due to missing data.

The COI-based NJ and ML phylogenetic analyses produced trees with similar topologies (Fig. 3-2) showing three main clades, or haplogroups, that when mapped show distinct phylogeographic structure (Figs. 3-1 and 3-2).

Although the haplogroups appear to be generally geographically separated, they do not clearly coincide with drainages or basins. Haplogroup A (h35-h39) comprises *O. virilis* in spatially separated areas along both the eastern and western margins of the study area, including the Pacific watershed of western Montana, the upper Missouri River watershed, upper South Saskatchewan River drainage, and single site representatives from the St. Lawrence and Ohio River watersheds and the lower reach of the upper Mississippi River drainage. Haplogroup B (h1-h34) includes the majority of individuals sampled throughout the Mackenzie, Churchill, Nelson, upper- to mid-Missouri, and upper Mississippi watersheds. Haplogroup C (h40-h48) includes *O. virilis* from a single site sampled by us (no 99) in the Nelson watershed and locations in the lower Missouri River watershed sampled by Mathews et al. (2008).

The parsimony network of COI haplotypes supported the existence of three haplogroups, each separated by ≥ 10 mutational steps (Fig. 3-3). Haplogroup A reflects low haplotype diversity (see below), despite the large number of representative individuals and sites (157 and 27, respectively). One high frequency haplotype, h34, is separated by 1 mutation step from h36 and h37, but shows larger divergence from h38 and h39. Haplogroup B displayed a star-like pattern with 32 haplotypes showing low divergence (1-2 bp mutation steps) from a high-frequency putative ancestral haplotype, h1, found in 146 of 324 individuals and 48 of 82 sites within the clade. A subnetwork was also found in haplogroup B (Fig. 3-3), comprising the majority of crayfish collected from the northernmost drainages (Fig. 3-1). No clear phylogeographic structure was displayed within haplogroup C, although haplotype diversity and intra-clade divergence was higher than in haplogroups A and B per sample size (Table 3-3).

The nuclear GAPDH dataset was composed of 715 unambiguous aligned positions from 211 crayfish sampled across 46 sites (Table 3-2). I identified 15 polymorphic nucleotide positions and 30 unique alleles. The greatest allelic diversity was found in haplogroup C (24 alleles). Likewise, 11 of the 30 unique alleles were observed only in haplogroup C. Haplogroups A and B differed only by four polymorphic sites that were largely homozygous (g1 and g2; Table 3-2,

Fig. 3-2). Heterozygous allelic states within haplogroups A and B (g3; Table 3-2, Fig. 3-2) appeared to be largely geographically structured, observed in areas of the upper South Saskatchewan River drainage, one site in north-central Montana (no. 76), and two sites in north-eastern Minnesota (nos. 99 and 102).

3.4.2 Genetic diversity and historical demography

Haplotype diversity was moderately high within haplogroup B, low within haplogroup A, and lowest in haplogroup A when the non-contiguous Minnesota and Illinois samples (sites 99, 107, 108) were removed (Table 3-3). Haplotype diversity in haplogroup B was similar to that reported by Mathews et al. (2008) for each of the two subclades within haplogroup C (0.710 and 0.621). Nucleotide diversity was also much lower in haplogroup A than in haplogroup B. Nucleotide diversity in haplogroup B was again similar to values reported by Mathews et al. (2008) for haplogroup C subclades (0.0025 and 0.0034).

I observed low average intra-population diversity (Clade A: $h = 0.033$, $\pi = 0.00079$; Clade B: $h = 0.264$, $\pi = 0.00006$) as a result of single haplotypes at 19 of 20 and 41 of 69 multi-sample sites in haplogroups A and B, respectively. This pattern suggests that one haplotype was frequently fixed at a location, although due to generally low sample sizes, it could be due to unsampled diversity. In addition, 28 of the 49 observed haplotypes were detected at single sites only, suggesting that many haplotypes were restricted geographically. In contrast, 2-3 different haplotypes within haplogroup C were observed at each of three closely located sites in Kansas sampled for Mathews et al. (2008), suggesting greater diversity and structure may exist in this region than found in areas inhabited by haplogroups A and B.

In both haplogroups A and B the mismatch distribution did not differ significantly from the expected distribution of either the demographic or range expansion model ($P > 0.10$), regardless of whether all samples in haplogroup A were considered or only those restricted to the western portion of the study area. Similarly, results of the neutrality tests indicated population growth in both

haplogroups A and B (Table 3-3); however, no significant values were observed for haplogroup A when its three easternmost sites were removed.

3.4.3 Comparison of sequence data

Two COI haplogroups co-occurred in close proximity within two separate drainages, the South Saskatchewan River (Nelson watershed) and the Milk River (Missouri River watershed). In the former drainage, individuals representing both haplogroups were collected from two neighbouring sites (numbers 36 and 37 along the South Saskatchewan River; Fig. 3-1, Table 3-1). All crayfish within the South Saskatchewan River drainage clearly clustered within either COI haplogroup A or haplogroup B.

The spatial distribution and frequency of GAPDH genotypes (g1 – g3) differed among sites (Fig. 3-4). Although g2 was predominantly associated with haplogroup B, both g1 and g3 were found associated with haplogroup A, suggesting potential hybridization and/or asymmetrical introgression across several sites.

3.5 Discussion

3.5.1 Phylogeography of northern crayfish in the central Interior Plains

Phylogenetic analyses recovered three distinct haplogroups within *O. virilis* across a large portion of its core range, including the central Interior Plains, western Canadian Shield, and western Laurentian and Central Interior region. At first glance, the geographic distribution of the three haplogroups is suggestive of allopatric or parapatric divergence. There was little observed overlap, with individuals from more than one distinct clade found only at two sites (36 and 37) in the South Saskatchewan River in Alberta. However, in several cases members of different haplogroups were found in close proximity within the same watershed with few or no clear landscape boundaries, and were not completely coincident with expectations of genetic structure based on regional historic processes.

Phylogeographic assessments of freshwater organisms in northern latitudes of North America have predominately focused on fishes (e.g.,

Bernatchez and Wilson 1998 and references within, Gagnon and Angers 2006, Elmer et al. 2008). These fish species generally share several common patterns indicative of high active dispersal ability and shared responses to major geological processes. Genetic diversity is generally low relative to populations found in unglaciated regions to the south, implying low effective population sizes in refugial areas and sequential bottlenecks along linear colonization pathways (Bernatchez and Wilson 1998). During glacial periods, freshwater organisms were forced into refugial areas along the southern margins of the Laurentide ice sheet. In the prairie region, these refugial areas are collectively called the Great Plains refugium; however, distinct genetic signatures have been recovered from species restricted to specific locales within this area (e.g., specific fish showing signature of Missouri refugium, Van Houdt et al. 2005). Genetic divergence among glacial refugial groups also appears to be low (Billington and Hebert 1991, Bernatchez and Wilson 1998), presumably as a result of frequent mixing among lineages during interglacial periods throughout the Pleistocene. Indeed, geographic overlap of different refugial lineages within present-day fish distributions is frequently observed (e.g., Bernatchez and Wilson 1998, Turgeon and Bernatchez 2001). In contrast, refugial lineages of a passively dispersed cladoceran crustacean showed greater inter-lineage divergence and geographic separation and greater intra-lineage diversity than observed in similarly distributed fishes (Cox and Hebert 2001), highlighting the likely influence of taxon-specific colonisation patterns and ability on genetic structure.

Haplogroup C, despite relatively limited geographic sampling and small number of individuals compared to haplogroups A and B, displayed the highest intra-clade genetic divergence, structure, and diversity (9 COI haplotypes and 24 GAPDH alleles over 36 individuals). These patterns are consistent with stable, glacially unsundered populations shown in several aquatic organisms (Bernatchez and Wilson 1998, Elderkin et al. 2008), including crayfishes (Fetzner and Crandall 2003). Although additional sampling is needed to support phylogeographic interpretation of this clade, I expect the haplogroup to roughly encompass the south-central Ozark region, based on biogeographic patterns

observed for several freshwater fishes (e.g., Mayden 1988, Mathews and Robison 1998). Although it is possible that this clade extends into the central Mississippi drainage, the presence of *O. virilis* belonging to haplogroup C in northeastern Minnesota (site 99) is likely a result of human-mediated introduction.

The geographic distribution, star-shaped haplotype network, shallow phylogeny, and haplotype dispersion of haplogroup B are strongly indicative of a rapid range expansion, as expected from colonisation following retreat of the Laurentide ice sheet approximately 15,000 ya. Continuity in distribution across the upper Mississippi and Nelson watersheds is consistent with a connection during glacial recession, and shared by several fish taxa (Scott and Crossman 1973). In general, I found higher haplotype diversity in populations in southeastern areas of the haplogroup, suggesting that colonisation routes leading into and across the Nelson watershed originated from the Mississippi refugium. Haplogroup B is also present across the mid-Missouri watershed, and in several Prairie headwater streams in the upper Missouri watershed, consistent with additional glacial refugia or connections along the southern edge of the Laurentide ice sheet. However, haplogroup B in the mid- and upper-Missouri watershed is bisected by a large and apparently geographically restricted cluster belonging to haplogroup A.

Haplogroup A comprised several disjunct populations in the Clearwater River drainage in the Pacific watershed, upper South Saskatchewan River drainage in the Nelson watershed, and the upper Missouri River drainage throughout the state of Montana, Great Lakes/St. Lawrence watershed, and southern Upper Mississippi and lower Ohio watersheds. Haplogroup A is consistent across *O. virilis* sampled in the eastern St. Lawrence and Atlantic watersheds (Mathews et al. 2008), and so is presumably the dominant haplogroup throughout the eastern portion of the species' range, including the Great Lakes region. If this is the case, the presence of haplogroup A in southern Illinois and in the Great Lakes watershed of northeastern Minnesota represents the western edge of the haplogroup. Genetically distinct populations spanning both the upper Missouri watershed and upper South Saskatchewan basin in the Nelson drainage

have been considered a signature of restriction to the Missouri refugium during the last glacial maximum (e.g., Wilson and Hebert 1998). However, this is unlikely to be the case for *O. virilis* given the genetic similarity of crayfish in the upper Missouri River watershed and upper South Saskatchewan basin to individuals sampled large distances to the east and to populations in the Clearwater drainage west of the Continental Divide. Virile crayfish from Wisconsin were deliberately introduced by Montana Department of Fish, Wildlife and Parks (MTFWP) personnel into the Clearwater drainage in Montana as a food supplement for sport fish (Sheldon 1989; Anne Tews, MTFWP, pers. comm). The lack of genetic diversity and structure in the four populations sampled in the Clearwater drainage suggests use of one or a few closely located sources and/or a small number of founders. Alternatively, these patterns could indicate a selective advantage for survival or establishment of crayfish bearing a particular haplotype. However, as the dominant haplotype, h35, is also found at two of the four sparsely sampled haplogroup A sites in the Great Lakes drainage, it is likely to be a widespread high-frequency 'parental' haplotype throughout the extent of the distribution of haplogroup A, similar to h1 within haplogroup B. Additional sampling is necessary to establish the distribution and phylogeographic patterns of haplogroup B across the range of *O. virilis*. Low genetic diversity and structure is also found throughout the upper Missouri watershed in Montana and upper South Saskatchewan River drainage in Alberta, suggesting that these areas were serially stocked from source locations in the Clearwater drainage or from the same source in Wisconsin used for the initial Clearwater introduction.

Similar to previous phylogenetic analyses of *Orconectes virilis* (Mathews et al. 2008, Filipova et al. 2010), I found substantial genetic divergence among haplogroups, but have a different interpretation of inter-haplogroup phylogenetic structure. Clades 3 and 4 of Mathews et al. (2008) do not appear distinctly different in our analysis, and instead are collapsed into our haplogroup C. Also included in haplogroup C is haplotype h40, found at a single site in northeastern MN, similar to (1 bp difference) haplotypes observed from *O. virilis* introduced into the UK and the Netherlands from an unknown source (Filipova et al. 2010).

Although the inter-haplogroup genetic divergence I observed was similar to divergence values calculated by Mathews et al. (2008) and Filipova et al. (2010), I did not find significant morphological differences among these haplogroups (unpubl. data).

3.5.2 Ramifications of introduction

It is evident that human-mediated introduction of *O. virilis* is common, and that this has had widespread effects on the distribution and genetic structure of the species. Although haplogroup B as a whole displays a strong signature of both demographic and spatial expansion, several sites along the haplogroup's western range margin differ from the genetic signatures within neighbouring areas. Interestingly, these sites include isolated lakes (sites 1, 2, 8), an urban/suburban creek (45), and man-made suburban ponds (9 and 11), and all share the same haplotype (h1), which was not detected within a 300 km distance, as the crow flies, of any of these sites.

Although the existence of haplogroup B in several headwater streams along the upper Missouri watershed might be the result of multiple stocking events from more northerly or easterly locales, it is unlikely. The northern crayfish is not widely marketed for consumption in the Great Plains region, and no aquaculture industry exists. Live crayfish are a popular bait item for several sport fishes, including largemouth bass and pike, neither of which would be targeted in Prairie headwater streams. Thus, our interpretation is that populations of haplogroup B in these headwater streams likely represent remnants of a previous distribution throughout portions of the upper Missouri watershed that has largely been supplanted by the introduction, establishment, and spread of members of haplogroup A.

The history of *O. virilis* in the Plains area, including Montana, is poorly documented. Early records in the Plains were from the Red River of the North and Souris River in North Dakota (Nelson Drainage) and the Saskatchewan River in Manitoba (Hagen 1870, Faxon 1885). The furthest upstream observations in the Missouri River were documented as Omaha, Nebraska (Faxon 1885). The first

report in Montana was from an unnamed Missouri River tributary in the western part of the state in 1947, followed by observations in 1960 from the Poplar River in northeastern MT (near site 80), and from Sun River, a Missouri River tributary downstream of the initial 1947 record (Holthuis 1962).

Timing and direction of expansion and/or the presence of dams or similar barriers to upstream dispersal have likely maintained the signature of haplogroup B in the prairie headwater streams of the Missouri watershed. Given observations of members of haplogroup B in undammed tributaries of the mid-Missouri watershed (sites 89 – 91), it is probable that spread of introduced haplogroup A *O. virilis* had been confined by one of the major dams along the Missouri River (e.g., Fort Peck Dam in eastern Montana southwest of site 77, or the Garrison Dam in central North Dakota immediately west of site 89).

The most similar barriers to crayfish dispersal in the South Saskatchewan River drainage of southern Alberta and Saskatchewan are the Gardiner and Qu'Appelle dams, which retain water in Lake Diefenbaker, approximately 250 km downstream of the easternmost instance of haplogroup A members. Accordingly, continued downstream expansion of haplogroup A within the Saskatchewan River drainage east of Lake Diefenbaker may result in a similar pattern of displacement of haplogroup B as I hypothesize occurred in the upper Missouri watershed in Montana.

3.5.3 Hybridization, introgression, and application of multiple markers

Inter-specific hybridization has been reported among several *Orconectes* species, (e.g., Perry et al. 2001a; 2001b; 2002), including *O. virilis*. Therefore, intraspecific inter-haplogroup hybridization would be a predicted result of secondary contact. In the upper Missouri watershed, putative displacement of endemic haplogroup B *O. virilis* by introduced haplogroup A has left no clear genetic signal of hybridization or introgression, suggesting complete displacement. In contrast, combined analysis of mitochondrial COI and nuclear GAPDH sequences suggests that hybridization is common within sites along the South Saskatchewan River in southern Alberta, with asymmetrical introgression

of haplogroup A mtDNA into the endemic lineage. Although this direction of introgression (mtDNA from an invasive lineage into that of the endemic) is rare (e.g., Currat et al. 2008), hybridization is not uncommon between closely related species (e.g., Seehausen 2004), and certainly not within species.

Reliance on single markers, primarily mtDNA sequences, in phylogeographic analyses has well-known limitations (e.g., Ballard and Whitlock 2004). Consequently, an increasing number of studies are complementing mtDNA data with nuclear markers. The nuclear gene GAPDH has been employed in previous phylogenetic and phylogeographic analyses of crayfishes (e.g., Buhay et al. 2007; Mathews et al. 2008; Schultz et al. 2009). Intraspecific variation in GAPDH was observed in *O. virilis sensu lato*, both in the current study and in Mathews et al. (2008). However, overlap in GAPDH allelic states among distinct haplogroups is suggestive of incomplete lineage sorting of the nuclear marker. As a result, caution must be taken in interpretation of phylogeographic patterns of *O. virilis* based on GAPDH. For example, the high frequency of the heterozygote genotype g3 found in the upper South Saskatchewan River drainage is likely due to a high frequency of that genotype in the founding individuals, and not hybridization. This marker might be best applied to elucidate interspecific or deeper phylogenetic relationships.

3.6 Conclusion

Phylogeographic assessment of the widespread virile crayfish reveals a complex evolutionary history. Distinct genetic divisions among several clades are indicative of regional responses to the cyclic climatic fluctuations throughout the Quaternary, expected from a wide-ranging taxon. However, genetic signatures of historical processes are rapidly becoming obscured by direct human actions (i.e., introduction) across a much larger spatial scale than anticipated. Further, many introduced *O. virilis* populations have a limited number of COI haplotypes and GAPDH alleles, suggesting that the displacement of endemic lineages may have an additional consequence of large-scale loss of genetic diversity and structure.

Table 3-1. Primers screened and used for polymerase chain reaction amplification and sequencing.

Gene	Primer	Sequence (5'-3')	Reference
COI	orcoCOIF	GTGGTAGTTACAGCYCATGC	(Mathews et al. 2008)
	orcoCOIR	CCAGACTCTTGAACTACAAT	(Mathews et al. 2008)
GAPDH	G3PCq157F	TGACCCCTTCATTGCTCTTGACTA	(Buhay et al. 2007)
	G3PCq981R	ATTACACGGGTAGAATAGCCAAACTC	(Buhay et al. 2007)
28S rDNA	Rdla	CCCSCGTAAYTTAAGCATAT	(Crandall et al. 2000)
	Rd4b	CCTTGGTCCGTGTTTCAAGAC	(Crandall et al. 2000)
Histone H3	H3af	ATGGCTCGTACCAAGCAGACVGC	(Colgar et al. 1998)
	H3ar	ATATCCTTRGGCATRGTGAC	(Colgar et al. 1998)
ITS1	ITS1F	GTAAAAGTCGTAACAAGG	(Harris and Crandall 2000)
	ITS1R	TCCTCCGCTWAWTGATATGC	(Harris and Crandall 2000)
16S rDNA	16S-1472	AGATAGAAACCAACCTGG	(Schubart et al. 2000)
	16s-L2	TGCCTGTTTATCAAAAACAT	(Mathews et al. 2002)

Table 3-2. List of sites sampled for this study including number of individuals included in the COI and GAPDH analyses, COI haplotypes and haplogroups, and GAPDH genotypes. Site numbers and watershed correspond to those shown in Figure 3-1.

Site no.	Watershed	Lat.	Long.	Waterbody or waterway*	Sample size (COI, GAPDH)	COI Haplotypes	COI Haplogroup	GAPDH genotypes
1	Mackenzie	54.293	-115.651	McLeod Lake, AB	3, 0	h1	Clade B	
2	Mackenzie	54.592	-114.199	Beyette Lake, AB	3, 0	h1	Clade B	
3	Churchill	54.604	-112.647	Amisk Lake, AB	5, 5	h20	Clade B	g2
4	Churchill	54.389	-110.755	Beaver River, AB	5, 5	h21, h22	Clade B	g2
5	Churchill	54.260	-109.221	Beaver River, SK	5, 0	h20, h21, h23	Clade B	
6	Churchill	55.153	-107.598	Beaver River, SK	1, 0	h20	Clade B	
7	Churchill	55.418	-104.561	Churchill River, SK	5, 1	h20	Clade B	g2
8	Nelson	53.584	-114.464	East Pit Lake, AB	3, 0	h1	Clade B	
9	Nelson	53.637	-113.653	Lacombe Lake, AB	3, 0	h1	Clade B	
10	Nelson	53.370	-113.751	North Saskatchewan River, AB	5, 5	h20	Clade B	g2
11	Nelson	53.443	-113.505	Bearspaw Lake, AB	3, 0	h1	Clade B	
12	Nelson	53.530	-113.521	North Saskatchewan River, AB	1, 0	h20	Clade B	
13	Nelson	53.659	-110.337	North Saskatchewan River, AB	3, 0	h20	Clade B	
14	Nelson	53.396	-109.293	North Saskatchewan River, SK	1, 0	h20	Clade B	
15	Nelson	53.024	-108.828	North Saskatchewan River, SK	3, 0	h20	Clade B	
16	Nelson	52.743	-108.284	North Saskatchewan River, SK	1, 0	h20	Clade B	
17	Nelson	52.409	-111.810	Battle River, AB	1, 0	h20	Clade B	
18	Nelson	52.907	-108.949	Battle River, SK	1, 0	h20	Clade B	
19	Nelson	52.946	-106.435	North Saskatchewan River, SK	5, 0	h20	Clade B	
20	Nelson	53.245	-105.433	North Saskatchewan River, SK	6, 0	h20	Clade B	
21	Nelson	53.244	-105.072	Saskatchewan River, SK	5, 0	h1, h20	Clade B	
22	Nelson	53.237	-104.464	Saskatchewan River, SK	5, 5	h1, h20	Clade B	g2
23	Nelson	53.137	-104.023	Carrot River, SK	2, 0	h20	Clade B	

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Site no.	Watershed	Lat.	Long.	Waterbody or waterway*	Sample size (COI, GAPDH)	COI Haplotypes	COI Haplogroup	GAPDH allelic states
24	Nelson	53.323	-104.041	Saskatchewan River, SK	3, 0	h20	Clade B	
25	Nelson	53.182	-105.162	South Saskatchewan River, SK	5, 0	h1, h20, h28	Clade B	
26	Nelson	52.923	-105.805	South Saskatchewan River, SK	5, 0	h1, h20, h28	Clade B	
27	Nelson	52.491	-106.283	South Saskatchewan River, SK	5, 0	h1	Clade B	
28	Nelson	52.319	-106.455	South Saskatchewan River, SK	5, 0	h1, h20, h28	Clade B	
29	Nelson	52.137	-106.646	South Saskatchewan River, SK	5, 1	h1	Clade B	g2
30	Nelson	51.613	-107.008	South Saskatchewan River, SK	5, 0	h1	Clade B	
31	Nelson	51.438	-107.089	South Saskatchewan River, SK	5, 0	h1	Clade B	
32	Nelson	51.259	-106.896	South Saskatchewan River, SK	6, 0	h3	Clade B	
33	Nelson	50.905	-106.917	South Saskatchewan River, SK	5, 0	h1	Clade B	
34	Nelson	50.308	-107.769	Swift Current Creek, SK	5, 0	h7	Clade B	
35	Nelson	51.024	-109.134	South Saskatchewan River, SK	10, 10	h7, h14	Clade B	g2
36	Nelson	50.732	-110.075	South Saskatchewan River, AB	19, 20	h7, h14, h35	Clade A + B	g2, g3
37	Nelson	50.399	-110.589	South Saskatchewan River, AB	13, 13	h7, h14, h35	Clade A + B	g1, g2, g3
38	Nelson	50.045	-110.674	South Saskatchewan River, AB	16, 6	h35	Clade A	g1
39	Nelson	49.904	-111.476	South Saskatchewan River, AB	20, 7	h35	Clade A	g1, g3
40	Nelson	50.379	-111.911	Lake Newell Reservoir, AB	20, 20	h7	Clade B	g2
41	Nelson	50.246	-112.079	Bow River, AB	20, 17	h35	Clade A	g1, g2, g3
42	Nelson	49.856	-112.625	Oldman River, AB	16, 17	h35	Clade A	g1, g3
43	Nelson	49.688	-112.790	Henderson Lake, AB	20, 6	h35	Clade A	g1
44	Nelson	50.503	-112.882	McGregor Lake, AB	5, 3	h35	Clade A	g1
45	Nelson	51.086	-114.047	Nose Creek, AB	4, 5	h1	Clade B	g2
46	Nelson	50.985	-106.416	Qu'Appelle River, SK	3, 0	h1, h7	Clade B	
47	Nelson	50.595	-105.411	Qu'Appelle River, SK	5, 0	h1, h7	Clade B	
48	Nelson	50.642	-102.847	Qu'Appelle River, SK	5, 0	h1, h13	Clade B	
49	Nelson	50.499	-101.728	Qu'Appelle River, SK	3, 3	h1, h19	Clade B	g2

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Site no.	Watershed	Lat.	Long.	Waterbody or waterway*	Sample size (COI, GAPDH)	COI Haplotypes	COI Haplogroup	GAPDH allelic states
50	Nelson	51.533	-101.877	Assiniboine River, SK	3, 0	h1, h17	Clade B	
51	Nelson	51.998	-102.075	Swan River, SK	3, 0	h1	Clade B	
52	Nelson	50.044	-101.677	Pipestone Creek, SK	5, 0	h1	Clade B	
53	Nelson	49.193	-101.711	Antler River, SK	4, 0	h1, h32	Clade B	
54	Nelson	49.141	-101.654	Antler River, SK	3, 0	h5, h32	Clade B	
55	Nelson	49.079	-102.399	Souris River, SK	3, 0	h1, h27	Clade B	
56	Nelson	48.923	-102.271	Northgate Dam, ND	4, 0	h1	Clade B	
57	Nelson	48.992	-102.784	Short Creek Dam, ND	12, 0	h1	Clade B	
58	Nelson	49.062	-103.498	Long Creek, SK	1, 0	h1	Clade B	
59	Nelson	49.145	-103.098	Rafferty Dam Reservoir, SK	3, 0	h1, h26	Clade B	
60	Pacific	47.320	-114.314	Flathead River, MT	3, 3	h35	Clade A	g1
61	Pacific	48.121	-114.036	Echo Lake, MT	3, 3	h35	Clade A	g1
62	Pacific	47.316	-113.582	Lake Alva, MT	3, 3	h35	Clade A	g1
63	Pacific	46.945	-113.431	Clearwater River, MT	3, 3	h35	Clade A	g1
64	Missouri	48.285	-112.264	Lake Frances, MT	3, 3	h35	Clade A	g1
65	Missouri	47.557	-112.443	Willow Creek Reservoir, MT	3, 3	h35	Clade A	g1
66	Missouri	47.220	-112.243	Dearborn River, MT	1, 1	h35	Clade A	g1
67	Missouri	47.019	-112.012	Missouri River, MT	3, 3	h35	Clade A	g1
68	Missouri	47.626	-111.034	Missouri River, MT	4, 1	h35	Clade A	g1
69	Missouri	48.342	-111.163	Tiber Reservoir, MT	3, 3	h35	Clade A	g1
70	Missouri	48.685	-110.008	Fresno Reservoir, MT	2, 2	h35	Clade A	g1
71	Missouri	49.412	-109.744	Battle Creek, SK	1, 0	h35	Clade A	
72	Missouri	49.495	-109.223	Frenchman River, SK	2, 0	h7	Clade B	
73	Missouri	49.503	-109.220	Fairwell Creek, SK	1, 0	h7	Clade B	
74	Missouri	49.507	-109.047	Conglomerate Creek, SK	4, 0	h7	Clade B	
75	Missouri	49.250	-107.717	Frenchman River, SK	3, 1	h7	Clade B	g2

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Site no.	Watershed	Lat.	Long.	Waterbody or waterway*	Sample size (COI, GAPDH)	COI Haplotypes	COI Haplogroup	GAPDH allelic states
76	Missouri	48.495	-107.546	Nelson Reservoir, MT	2, 2	h36	Clade A	g1, g3
77	Missouri	49.093	-106.738	Weatherall Creek, SK	1, 0	h8	Clade B	
78	Missouri	49.069	-106.531	Rock Creek, SK	3, 0	h1	Clade B	
79	Missouri	49.008	-106.718	Rock Creek, SK	3, 0	h1	Clade B	
80	Missouri	49.030	-105.894	Poplar River, SK	1, 1	h8	Clade B	g2
81	Missouri	46.339	-109.426	Deadmans Basin, MT	2, 1	h35	Clade A	g1
82	Missouri	45.228	-108.072	Big Bull Elk Bay, MT	5, 0	h35	Clade A	
83	Missouri	46.387	-105.867	Miles City, MT	2, 2	h35	Clade A	g1
84	Missouri	48.433	-103.735	Blacktail Dam, ND	3, 0	h1	Clade B	
85	Missouri	48.256	-103.430	Epping-Springbrook Dam, ND	3, 3	h1	Clade B	g2
86	Missouri	48.239	-103.143	Kota-Ray Dam, ND	3, 3	h1	Clade B	g2
87	Missouri	48.456	-102.744	White Earth Dam, ND	3, 0	h1	Clade B	
88	Missouri	48.583	-102.936	McGregor Dam, ND	3, 1	h1	Clade B	g2
89	Missouri	47.514	-100.461	South Hoffer McClusky, ND	3, 0	h1	Clade B	
90	Missouri	46.297	-99.866	Beaver Creek, ND	6, 0	h1, h6	Clade B	
91	Missouri	45.412	-97.425	Waubay Lake, SD	2, 1	h15, h33	Clade B	g2
92	Nelson	49.723	-97.173	LaSalle River, MB	2, 0	h29, h31	Clade B	
93	Nelson	49.876	-97.232	Assiniboine River, MB	4, 4	h1, h2, h3, h4	Clade B	g2
94	Nelson	50.160	-95.867	Pinawa diversion, MB	1, 1	h25	Clade B	g2
95	Nelson	49.317	-96.945	Rat River, MB	5, 0	h1, h16	Clade B	
96	Nelson	48.899	-95.240	Lake of the Woods, MN	3, 3	h1, h9	Clade B	g2
97	Nelson	48.908	-95.239	Muskeg Bay, MN	4, 0	h1, h12	Clade B	
98	Nelson	48.459	-93.017	Kabetogema Lake, MN	2, 0	h1, h18	Clade B	
99	Nelson	47.842	-92.289	Lake Vermillion, MN	3, 3	h40	Clade C	g1, g3
100	Nelson	48.229	-90.840	Granite River, MN	4, 4	h1	Clade B	g2
101	Nelson	48.112	-90.620	Little Gunflint Lake, MN	1, 0	h1	Clade B	

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Site no.	Watershed	Lat.	Long.	Waterbody or waterway*	Sample size (COI, GAPDH)	COI Haplotypes	COI Haplogroup	GAPDH allelic states
102	Great Lakes	48.062	-90.164	Pine Lake, MN	4, 0	h35, h37	Clade A	g1, g3
103	Mississippi	47.432	-94.209	Lake Winnibigoshish, MN	3, 0	h1	Clade B	
104	Mississippi	46.211	-93.528	Mille Lacs, MN	7, 0	h1, h10, h11, h24, h30	Clade B	
105	Mississippi	45.027	-93.036	Markham Pond, MN	1, 0	h1	Clade B	
106	Mississippi	41.967	-91.667	Squaw Creek, IA	1, 0	h34	Clade A	
107	Mississippi	37.617	-89.210	Drury Creek, IL	1, 0	h38	Clade A	
108	Ohio	38.802	-88.476	Little Wabash River, IL	1, 0	h39	Clade A	
109†	Missouri	39.109	-96.608	King's Creek, KS	11, 6	h41, h42	Clade C	g4
110†	Missouri	39.008	-96.739	Clark's Creek, KS	10, 7	h43 h44, h45	Clade C	g4, g5
111†	Missouri	38.964	-95.938	Mission Creek, KS	12, 8	h46, h47, h48	Clade C	g6 – g12

* Waterbody or waterway includes province or state. Canada: AB = Alberta; SK = Saskatchewan; MB = Manitoba. U.S.A: MT = Montana; ND = North Dakota; SD = South Dakota; MN = Minnesota; IA = Iowa; IL = Illinois; KS = Kansas.
† Based on sequences from Mathews et al. (2008)

Table 3-3. Diversity measures for the three major clades identified through phylogenetic analyses, including number of sequences (*N*), number of haplotypes (N_{hap}), haplotype diversity (*h*), number of polymorphic sites (N_P), nucleotide diversity (π), and the mean number of paired differences (*k*). Estimates were not calculated for clade C. Results of neutrality tests are also provided, with significant values in bold (P < 0.05).

Clade	*N*	N_{hap}	N_P	*h*	π	k	Fu and Li's D*	Fu and Li's F*	Fu's Fs[a]	Tajima's D
Clade A	157	5	7	0.075	0.00022	0.127	**-2.546**	**-2.790**	**-5.390**	**-1.970**
Clade A west[b]	151	2	1	0.026	0.00004	0.026	0.469	0.056	-1.858	-0.904
Clade B	324	34	32	0.739	0.00238	1.385	**-5.125**	**-4.566**	**-28.129**	**-1.979**
Clade C	36[c]	9	17							
Total	507	48	58							

[a] Fu's Fs considered significant at P < 0.02 (Fu 1997)

[b] Includes only members of clade A restricted to the western portion of the study area (sites 36-39, 41-44, 60-71, 76, 81-83).

[c] Sample size reflects total number of individuals sequenced in Mathews et al. (2008) and the current study; however, only 21 sequences were used in this study due to a lack of individual-based information available from Mathews et al. (2008).

Table 3-4. Mean distances (K2-P) among haplogroups identified by phylogenetic analyses for the COI dataset. Mean within-haplogroup distances (K2-P) are shown along the diagonal.

	Clade A	Clade B	Clade C
Clade A	0.004	0.037	0.031
Clade B		0.005	0.029
Clade C			0.011

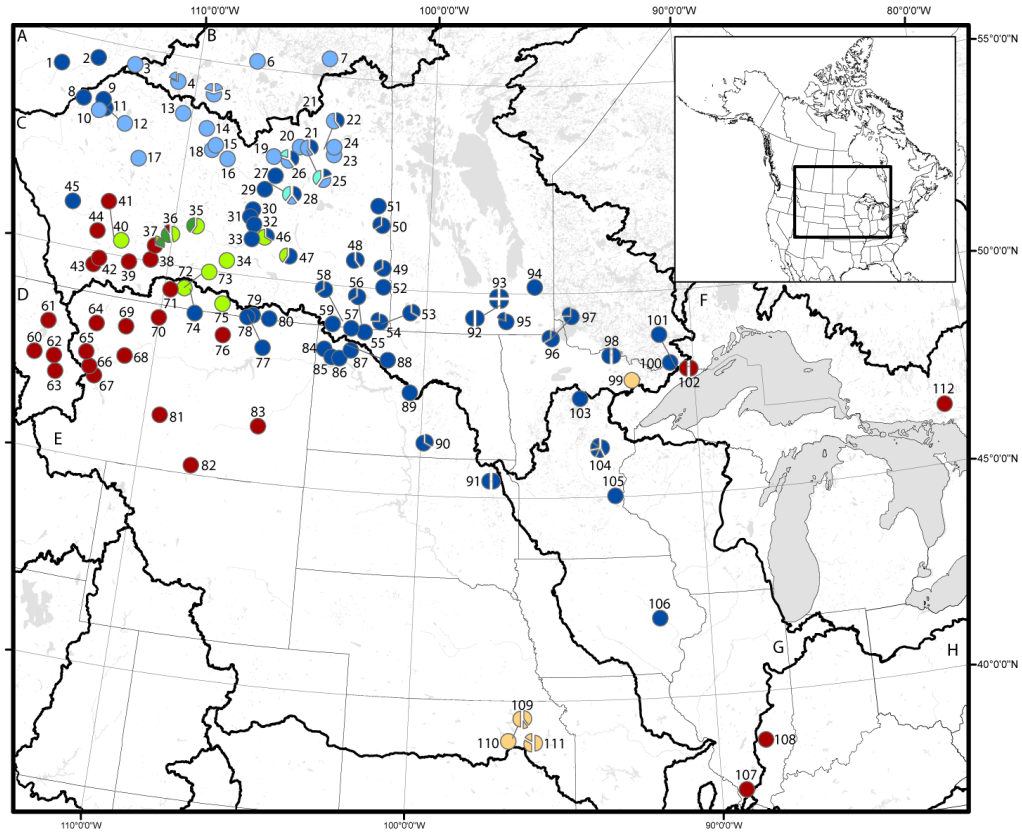

Figure 3-1. Distribution of the three major COI haplogroups of *Orconectes virilis* across a total of 110 sampling locations in the central Interior Plains of North America. Colour-coding of haplotypes and haplogroups are as found in Figure 3-3: south-central Interior Plains, yellow; Montana and eastern sites, red; north-central Interior Plains, assorted colours based on frequencies. Major watershed subdivisions shown are as follows: Mackenzie (A), Churchill (B), Nelson (C), Pacific (D), Missouri (E), Great Lakes (F), [Upper] Mississippi (G), Ohio (H).

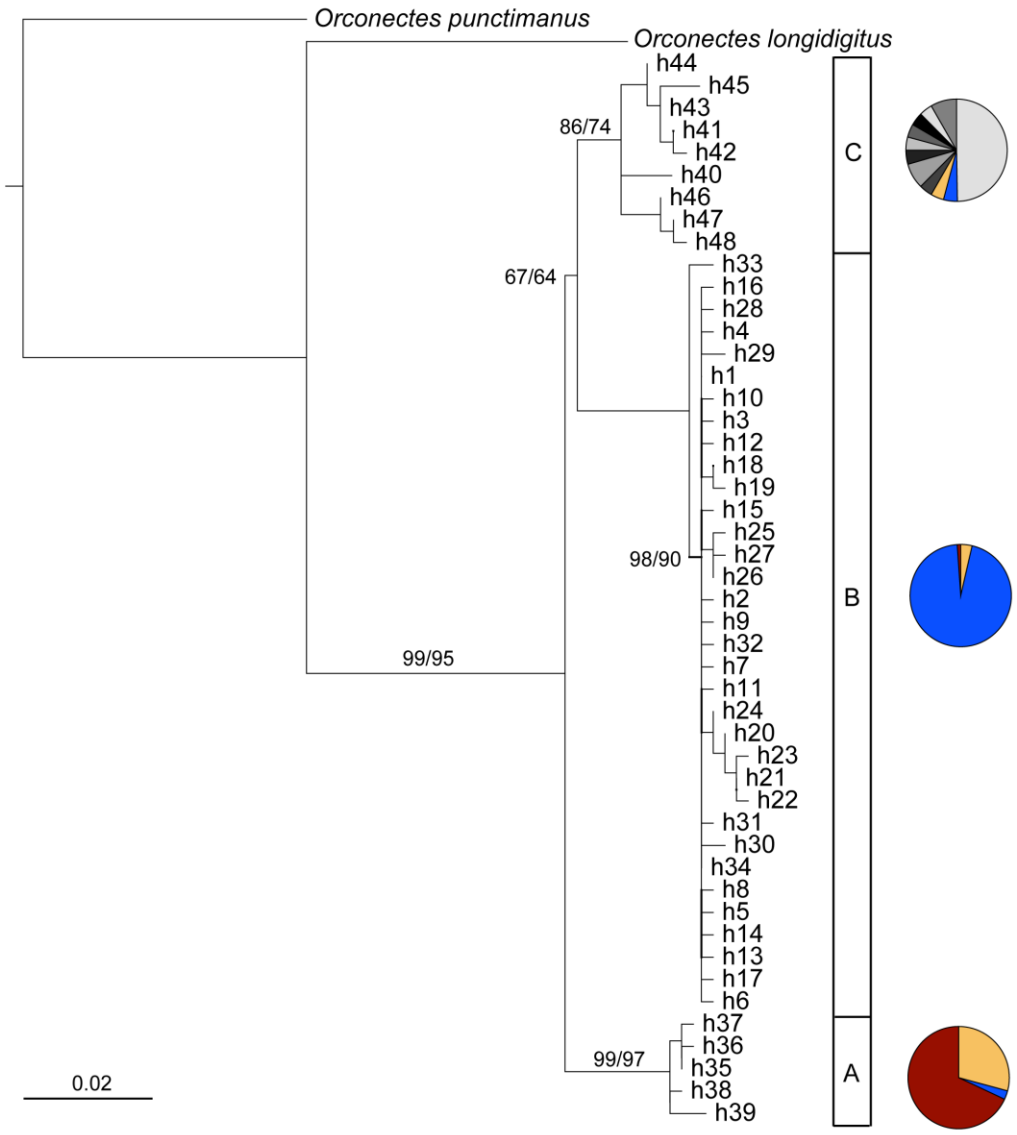

Figure 3-2. Maximum likelihood (ML) tree showing relationships among 48 unique *Orconectes virilis* COI haplotypes. Bootstrap support (1000 replicates) values providing nodal support of > 50% are shown (NJ/ML). Nuclear GAPDH genotype frequencies are shown for each haplogroup (red = g1; blue = g2; orange = g3; grey shades = g4-g12).

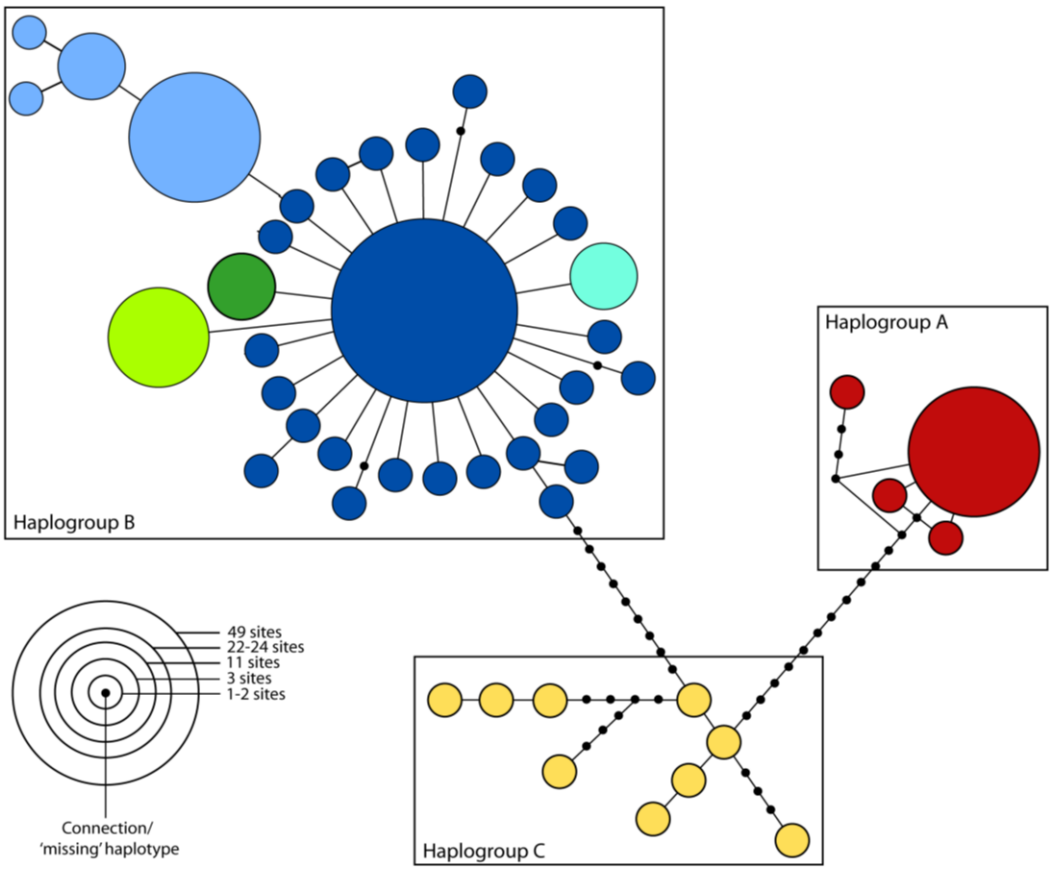

Figure 3-3. Parsimony network of *Orconectes virilis* COI haplotypes. Coloured circles represent haplotypes and size is based on number of sites at which each haplotype was observed. Colour-coding of haplotypes and haplogroups are as found in Figure 3-2. The three subnetworks are congruent with the three major haplogroups in the NJ and ML analyses.

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Appendix 3-I. Mitochondrial COI-based diversity measures for each site including number of sequences (*N*), number of haplotypes (N_{hap}), haplogroup designation, number of polymorphic sites (N_P), haplotype diversity (*h*), nucleotide diversity (π), and the mean number of paired differences (*k*). Estimated measures are provided only for sites where $N \geq 2$.

Site no.	Watershed	Waterway/Waterbody	*N*	N_{hap}	Haplogroup	N_P	*h*	π	*k*
1	Mackenzie	McLeod Lake, AB	3	1	Clade B	0	0.00	0.0000	0.00
2	Mackenzie	Beyette Lake, AB	3	1	Clade B	0	0.00	0.0000	0.00
3	Churchill	Amisk Lake, AB	5	1	Clade B	0	0.00	0.0000	0.00
4	Churchill	Beaver River, AB	5	2	Clade B	1	0.40	0.0007	0.40
5	Churchill	Beaver River, SK	5	3	Clade B	2	0.70	0.0014	0.80
6	Churchill	Beaver River, SK	1	1	Clade B				
7	Churchill	Churchill River, SK	5	1	Clade B	0	0.00	0.0000	0.00
8	Nelson	East Pit Lake, AB	3	1	Clade B	0	0.00	0.0000	0.00
9	Nelson	Lacombe Lake, AB	3	1	Clade B	0	0.00	0.0000	0.00
10	Nelson	North Saskatchewan River, AB	5	1	Clade B	0	0.00	0.0000	0.00
11	Nelson	Bearspaw Lake, AB	3	1	Clade B	0	0.00	0.0000	0.00
12	Nelson	North Saskatchewan River, AB	1	1	Clade B				
13	Nelson	North Saskatchewan River, AB	3	1	Clade B	0	0.00	0.0000	0.00
14	Nelson	North Saskatchewan River, SK	1	1	Clade B				
15	Nelson	North Saskatchewan River, SK	3	1	Clade B	0	0.00	0.0000	0.00
16	Nelson	North Saskatchewan River, SK	1	1	Clade B				
17	Nelson	Battle River, AB	1	1	Clade B				
18	Nelson	Battle River, SK	1	1	Clade B				
19	Nelson	North Saskatchewan River, SK	5	1	Clade B	0	0.00	0.0000	0.00
20	Nelson	North Saskatchewan River, SK	6	1	Clade B	0	0.00	0.0000	0.00
21	Nelson	Saskatchewan River, SK	5	2	Clade B	2	0.60	0.0021	1.20
22	Nelson	Saskatchewan River, SK	5	2	Clade B	2	0.60	0.0021	1.20
23	Nelson	Carrot River, SK	2	1	Clade B	0	0.00	0.0000	0.00

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Site no.	Watershed	Waterway/Waterbody	N	N_{hap}	Haplogroup	N_P	h	π	k
24	Nelson	Saskatchewan River, SK	3	1	Clade B	0	0.00	0.0000	0.00
25	Nelson	South Saskatchewan River, SK	5	3	Clade B	3	0.80	0.0031	1.80
26	Nelson	South Saskatchewan River, SK	5	3	Clade B	3	0.80	0.0027	1.60
27	Nelson	South Saskatchewan River, SK	5	1	Clade B	0	0.00	0.0000	0.00
28	Nelson	South Saskatchewan River, SK	5	3	Clade B	3	0.80	0.0024	1.40
29	Nelson	South Saskatchewan River, SK [a]	5	1	Clade B	0	0.00	0.0000	0.00
30	Nelson	South Saskatchewan River, SK [a]	5	1	Clade B	0	0.00	0.0000	0.00
31	Nelson	South Saskatchewan River, SK [a]	5	1	Clade B	0	0.00	0.0000	0.00
32	Nelson	South Saskatchewan River, SK	5	1	Clade B	0	0.00	0.0000	0.00
33	Nelson	South Saskatchewan River, SK	5	1	Clade B	0	0.00	0.0000	0.00
34	Nelson	Swift Current Creek, SK	5	1	Clade B	0	0.00	0.0000	0.00
35	Nelson	South Saskatchewan River, SK	10	2	Clade B	2	0.53	0.0018	1.07
36	Nelson	South Saskatchewan River, AB	19	3	Clade A + B	21	0.62	0.0083	4.82
37	Nelson	South Saskatchewan River, AB [b]	13	3	Clade A + B	21	0.50	0.0160	9.31
38	Nelson	South Saskatchewan River, AB	16	1	Clade A	0	0.00	0.0000	0.00
39	Nelson	South Saskatchewan River, AB	20	1	Clade A	0	0.00	0.0000	0.00
40	Nelson	Lake Newell Reservoir, AB	20	1	Clade B	0	0.00	0.0000	0.00
41	Nelson	Bow River, AB	20	1	Clade A	0	0.00	0.0000	0.00
42	Nelson	Oldman River, AB	16	1	Clade A	0	0.00	0.0000	0.00
43	Nelson	Henderson Lake, AB	20	1	Clade A	0	0.00	0.0000	0.00
44	Nelson	McGregor Lake, AB [b]	5	1	Clade A	0	0.00	0.0000	0.00
45	Nelson	Nose Creek, AB	4	1	Clade B	0	0.00	0.0000	0.00
46	Nelson	Qu'Appelle River, SK [a]	3	2	Clade B	1	0.67	0.0011	0.67
47	Nelson	Qu'Appelle River, SK	5	2	Clade B	1	0.60	0.0010	0.60
48	Nelson	Qu'Appelle River, SK	5	2	Clade B	1	0.60	0.0010	0.60
49	Nelson	Qu'Appelle River, SK	3	2	Clade B	2	0.67	0.0023	1.33
50	Nelson	Assiniboine River, SK	3	2	Clade B	1	0.67	0.0011	0.67

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Site no.	Watershed	Waterway/Waterbody	N	N_{hap}	Haplogroup	N_P	h	π	k
51	Nelson	Swan River, SK	3	1	Clade B	0	0.00	0.0000	0.00
52	Nelson	Pipestone Creek, SK	5	1	Clade B	0	0.00	0.0000	0.00
53	Nelson	Antler River, SK [a]	4	2	Clade B				
54	Nelson	Antler River, SK [a]	3	2	Clade B	2	0.67	0.0023	1.33
55	Nelson	Souris River, SK	3	2	Clade B	2	0.67	0.0023	1.33
56	Nelson	Northgate Dam, ND [c]	4	1	Clade B	0	0.00	0.0000	0.00
57	Nelson	Short Creek Dam, ND [c]	12	1	Clade B	0	0.00	0.0000	0.00
58	Nelson	Long Creek, SK	1	1	Clade B				
59	Nelson	Rafferty Dam Reservoir, SK	3	2	Clade B	1	0.67	0.0011	0.67
60	Pacific	Flathead River, MT [d]	3	1	Clade A	0	0.00	0.0000	0.00
61	Pacific	Echo Lake, MT [d]	3	1	Clade A	0	0.00	0.0000	0.00
62	Pacific	Lake Alva, MT [d]	3	1	Clade A	0	0.00	0.0000	0.00
63	Pacific	Clearwater River, MT [d]	3	1	Clade A	0	0.00	0.0000	0.00
64	Missouri	Lake Frances, MT [g]	3	1	Clade A	0	0.00	0.0000	0.00
65	Missouri	Willow Creek Reservoir, MT [g]	3	1	Clade A	0	0.00	0.0000	0.00
66	Missouri	Dearborn River, MT [d]	1	1	Clade A				
67	Missouri	Missouri River, MT [d]	3	1	Clade A	0	0.00	0.0000	0.00
68	Missouri	Missouri River, MT [g]	4	1	Clade A	0	0.00	0.0000	0.00
69	Missouri	Tiber Reservoir, MT [g]	3	1	Clade A	0	0.00	0.0000	0.00
70	Missouri	Fresno Reservoir, MT [g]	2	1	Clade A	0	0.00	0.0000	0.00
71	Missouri	Battle Creek, SK [a]	1	1	Clade A				
72	Missouri	Frenchman River, SK [a]	2	1	Clade B	0	0.00	0.0000	0.00
73	Missouri	Fairwell Creek, SK [a]	1	1	Clade B				
74	Missouri	Conglomerate Creek, SK [a]	4	1	Clade B	0	0.00	0.0000	0.00
75	Missouri	Frenchman River, SK [i]	3	1	Clade B	0	0.00	0.0000	0.00
76	Missouri	Nelson Reservoir, MT [g]	2	1	Clade A	0	0.00	0.0000	0.00
77	Missouri	Weatherall Creek, SK [a]	1	1	Clade B				

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Site no.	Watershed	Waterway/Waterbody	N	N_{hap}	Haplogroup	N_P	h	π	k
78	Missouri	Rock Creek, SK	3	1	Clade B	0	0.00	0.0000	0.00
79	Missouri	Rock Creek, SK	3	1	Clade B	0	0.00	0.0000	0.00
80	Missouri	Poplar River, SK [a]	1	1	Clade B				
81	Missouri	Deadmans Basin, MT [g]	2	1	Clade A	0	0.00	0.0000	0.00
82	Missouri	Big Bull Elk Bay, MT [g]	5	1	Clade A	0	0.00	0.0000	0.00
83	Missouri	Miles City, MT [g]	2	1	Clade A	0	0.00	0.0000	0.00
84	Missouri	Blacktail Dam, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
85	Missouri	Epping-Springbrook Dam, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
86	Missouri	Kota-Ray Dam, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
87	Missouri	White Earth Dam, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
88	Missouri	McGregor Dam, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
89	Missouri	South Hoffer McClusky, ND [c]	3	1	Clade B	0	0.00	0.0000	0.00
90	Missouri	Beaver Creek, ND [c]	6	2	Clade B	1	0.53	0.0009	0.53
91	Missouri	Waubay Lake, SD [h]	2	2	Clade B	4	1.00	0.0069	4.00
92	Nelson	LaSalle River, MB [f]	2	2	Clade B	3	1.00	0.0052	3.00
93	Nelson	Assiniboine River, MB [f]	4	4	Clade B	3	1.00	0.0026	1.50
94	Nelson	Pinawa diversion, MB [f]	1	1	Clade B				
95	Nelson	Rat River, MB [f]	5	2	Clade B	1	0.40	0.0007	0.40
96	Nelson	Lake of the Woods, MN [e]	3	2	Clade B	1	0.67	0.0011	0.67
97	Nelson	Muskeg Bay, MN [e]	4	2	Clade B	1	0.50	0.0009	0.50
98	Nelson	Kabetogema Lake, MN [e]	2	2	Clade B	1	1.00	0.0017	1.00
99	Nelson	Lake Vermillion, MN [e]	3	1	Clade C	0	0.00	0.0000	0.00
100	Nelson	Granite River, MN [e]	4	1	Clade B	0	0.00	0.0000	0.00
101	Nelson	Little Gunflint Lake, MN [e]	1	1	Clade B				
102	Great Lakes	Pine Lake, MN [e]	4	2	Clade A	1	0.67	0.0011	0.67
103	Mississippi	Lake Winnibigoshish, MN [e]	3	1	Clade B	0	0.00	0.0000	0.00
104	Mississippi	Mille Lacs, MN [e]	7	5	Clade B	5	0.86	0.0035	1.43

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Site no.	Watershed	Waterway/Waterbody	N	N_{hap}	Haplogroup	N_P	h	π	k
105	Mississippi	Markham Pond, MN [j]	1	1	Clade B				
106	Mississippi	Squaw Creek, IA †	1	1	Clade B				
107	Mississippi	Drury Creek, IL [k]	1	1	Clade A				
108	Ohio	Little Wabash River, IL [k]	1	1	Clade A				
109	Missouri	King's Creek, KS*	11	2	Clade C				
110	Missouri	Clark's Creek, KS*	10	3	Clade C				
111	Missouri	Mission Creek, KS*	12	3	Clade C				

* Sequences from Mathews et al. (2008)
† Sequence from Filipova et al. (2010)
[a] Provided by Iain Phillips, Saskatchewan Watershed Authority
[b] Provided by Terry Clayton, Alberta Sustainable Resource Development, Fish and Wildlife Division
[c] Provided by Fred Ryckman, Lynn Schlueter, North Dakota Fish and Game
[d] Provided by Susan Adams, U.S. Forest Service, Southern Research Station
[e] Provided by Konrad Schmidt, Tom Heinrich, Patrick Schmalz, Melissa Drake, Minnesota Department of Natural Resources
[f] Provided by Terry Galloway, Lane Graham, University of Manitoba
[g] Provided by Bill Gardner, Mike Ruggles, Cody Nagel, Mike Backes, Dave Yerk, Montana Fish, Wildlife and Parks
[h] Provided by Brian Blackwell, South Dakota Game, Fish and Parks
[i] Provided by Felix Sperling, University of Alberta
[j] Provided by David Huff, University of Minnesota
[k] Provided by Chris Taylor, Illinois Natural History Survey

Chapter 4. Isolation and characterization of nine polymorphic microsatellite loci in the northern crayfish (*Orconectes virilis*)[1]

4.1 Abstract

Nine novel polymorphic microsatellite loci were isolated from *Orconectes virilis* and characterized in 46 individuals from 2 major rivers in Alberta, Canada. Number of alleles per locus per site ranged from 1 to 5. Observed and expected heterozygosity per site ranged from 0.000 to 0.900 and 0.000 to 0.740, respectively. All 9 microsatellite loci conformed to expectations of Hardy-Weinberg and linkage equilibrium. These markers will be useful in the study of movement patterns, genetic diversity, and population structure of native and invading *O. virilis*.

KEYWORDS: Biodiversity, Invasive species, Microsatellite, Northern crayfish, *Orconectes virilis*

4.2 Main text

The northern crayfish, *Orconectes virilis*, is among the most wide-ranging crayfish species in North America, occurring from Alberta and Montana in the west to Quebec in the east, and south through the upper Mississippi River drainage (Schwartz et al. 1963; Williams et al. 2009). The species is considered invasive in areas of western and eastern North America (e.g., California, Riegel 1959; Maryland, Schwartz et al. 1963), and Europe (e.g., Souty-Grosset et al. 2006). In Alberta, *O. virilis* has undergone a recent and rapid expansion into previously uninhabited rivers (Williams et al. 2009). As part of a larger study to identify source populations and mode of expansion (i.e., natural vs human-mediated movement), we developed 9 microsatellite markers from *O. virilis*.

We constructed a partial genomic library using the enrichment protocol of Hamilton *et al.* (1999). We isolated genomic DNA from crayfish cheliped or

[1] A version of this chapter has been published. Williams, B.W., C.S. Davis, and D.W. Coltman. 2010. Conservation Genetics Resources 2:235-237. Formatting follows this journal's guidelines.

abdominal muscle tissue using phenol-chloroform extraction (Sambrook and Russell 2001). Isolated genomic DNA was digested using *Rsa I, Alu I,* and *Nhe I* restriction enzymes and ligated to double-stranded SNX linkers. Four pooled oligonucleotide repeat probes (GT, CT, GACA, and GATC) were hybridized to linker ligated genomic fragments, separated from non-hybridized fragments using magnetic beads coated with streptavidin, and subjected to a series of stringency washes. Remaining fragments were eluted and amplified using the SNX forward primer. Amplified fragments were digested with *Nhe I,* ligated into *Xba I* digested pBSII SK+ vector and cloned into XLI-Blue *Escherichia coli* cells. Positive clones were cultured and used as template for colony PCR to determine presence of insert. Confirmed insert-bearing PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced using the BigDye v3.1 sequencing kit (Applied Biosystems) on an ABI 3730 DNA Analyzer (Applied Biosystems).

We designed 70 primer pairs using *PRIMER* 3 (Rozen & Skaletsky 1998) and conducted initial screening using the M13 dye system (Schuelke 2000). Nine microsatellite loci amplified consistently and clearly with > 1 allele. We obtained 5' fluorescently labeled primers for these nine loci and genotyped 46 crayfish, consisting of *O. virilis* from the Bow (N = 20) and North Saskatchewan Rivers (N = 26) in Alberta.

We performed PCR amplification in 10 µL reactions consisting of 1x PCR reaction buffer (10 mM Tris pH 8.8, 0.1 % Triton X-100, 50 mM KCl, 0.16 mg/ml BSA) 0.16 µM forward and reverse primers, 0.2 µM dNTPs, 1.5 mM $MgCl_2$, 25-100 ng template DNA, and 0.3 U *Taq* DNA polymerase. The thermal profile for PCR amplification was 94°C for 1 minute, 3 cycles of 94°C for 30 seconds, 52°C for 20 seconds, and 72°C for 5 seconds followed by 33 cycles of 94°C for 15 seconds, 52°C for 20 seconds, and 72°C for 1 second, with a final 72°C extension for 30 minutes. Fragments were coloaded with GS600LIZ size standard (Applied Biosystems), run on an ABI 3730 DNA Analyzer, and genotyped using GeneMapper ® version 4.0 (Applied Biosystems).

We used GENEPOP version 3.4 (Raymond & Rousset 1995) to test for linkage disequilibrium and departure from Hardy-Weinberg equilibrium (HWE)

overall and for each sampling site. We used Microsatellite Analyser (MSA) version 4.05 (Dieringer & Schlötterer 2003) to calculate observed and expected heterozygosity. Following Bonferroni correction, significant deviation from HWE occurred in 6 loci over all samples. Linkage disequilibrium was detected in 26 of 36 inter-locus comparisons when all samples were combined. In contrast, no loci deviated from HWE and linkage disequilibrium was not detected in any pair of loci when the Bow and North Saskatchewan River crayfish were considered separately. Number of alleles per locus per site ranged from 1 to 5 (Table 4-1). Observed heterozygosity ranged from 0.200 to 0.900 in the Bow River crayfish and 0.000 to 0.539 in the North Saskatchewan River specimens. Expected heterozygosity ranged from 0.185 to 0.740 and 0.000 to 0.566 in the Bow and North Saskatchewan River crayfish, respectively.

The microsatellite loci are sufficient to detect significant differences among crayfish populations in different rivers of Alberta. We will be using these markers to determine movement patterns and resulting population genetic structure of *O. virilis* in the western Prairie Provinces of Canada.

4.3 Acknowledgements

We thank K. Williams, Dr. J. LaMontagne, and Dr. H. Proctor for assistance in collecting *O. virilis* specimens. Funding for this study was provided by a Natural Sciences and Engineering Research Council of Canada Discovery Grant (DWC), and Alberta Conservation Association Grant in Biodiversity (BWW).

Table 4-1. Characteristics, optimized PCR conditions, and summary statistics for nine microsatellite loci from *Orconectes virilis* collected from the Bow and North Saskatchewan Rivers, Alberta, Canada.

Locus/ Genbank No.	Primer sequence (5'-3')	Repeat motif	T_a (°C)	Bow River n = 20			North Saskatchewan River n= 26		
				N_a	H_O/ H_E	Size range (bp)	N_a	H_O/ H_E	Size range (bp)
Ov3	*NED*-AGTCTTCAACCACCGTCACCCTTG	$(CA)_5(AC)_{10}$	56	3	0.750	141-171	1	0.000	141
HM152475	TCCCACAGTCCTGCTCAAATGA				0.600			0.000	
Ov5	*FAM*-GCCCTTCCTCTCTTCCTGTTCT	$(AG)_{28}$	56	4	0.900	169-181	3	0.346	155-167
HM152476	GGTTGATGTCTCCTCCTAGCAAC				0.740			0.370	
Ov6	*VIC*-CGGCTGGCGTATGAGAGTCACA	$(TC)_{26}$	56	4	0.750	137-151	3	0.423	151-155
HM152477	GTGTCGGCCCCGCTTAATGT				0.696			0.540	
Ov15	*PET*-AGTGTGCAGACACATGGTGAGGAC	$(GA)_{18}(CAGA)_5$	48	3	0.250	174-192	5	0.462	182-204
HM152478	TGTATTTTTCTTGCCCCCTTC				0.304			0.502	
Ov24	*VIC*-TCACCCCCTTCGTTTCGTTATG	$(GTCT)_{13}$	48	2	0.200	198-240	2	0.192	232-240
HM152479	CGCCTTGTCTTAGTCCGTCTCA				0.185			0.177	
Ov34	*PET*-CTGTAGTGTTTCATGCGTCA	$(AG)_{24}$	48	2	0.400	181-191	5	0.539	173-211
HM152480	ATGAAAGCAACTCAATTCCA				0.328			0.566	
Ov3-48	*VIC*-AAGTTGCTGGTTTCAGGAAT	$(GT)_{32}$	56	2	0.350	191-195	2	0.077	195-197
HM152481	TCACTCTTTCTCTTCCTCGTT				0.358			0.075	
Ov5-07	*FAM*-GCAAGCACAAATAGGTGAGT	$(GT)_{21}$	58	3	0.550	162-184	3	0.115	160-166
HM152482	TTCTCTTCCTGGGACATACC				0.527			0.112	
Ov5-73	*PET*-ACAACCTGCATTTACCCTCT	$(TC)_9$	56	2	0.450	213-215	2	0.039	213-215
HM152483	CTGGGAAACAAAAACATCAA				0.409			0.039	

Primer sequence, repeat motif, and optimized annealing temperature (T_a) are provided for each locus. Number of individuals genotyped (n), number of alleles (N_a), observed (H_O) and expected (H_E) heterozygosity, and size range are provided for each locus in each river population. NED, FAM, VIC, and PET refer to ABI fluorescent dyes.

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Chapter 5. Multiple pathways to a recent, rapid range expansion of a widespread crayfish species

5.1 Abstract

Expansion of species' ranges into areas previously not occupied can be effected by movement of individuals under their own power, unusual wind or flooding events, or, increasingly, by deliberate or accidental introduction by humans. The ability to distinguish between 'natural' spread and spread associated with human-mediated introduction is not always simple, although these mechanisms may differ drastically in their ecological and evolutionary impacts, and in the way shifts in ranges are managed. The northern crayfish, *Orconectes virilis* (Hagen), has recently and rapidly expanded its range along several rivers in the Interior Plains of North America, moving over 200 km in upstream river distance in approximately 15 years. Here I use a suite of nine microsatellite markers to examine genetic patterns associated with upstream spread of *O. virilis* along three major river systems, or expansion axes. I collected 1354 northern crayfish from 83 sites along the North Saskatchewan, Battle, and South Saskatchewan River systems as well as the neighbouring Churchill and Missouri River Drainages. Crayfish were also collected from several sites disjunct from the core contiguous distribution. Due to the similar timing of expansion events, I expected that genetic patterns would be similar among the three expansion axes; however, I found that patterns were not consistent across the three axes, suggesting that the current distribution of *O. virilis* along its western range edge is a result of expansion via multiple pathways. Several areas along the South Saskatchewan River expansion axis showed spatial and geographic signatures of introduction. In contrast, introduction was not clearly implicated along the North Saskatchewan and Battle River expansion axes; however, genetic patterns were not entirely consistent with unassisted upstream movement. The Churchill River Drainage was the only waterway to display genetic patterns consistent with a stepping-stone colonization model. Although I could not identify the source of any disjunct population, genetic patterns were largely consistent with multiple releases or introduction

from a high-diversity source. Expansion by different mechanisms might be a pattern expected for species with diverse economic, ecological, or recreational value, such as crayfishes in areas undergoing substantial environmental change.

5.2 Introduction

Geographic limits to a species' range depend on factors that affect persistence and survival of members of that species in an area (e.g., climate, species interactions). Range shifts are a common occurrence in the evolutionary history of almost all species (Hewitt 1996; Davis & Shaw 2001), and in the past were usually the result of movement of individuals under their own power, or by transport via wind or water. But human activities have facilitated recent and rapid changes in the distribution of many taxa, both directly (via introduction) and indirectly (via modification of environmental characteristics or habitat) (e.g., Parmesan & Yohe 2003; Harley et al. 2006; Chen et al. 2011). The distinction between 'natural' or unassisted range expansion and human-mediated introduction (with or without subsequent spread) is often clear, with geographic distance among noncontiguous populations of human-introduced species substantially greater than the normal range of dispersal (e.g., Colautti et al. 2005; Therriault et al. 2005; Brown & Stepien 2009). However, contiguity of expanding populations does not necessarily indicate that organisms moved into new areas without human aid (e.g., Carlton 1996; Gopurenko et al. 2003). Large-scale contiguous expansions might result from unassisted dispersal, introduction, or a combination of mechanisms.

Large-scale movement of a species into novel habitats by either unassisted colonization or human-mediated introduction may differ drastically in ecological and evolutionary impacts, and in the way shifts in ranges are managed (Hoffmeister et al. 2005; Carlson 2008; Wilson et al. 2009). Consequently, identifying the pathways underlying range expansion is critical to understanding the ecological and evolutionary potential of a species facing changing abiotic and biotic factors.

Much of our understanding of genetic patterns of unassisted range expansion is derived from studies examining post-glacial spread (e.g., Hewitt 1996, 2004; Bernatchez & Wilson 1998; Excoffier et al. 2009). Common genetic signatures include gradients in allele frequency and diversity along axes of spread (Excoffier et al. 2009; Novembre & Di Rienzo 2009) and greater differentiation among edge or peripheral populations (Excoffier et al. 2009; Hallatscheck & Nelson 2009). A few recent studies have examined the genetic impacts of recent climate-driven expansions (Banks et al. 2010; Dawson et al. 2010; Garroway et al. 2011), highlighting effects of demographic characteristics (e.g., dispersal ability and speed, number of dispersers) and inter-population connectivity. Additional information on genetic patterns of rapid unassisted range expansion is provided by studies of spread following introduction of nonindigenous species (e.g., Darling & Folino-Rorem 2009; Ramakrishnan et al. 2009; Watts et al. 2010; Bronnenhuber et al. 2011). High motility of individuals within and among recently colonized areas would mitigate the expected decrease in genetic diversity and the increase in genetic divergence with distance from the original population (e.g., Banks et al. 2010; Dawson et al. 2010; but see Garroway et al. 2011). Conversely, small numbers of founding inviduals along an expansion axis are expected to result in a series of founder events, each resulting in reduced genetic diversity (Le Corre & Kremer 1998; Excoffier et al. 2009).

Genetic consequences of recent human-mediated introduction are well-documented (e.g., Colautti et al. 2005; Stepien & Tumeo 2006; Dlugosch & Parker 2008), and are highly dependent upon demographic characteristics of the introduction and sequential spread. Low diversity and signature of a bottleneck is expected with the introduction of few individuals. Unusually high diversity is expected with several independent introduction events. Unlike unassisted range expansion, recent introduction events should not be associated with gradients or clines in genetic patterns with geographic distance. Instead, we would expect abrupt changes in genetic structure and/or diversity.

The northern crayfish, *Orconectes virilis* (Hagen), is one of the most widespread crayfish in North America, with a core range spanning from Alberta

and Montana east to Maine and New Brunswick, and from northern Saskatchewan and Manitoba south to Texas (Crocker & Barr 1968; McAlpine et al. 1999; Hamr 2002; Taylor et al. 2005; Williams et al. 2011). More than half of the current distribution of *O. virilis* is in areas that were covered by the Laurentide ice sheet during the last glacial maximum (LGM) ~ 21,000 ya (Dyke et al. 2002), making the species the most successful post-LGM crayfish colonizer in the Holarctic in terms of area in which it has established. In addition to this historical spread, the species has undergone recent and rapid range expansion in areas of its core distribution (e.g., McAlpine et al. 1999), most notably along its western range edge, where spatio-temporal trends in sightings suggest upstream spread in several river systems of more than 200 km in river distance in approximately 15 years (Williams et al. 2011). Similarity in extent and timing of spread among these rivers suggests that range expansion might be a large-scale response to changing water characteristics (e.g., temperature, dissolved oxygen, flow rates). However, Alberta Sustainable Resource Development Fisheries reports indicate that patterns of spread differed among rivers, and that different dispersal mechanisms may have resulted in the current distribution of *O. virilis* (Williams et al. 2011). Human-mediated introduction has been common for *O. virilis* (e.g., Schwartz et al. 1963; Larson & Olden 2011) and has facilitated establishment of the species widely beyond the margins of core range. The northern crayfish is now present in 40 states, six provinces, and several areas of Europe (Souty-Grosset et al. 2006), and thus can be considered one of several highly invasive crayfishes.

In this chapter I use a suite of microsatellite markers to examine patterns of recent and rapid expansion of *O. virilis* along its western range edge. I focus on three major rivers, or expansion axes, along which substantial westward (upstream) spread of the northern crayfish has been documented. *Orconectes virilis* has clearly been introduced to portions of this region of the Interior Plains (Chapter 3); however, in the current chapter I employ a method complementary to the phylogeographic analysis presented earlier (Chapter 3) to examine spatial genetic structure; one that can be used to look at fine-scale patterns and thus

recent colonization history. The main objective of this chapter is to determine if genetic patterns along these three rivers match theoretical expectations of unassisted dispersal, of human-assisted dispersal, or of a combination of pathways. Specifically, I address four main expectations: (i) unassisted colonization will result in genetic homogeneity, or clines in genetic similarity, along each expansion axis whereas introduction will result in abrupt changes in genetic similarity; (ii) genetic diversity will decrease upstream along the expansion axis with unassisted dispersal whereas introduction will result in no obvious overall trend; (iii) significant genetic isolation by distance (IBD) is expected along expansion axes with unassisted dispersal following a stepping-stone colonization model, but will be weak to nonexistent along areas affected by rapid range expansion or introduction of genetically distinct individuals, and; (iv) patterns of IBD and genetic differentiation will vary over the length of each expansion axis so that IBD will be strongest (i.e., significant), and measures of differentiation greatest, among the longest established populations, or those found furthest downstream and in the Churchill River Drainage. In addition to assessing genetic patterns along the three major river-based expansion axes, I examine genetic characteristics of several sites disjunct from the core contiguous distribution, indicative of human-mediated introduction, to identify potential commonalities across introductions (e.g., source, numbers of founders).

5.3 Material and methods

5.3.1 Sample collection

A total of 1353 northern crayfish were collected from 82 sites (*n* ranging from 1-45 per site) across areas of the central Interior Plains and western Canadian Shield (Table 5-1, Figure 5-1). Sampling was done in ice-free conditions (April through November) across a five year period (2006 – 2010). Crayfish were either collected by hand or with baited Gee minnow traps, placed directly into individual vials of 95% ethanol, and stored at room temperature prior to DNA extraction.

Recent range expansion of *O. virilis* has occurred along three main river systems in the Nelson Drainage, namely the North Saskatchewan, Battle, and South Saskatchewan River systems. To examine genetic patterns associated with spread, I systematically sampled each of the three main rivers from the westernmost range edge downstream across a total river distance that was more than double that of the documented recent range expansion (Figure 5-1; Williams et al. 2011). This allowed for characterization of patterns among populations in areas of recent range expansion as well as eastern areas behind the latest wave of colonization. These latter reaches were expected to display genetic patterns indicative of historically established populations. The total sampled extent for the three expansion fronts each included more than one named river (Figure 5-1), but are hereafter termed the North Saskatchewan, Battle, and South Saskatchewan expansion axes. Due to river topology in this region, all three expansion axes also contain pre-expansion (downstream) sites in common (Figure 5-1). Additional sampling took place in rivers and lakes beyond the identified westernmost range edge sites and peripheral to the main courses of the focal rivers to (a) confirm the western extent of intra-river expansion, and (b) look for previously undetected colonization or disjunct populations that would suggest human-mediated introduction.

Crayfish were collected from the Churchill and Missouri River Drainages, immediately to the north and south, respectively, of the three expansion axes of interest in this study. Northern crayfish were reported from both drainages by the 1940s (Rawson & Moore 1944; Holthuis 1962), and these records provide the opportunity to examine genetic patterns (diversity and structure – Churchill; structure only – Missouri) of established populations at a similar longitude to my focal axes. In addition, based on geographic proximity, the Churchill and Missouri River Drainages are the most likely sources for human-mediated introduction into the areas of expansion.

5.3.2 DNA extraction and amplification

Total genomic DNA was extracted from gill tissue using the QIAGEN DNeasy kit (QIAGEN Inc.) using the standard protocol. I genotyped all crayfish at 9 polymorphic microsatellite loci, Ov3, Ov5, Ov6, Ov15, Ov24, Ov34, Ov3-48, Ov5-07, and Ov5-73 as described in Williams et al. (2010). Amplified fragments were run on an ABI 3730 DNA Analyzer (Applied Biosystems) in two poolplexes (A: Ov3, Ov5, Ov6, Ov15, Ov24; B: Ov34, Ov3-48, Ov5-07, Ov5-73) and analyzed in GeneMapper® v.4.0 (Applied Biosystems).

5.3.3 Standard genetic analyses

I tested for genotyping error (i.e., null alleles, stutter, and large allele drop-out) using MICRO-CHECKER (Van Oosterhout *et al.* 2004). Site and locus departures from Hardy-Weinberg equilibrium (HWE) and linkage equilibrium were assessed using GENEPOP v4 (Raymond & Rousset 1995) for all locations with a minimum sample size of ten, totaling 58 sites and 1296 individuals. I corrected for multiple tests using false discovery rates calculated in QVALUE (Storey 2002; Storey & Tibshirani 2003). I estimated the inbreeding coefficient, F_{IS}, and its significance with FSTAT 2.9.3 (Goudet 1995, 2001).

5.3.4 Inter- and intra-river population genetic structure

I used the Bayesian clustering algorithm of STRUCTURE 2.2 (Pritchard et al. 2000; Falush et al. 2003) to assess inter- and intra-river genetic connectivity and structure of all *O. virilis* sampled for this study. I ran ten independent iterations of *K* clusters ranging from 1-15, each with 10^6 MCMC replicates and a 10^5 iteration burnin period. In the simulations I used admixture ancestry models with correlated allele frequencies ($\lambda = 1$). I examined hierarchical clustering relationships among populations in two ways. I first considered assignments of individuals to *K* clusters beyond the initial maximal posterior probability of the data P(*K*|X), an approach that has been employed as a means to aid identification of historical processes that have resulted in the observed hierarchical structure (Rosenberg et al. 2005; Wang et al. 2007; Flanders et al. 2009; Bryja et al. 2010).

I combined the results of the ten iterations for each *K* using the Greedy algorithm in CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007), and used DISTRUCT v1.1 (Rosenberg 2004) to graphically display output from STRUCTURE. Secondly, I ran STRUCTURE independently for each identified discrete cluster in an iterative manner until $K = 1$.

5.3.5 Range expansion and genetic diversity

Upstream colonization in a stepping-stone manner is theorized to result in a series of founder events (Nei et al. 1975; Le Corre & Kremer 1998), with the strongest bottleneck signature in the most recently colonized areas and introduction sites. Recent decline in effective N_e results in reduction in number of alleles and heterozygosity. Bottlenecked populations experience more rapid reductions in allelic diversity than in heterozygosity via the loss of rare alleles. Consequently, bottlenecks are expected to result in significant heterozygosity excess in comparison to expectations of mutation-drift equilibrium (Cornuet & Luikart 1996). I used the Wilcoxon sign-rank test in BOTTLENECK v.1.2.02 (Piry et al. 1999) to test for differences in H_o and H_e at each site relative to observed number and frequency of alleles at each of the 9 loci. I implemented the two-phased mutation model (TPM) with 10% multi-step mutations and variance of 12.

I expected a steady decline in genetic diversity along each expansion axis with the lowest measures of diversity at range edge sites. I estimated expected and observed heterozygosity (H_e, H_o), number of alleles, and allelic richness for each site using MICROSATELLITE ANALYSER v.4.05 (Dieringer & Schlötterer 2003). Allelic richness was based on a minimum sample size of 10 (A_{10}) using rarefaction. I plotted A_{10} and H_e against river distance from the western range edge of each expansion axis and the Churchill River Drainage.

5.3.6 Range expansion and isolation by distance

I estimated genetic differentiation among sites along each expansion axis and the Churchill River Drainage by calculating pairwise estimates of F_{ST} among

sites using FSTAT with significance determined using 1000 permutations. As sample sizes for sites in the Missouri River Drainage were generally low (mean *n* = 4), I calculated pairwise F_{ST} values among the clusters that were identified using STRUCTURE. Disjunct populations were included in all sets of pairwise comparisons to facilitate source identification.

To assess patterns of genetic isolation by distance (IBD) across each expansion axis and the Churchill River Drainage I performed Mantel tests using the Isolation by Distance Web Service (Jensen et al. 2005) using river distances among sampling sites to construct the distance matrix. Significance of correlations was determined using 1000 randomizations.

Movement within each of the three expansion axes was rapid, with crayfish spreading across river distances of more than 200 km in approximately 15 years. However, this expansion, if unassisted, was expected to have originated from previously established populations in the downstream reaches of each axis. Therefore, I expected patterns of IBD to vary along the length of each axis. Specifically, I expected to see significant IBD in eastern, or lower reaches of each axis, but no significant IBD among populations within the areas of expansion. In addition, I expected to see lower levels of genetic differentiation among populations within the region of recent expansion than among historically established populations. To examine these intra-river patterns I performed a moving-window procedure whereby I iteratively calculated the parameters of IBD and mean pairwise F_{ST} values for every five consecutive west-to-east site-cluster along the length of the three expansion axes and the Churchill River Drainage.

5.4 Results

All nine microsatellite markers were polymorphic, ranging from three to 25 alleles per locus (mean = 14). Two sites in the lower South Saskatchewan River each displayed significant homozygote excess at a single locus (Site 63, Ov6; Site 64, Ov24). The low frequency of homozygote excess suggested null alleles were not a problem in my dataset. I detected no significant departures from HWE and linkage equilibrium following false discovery rate correction, although

it is interesting to note that nine of 36 (25%) locus-locus comparisons for disjunct site 44 showed departure from linkage equilibrium at a nominal significance value of P = 0.05. Significant ($P \leq 0.05$, uncorrected) F_{IS} values were observed at five sites (two positive, three negative, Table 5-1).

5.4.1 Genetic structure

Analysis of population genetic structure across the entire study area generally showed mixed individual assignments to several identified clusters with little discrete structure; however, the few discrete clusters were geographically restricted (Figure 5-1, Appendix 5-Ia). No additional discrete clustering was detected when $K > 7$ was inferred (Appendix 5-Ia). Iterative runs of STRUCTURE for each discrete cluster identified a further two genetically distinct clusters (Appendix 5-Ib).

No discrete clusters were found along the North Saskatchewan expansion axis, although mixed individual assignments appeared in high frequencies near the western range edge as well as sites near the eastern sampling extent (Figure 5-1, Appendix 5-Ia). Similarly, crayfish along the Battle River expansion axis appeared to be largely genetically homogeneous. In contrast, there are several genetically distinct clusters along the South Saskatchewan River expansion axis, with little to no overlap observed among clusters (Figure 5-1). Most clusters within the South Saskatchewan River expansion axis were shared with other rivers or drainages. The Churchill River Drainage comprised a genetically distinct cluster relative to all other sampled sites, but populations within the Drainage appeared genetically homogeneous. The Missouri River Drainage displayed four genetically and spatially distinct clusters.

Five sites identified as disjunct populations (sites 1, 2, 15-17) comprised a distinct genetic cluster (Figure 5-1). Two additional disjunct sites (45, 46) shared cluster assignment with most northern crayfish sampled from the Missouri River Drainage in Montana. The final identified disjunct site (44) displayed a pattern of mixed cluster assignment suggestive of introduction from more than one source and subsequent hybridization.

5.4.2 Expansion and genetic diversity

Three sites displayed significant heterozygosity excess (Wilcoxon signed-rank test; $P < 0.05$), including one disjunct lake (site 1) and two sites along the upper South Saskatchewan River (sites 51, 52). Despite the high overall per locus allelic diversity observed across all samples (see above), within-site diversity measures were low. Expected heterozygosity (H_e) and A_{10} ranged from 0.016 to 0.611 and from 1.11 to 4.68, respectively (Table 5-1). Genetic diversity measures also differed among river systems, with the lowest values of H_e and A_{10} found in the Churchill River Drainage and the highest in the lower reaches of the South Saskatchewan River (Table 5-1). There were no consistent patterns in genetic diversity along the expansion axis of either the North Saskatchewan or Battle River (Figure 5-2). The westernmost two genetically distinct clusters of the South Saskatchewan River expansion axis displayed relatively stable genetic diversity among sites, with a noticeable increase at the site where the clusters overlap. Sites along the lower South Saskatchewan River expansion axis showed no single consistent pattern in diversity (Figure 5-2). In contrast, genetic diversity (A_{10} and H_e) decreased linearly along the Churchill River Drainage (linear regression, t=10.60, P=1.452e-05; t=7.751, P=0.0001, respectively) from the easternmost sampling site to the western range edge (Figure 5-2).

Six of the eight disjunct sites had sufficient sample sizes to examine genetic diversity. Only one site (Henderson Lake, 45) displayed low diversity, suggestive of introduction of low number of founding individuals (Table 5-1). The remaining five sites displayed high diversity measures.

5.4.3 Range expansion and isolation by distance

Pairwise F_{ST} values among sites of the North Saskatchewan River expansion axis ranged from 0.000 to 0.094 (Appendix 5-IIa). Few of these pairwise comparisons were significant, although a significant, but weak, pattern of IBD was observed across the entire axis (Figure 5-3, Appendix 5-III). Pairwise F_{ST} values among sites of the Battle River expansion axis were low, ranging from

0.000 to 0.061, most being non-significant (Appendix 5-IIb). No significant pattern of IBD was observed across the Battle River expansion axis (Figure 5-3, Appendix 5-III). In contrast, strong IBD was displayed across the entire South Saskatchewan River expansion axis (Figure 5-3). Pairwise F_{ST} values among the three genetic clusters identified along the South Saskatchewan expansion axis were high, ranging from 0.100 to 0.424 (Appendix 5-IIc).

In the Churchill River Drainage, pairwise F_{ST} values ranged from 0.000 to 0.120 (Appendix 5-IId), following a significant overall pattern of IBD (Figure 5-3, Appendix 5-III). Although two of the four clusters identified by STRUCTURE in the Missouri River drainage were represented by small sample sizes, pairwise F_{ST} values among clusters were high, ranging from 0.182 to 0.614 (Appendix 5-IIe).

Using the moving-window approach, I found that characteristics of the IBD pattern (i.e., slope, r^2, and significance) were not consistent across different areas of both the North Saskatchewan and Battle River expansion axes (Appendix 5-III), and did not display the expected pattern of significant IBD among eastern-most sites. In contrast, significant and strong IBD was found in the eastern reach of the South Saskatchewan River (Appendix 5-III; sites 58-65, 33-35) and not consistently in upstream areas. The Churchill River Drainage displayed significant IBD for all subsets of sites, with a general pattern of decreasing r^2 and increasing slope from east to west (Appendix 5-III).

Mean F_{ST} across sites generally increased in an eastern, or downstream, direction along the North Saskatchewan and Battle River expansion axes and the Churchill River Drainage (Figure 5-4; Appendix 5-III). The opposite trend was observed along the South Saskatchewan River expansion axis, with mean F_{ST} decreasing in a downstream direction.

I found no clear genetic similarities between the disjunct sites (sites 1, 2, 15-17, 44- 46) and other sampled sites (Appendix 5-IIa, b, c, d). Consequently, source populations for these disjunct sites could not be identified.

5.5 Discussion

The northern crayfish has undergone substantial and recent westward range expansion along several river systems within the western Prairies. Although the spatial extent and timing of expansion along each axis are similar, suggesting that *O. virilis* is responding to a common longitudinal driving factor such as change in flow patterns or water temperature, my data indicate that genetic patterns along each expansion axis are not consistent with a single pathway of spread. Instead, both unassisted and human-mediated movements have contributed to the current western distribution of the species.

Genetic expectations of unassisted upstream spread of *O. virilis* based on a stepping-stone colonization model were: (a) populations across the expansion axis would be genetically homogeneous; (b) genetic diversity would gradually decrease along the axis towards the range edge; (c) a significant pattern of IBD would exist along the length of the axis; (d) patterns of IBD and genetic differentiation would vary along the axis, with significant IBD and greater differentiation among downstream, or historically established sites. Interestingly, populations along the Churchill River Drainage were the only ones consistent with all of the above expectations. Crayfish within this drainage displayed a homogeneous clustering pattern, decreasing diversity from east to west, significant IBD across the entire sampled waterway, decreasing fit of the IBD pattern in westernmost areas, and increasing average genetic differentiation from west to east. These results support natural colonization of this drainage, despite historical records suggesting that crayfish were introduced to locations near the Alberta-Saskatchewan border and restricted to upstream reaches of the drainage (Rawson & Moore 1944; Aiken 1968). Crayfish were not reported in downstream areas of the drainage until an assessment was made of the suitability for Saskatchewan waterbodies to sustain potential crayfish harvest (Sawchyn 1986). However, genetic patterns in the Churchill River Drainage are consistent with gradual spread, as expected with post-glacial colonization.

As *O. virilis* within Missouri River Drainage was assumed to have been historically established based on early reports (Holthuis 1962), I expected genetic

patterns to be similar to what I observed in the Churchill River Drainage. However, genetic patterns in the Missouri River Drainage are suggestive of human-mediated introduction, with four highly differentiated genetic clusters. The northern crayfish was first recorded in the upper Missouri River Drainage near Bozeman, Montana, in the 1940s (Holthuis 1962). As such, the species was likely already established throughout the drainage. However, reports from Montana (Virile Crayfish — *Orconectes virilis*. Montana Field Guide. Montana Natural Heritage Program. Retrieved on April 29, 2012, from http://FieldGuide.mt.gov/detail_ICMAL11670.aspx) show a large increase in the number of observations of *O. virilis* across the state since 2000, suggesting that either densities have increased or that fisherpersons and biologists have become more observant or aware of the species across the state. The northern crayfish was introduced to areas in the Clark Fork region (Pacific Drainage) of western Montana in the 1960s by Montana Fish, Wildlife and Parks as forage for sport fishes (Sheldon 1989), with the crayfish reportedly taken from Wisconsin. Northern crayfish from the Clark Fork region are genetically similar to those found in much of the upper Missouri River Drainage of Montana (B. Williams, unpubl. data), suggesting that *O. virilis* initially introduced into western Montana were subsequently translocated to areas of the upper Missouri River. The presence of distinctly different *O. virilis* in headwater streams of the Missouri River Drainage (sites 77-82) may represent remnants of previously established *O. virilis* populations that have been supplanted by introduced crayfish.

The genetic cluster found at the western edge of the South Saskatchewan River expansion axis is also clearly a result of introduction, but its origin is unclear because it is highly differentiated from all other sampled sites. Genetic diversity within this cluster is relatively high (Sites 46-52, Table 5-1) and consistent across representative sites. This, with the low genetic differentiation among sites (pairwise F_{ST} ranging from 0.000-0.034) suggests these crayfish likely represent a single introduction from a high diversity source that has subsequently spread. The adjacent genetic cluster in the mid South Saskatchewan River expansion axis (sites 52-55) shows similar characteristics among

representative sites (i.e., retention of diversity and low genetic differentiation). In this case, genetic similarity to crayfish sampled in the Frenchman River, a headwater stream to the Milk and ultimately Missouri River, indicates a potential source.

In contrast to the South Saskatchewan River, the North Saskatchewan and Battle Rivers display no clustering patterns that clearly indicate human-mediated introduction. Individual-based assignments within each river show that crayfish are relatively genetically homogeneous. In addition, genetic differentiation decreases towards the western range edge. However, neither river displays all of the patterns expected from entirely unassisted colonization from downstream sources. Genetic diversity does not decrease consistently along each river, but instead fluctuates. The westernmost site does not display the lowest diversity in either river. Indeed, in both the North Saskatchewan and Battle River, the lowest diversity is found ~ 200 km downstream of the western range edge (Figure 5-3). The high genetic diversity observed in the range edge sites of the North Saskatchewan River expansion axis might be explained by movement, likely human-mediated, of crayfish from one or more of the suburban ponds identified as disjunct sites (16, 17) into either the nearby main stem or tributaries of the North Saskatchewan River. Modeling allele frequencies along this expansion axis should allow for better determination of the cause of this pattern.

Lack of genetic isolation by distance (IBD) is expected in areas of recent expansion as these populations have not had sufficient time to achieve drift-migration equilibrium. Indeed, the only areas where strong IBD is present with a pattern of increasing slope towards the western range edge are in the Churchill River Drainage, and to some extent the downstream part of South Saskatchewan River expansion axis. Both of these areas are assumed to have been established well prior to the recent westward expansion of interest. The lack of an IBD pattern along the eastern reach of the North Saskatchewan and Battle River expansion axes was surprising, and suggests that expansion has occurred over a much large distance than initially presumed. The former explanation is unlikely, as although A_{10} and H_e are low along these expansion axes, structure is evident in

the Churchill River despite very low diversity measures. Apportionment of genetic diversity across the study area generally follows a decreasing trend along a south-north axis, as expected with post-glacial colonization from the Mississippi glacial refugium, found to the southeast of the study area. Given this pattern, and the historical establishment of *O. virilis* in the northern Churchill River Drainage, it is surprising that the species does not show a genetic signature indicative of previous establishment in downstream portions of the North Saskatchewan and Saskatchewan Rivers. The northern crayfish was reported in the easternmost reaches of the Saskatchewan River in the 1800s (Hagen 1870, Faxon 1885). Although several major dams exist along this river system (e.g., E.B. Campbell Dam, Grand Rapids Dam, they were constructed relatively recently (early 1960s). It may be that until recently, major rivers in Alberta were too cold or too fast to allow colonization and establishment of crayfish.

The inconsistent patterns of diversity and structure in the North Saskatchewan and Battle River expansion axes could also have resulted from severe declines in, or extirpation of, previously established *O. virilis* populations along lower reaches of these axes. Over a period of forty years (1948 through the 1980s), first DDT, then methoxychlor, was applied to several sites along both the North and South Saskatchewan Rivers as a means to control black fly populations (Fredeen et al. 1971; Fredeen 1975; Dosdall & Lehmkuhl 1989). These larvicide applications were made along segments of the North Saskatchewan River that correspond to unexpected variation in *O. virilis* genetic diversity and structure (i.e., sites 29-32). This reach of the North Saskatchewan River was expected to display pre-expansion genetic patterns, or those of historically established populations, but rather showed results similar to areas near the western range edge. Although the effects of DDT and methoxychlor on crayfish fitness and survival are not clear (but see observations of juvenile *O. virilis* mortality; Sebastien & Brust 1989), it is possible that repeated exposure may have affected population persistence during this time period. As a result, colonization of *O. virilis* along the North Saskatchewan and Battle River expansion axes may have been much more extensive and earlier than previously thought.

Current western range limits within each of the three focal river expansion axes are similar in longitude. However, while it appears that dams (Oldman and Battle Rivers) and a weir (Bow River) delineate the western limits in two of these expansion axes, no known physical barrier defines the current western limit in the North Saskatchewan River expansion axis. This suggests that spread may continue by natural upstream movement of the crayfish until an as yet unrecognized ecological threshold has been reached. Laboratory studies of the northern crayfish suggest that the species is likely limited by low temperature (Aiken 1969). Females cannot release fertilized eggs until water temperature has reached 11°C. Thus, the spread of *O. virilis* will be restricted in upper reaches of glacial-fed rivers. Temperature also likely restricts the northern range limits of the species. Although the northernmost records of *O. virilis* are from the Churchill River Drainage (Sawchyn 1986; Williams et al., 2011), the northern extent to which the species is found along the drainage, which flows northeastward into Hudson Bay, remains unknown.

Human-mediated introduction has clearly extended the distribution of *O. virilis* beyond the current limits reached within the major river systems. Several sampled sites within the study area but outside the focal rivers showed a clear signature of human-mediated introduction. Diversity measures within these disjunct sites were not low relative to other sites sampled across the study area, suggesting that either the source of introduction had high genetic diversity or that several different releases comprised each introduction. Only one of the disjunct sites (site 1) displayed the significant bottleneck signature expected from the founder events that populated these disjunct locations. Likewise, only one site, Nose Creek (site 43), showed both clustering and linkage disequilibrium evidence of founding by more than one genetically distinct population. Disjunct site 46, Henderson Lake, displayed very low levels of genetic diversity indicative of either introduction from a low diversity source or introduction of very few founding individuals. Together with crayfish sampled from McGregor Lake (site 45), a disjunct population in a reservoir within the upper South Saskatchewan River system, the Henderson Lake population is genetically similar to most

crayfish sampled in the upper Missouri River Drainage in Montana. Although low sample sizes throughout Montana do not allow for identification of location of origin, these data suggest that the Upper Missouri River Drainage is the source of these two disjunct populations.

One of the most common pathways for crayfish invasion is as live bait, which likely explains the introduction of *O. virilis* into the upper South Saskatchewan River. Crayfish are a popular bait item for the capture of pike (*Esox lucius* Linnaeus), which are common in several reservoirs within the South Saskatchewan River Drainage. Crayfish are also used as bait for lake sturgeon (*Acipenser fulvescens* Rafinesque), also found along the South Saskatchewan River Drainage. Intriguingly, fisherpersons who have, either purposefully or unwittingly, introduced populations of *O. virilis* into novel water bodies may have precipitated a chain of events that could lead to changes in population structure, or even decline, of the very fishes they aim to catch (e.g., Hobbs et al. 1989; Dorn & Mittelbach 1999, 2004; Wilson et al. 2004). Although this outcome is unlikely in large rivers given the dynamic nature of these systems, introduced crayfish could very well cause noticeable ecosystem shifts in lakes or reservoirs.

The prior absence of northern crayfish and current contiguous distribution along the three river systems studied here strongly suggest changes to water characteristics or habitat that have allowed *O. virilis* to persist in these previously unoccupied areas, regardless of mode of spread. More work is needed to determine potential environmental mechanisms underlying this recent expansion. It is clear that human-mediated introduction has been a major driver in shaping the current distribution of *O. virilis*, both in the Prairies region as well as globally, in many cases in spite of government regulations banning the transport of live crayfish or use of live crayfish as bait. As such actions are expected for species of economic or recreational value, including crayfishes, future management of these species needs to account for potential pathways of spread.

Table 5-1. List of sites sampled for this study including number of individuals (n), number of observed alleles (N_A), allelic richness based on a minimum sample size of 10 (A_{10}), expected (H_e) and observed (H_o) heterozygosity, the inbreeding coefficient (F_{IS}), and number of locus-locus comparisons displaying linkage disequilibrium (LD). Also provided are results of two bottleneck tests: the P-value resulting from the Wilcoxon signed-rank test (P_{Wil}) performed using program BOTTLENECK. Site numbers correspond to those shown in Figure 5-1.

Site	Waterbody/Waterway	Drainage	n	N_A	A_{10}	H_e	H_o	F_{IS}	LD	P_{Wil}
1	McLeod Lake, AB	Athabasca	34	2.89	2.56	0.499	0.520	-0.039	1	**0.00**
2	Beyette Lake, AB	Athabasca	15	3.33	2.54	0.434	0.415	0.033	2	0.90
3	Amisk Lake, AB	Churchill	22	1.11	1.11	0.026	0.029	-0.125	0	1.00
4	Amisk River, AB	Churchill	23	1.56	1.28	0.029	0.030	-0.014	0	1.00
5	Beaver River, AB	Churchill	21	1.33	1.08	0.016	0.016	0.000	0	1.00
6	Beaver River, AB	Churchill	20	1.44	1.13	0.027	0.028	-0.012	0	1.00
7	Beaver River, SK	Churchill	26	1.78	1.22	0.049	0.051	-0.024	0	1.00
8	Beaver River, SK	Churchill	1							
9	Beaver River, SK	Churchill	1							
10	Beaver River, SK	Churchill	12	1.56	1.26	0.053	0.056	-0.029	0	1.00
11	Beaver River, SK	Churchill	6	1.67						
12	Churchill River, SK	Churchill	1							
13	Churchill River, SK	Churchill	30	4.78	2.09	0.240	0.241	-0.011	1	1.00
14	Churchill River, SK	Churchill	28	4.44	2.06	0.218	0.230	-0.041	0	1.00
15	East Pit Lake, AB	Saskatchewan	5	2.00						
16	Lake Lacombe, AB	Saskatchewan	25	3.67	2.85	0.485	0.471	0.001	5	0.50
17	Bearspaw Lake, AB	Saskatchewan	26	3.44	2.98	0.542	0.534	-0.009	1	0.06
18	North Saskatchewan River, AB	Saskatchewan	16	4.22	2.71	0.392	0.403	-0.040	1	1.00

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Site	Waterbody/Waterway	Drainage	n	N_A	A_{10}	H_e	H_o	F_{IS}	LD	P_{Wil}
19	North Saskatchewan River, AB	Saskatchewan	28	4.44	2.87	0.465	0.429	0.055	1	0.99
20	North Saskatchewan River, AB	Saskatchewan	13	2.89	2.00	0.247	0.239	0.022	0	1.00
21	North Saskatchewan River, AB	Saskatchewan	26	2.89	1.98	0.265	0.244	0.035	1	0.99
22	North Saskatchewan River, AB	Saskatchewan	20	2.56	1.90	0.254	0.239	0.023	1	1.00
23	North Saskatchewan River, AB	Saskatchewan	23	3.22	1.97	0.241	0.237	-0.011	1	1.00
24	North Saskatchewan River, AB	Saskatchewan	23	3.33	2.12	0.276	0.251	0.068	1	1.00
25	North Saskatchewan River, SK	Saskatchewan	19	3.67	2.43	0.333	0.351	0.010	1	1.00
26	North Saskatchewan River, SK	Saskatchewan	29	3.67	2.32	0.326	0.318	0.004	1	1.00
27	North Saskatchewan River, SK	Saskatchewan	20	3.78	2.45	0.336	0.356	-0.036	1	1.00
28	North Saskatchewan River, SK	Saskatchewan	13	3.44	2.41	0.326	0.342	-0.037	1	1.00
29	North Saskatchewan River, SK	Saskatchewan	17	3.00	2.10	0.283	0.268	0.040	0	1.00
30	North Saskatchewan River, SK	Saskatchewan	18	2.89	2.08	0.288	0.302	-0.052	0	0.99
31	North Saskatchewan River, SK	Saskatchewan	24	2.67	1.90	0.250	0.250	-0.018	0	0.96
32	North Saskatchewan River, SK	Saskatchewan	22	2.89	2.03	0.257	0.273	-0.067	2	0.98
33	Saskatchewan River, SK	Saskatchewan	25	4.89	2.74	0.403	0.409	-0.016	3	0.99
34	Saskatchewan River, SK	Saskatchewan	17	4.44	2.63	0.354	0.353	0.042	1	1.00
35	Saskatchewan River, SK	Saskatchewan	20	3.11	2.18	0.285	0.278	0.027	0	0.96
36	Battle River, AB	Saskatchewan	20	2.56	1.95	0.258	0.256	-0.013	0	0.95
37	Battle River, AB	Saskatchewan	19	2.56	1.93	0.253	0.240	0.040	0	0.96
38	Battle River, AB	Saskatchewan	28	2.89	2.03	0.270	0.262	0.027	0	0.95
39	Battle River, AB	Saskatchewan	25	2.67	1.89	0.237	0.236	0.070	1	0.99
40	Battle River,SK	Saskatchewan	25	3.11	1.91	0.231	0.222	0.122	1	1.00
41	Battle River,SK	Saskatchewan	1							

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Site	Waterbody/Waterway	Drainage	n	N_A	A_{10}	H_e	H_o	F_{IS}	LD	P_{Wil}
42	Battle River,SK	Saskatchewan	20	3.89	2.42	0.328	0.344	-0.054	1	1.00
43	Battle River,SK	Saskatchewan	45	4.00	2.33	0.314	0.331	-0.055	2	1.00
44	Nose Creek, AB	Saskatchewan	16	2.89	2.48	0.401	0.389	0.085	9	0.63
45	Henderson Lake, AB	Saskatchewan	24	1.44	1.37	0.155	0.190	**-0.222**	0	0.06
46	McGregor Lake, AB	Saskatchewan	3							
47	Oldman River, AB	Saskatchewan	17	3.00	2.53	0.470	0.464	0.022	1	0.33
48	Oldman River, AB	Saskatchewan	1							
49	Little Bow River, AB	Saskatchewan	1							
50	Bow River, AB	Saskatchewan	20	2.78	2.48	0.461	0.511	**-0.091**	0	0.10
51	South Saskatchewan River, AB	Saskatchewan	25	3.11	2.56	0.487	0.498	-0.030	1	0.10
52	South Saskatchewan River, AB	Saskatchewan	16	2.78	2.48	0.478	0.458	0.029	0	**0.00**
53	South Saskatchewan River, AB	Saskatchewan	13	3.89	3.24	0.611	0.615	-0.025	3	**0.01**
54	South Saskatchewan River, AB	Saskatchewan	28	3.78	2.48	0.397	0.397	0.013	3	0.99
55	South Saskatchewan River, SK	Saskatchewan	36	2.78	2.20	0.353	0.349	-0.003	1	0.84
56	South Saskatchewan River, SK	Saskatchewan	27	2.89	2.19	0.352	0.350	0.003	1	0.81
57	South Saskatchewan River, SK	Saskatchewan	21	3.11	2.47	0.423	0.444	-0.047	2	0.47
58	South Saskatchewan River, SK	Saskatchewan	21	3.00	2.31	0.380	0.376	0.004	1	0.66
59	South Saskatchewan River, SK	Saskatchewan	40	3.67	2.43	0.360	0.394	**-0.077**	0	1.00
60	South Saskatchewan River, SK	Saskatchewan	7	3.78						
61	South Saskatchewan River, SK	Saskatchewan	14	4.56	3.20	0.500	0.540	-0.095	2	0.99
62	South Saskatchewan River, SK	Saskatchewan	12	5.00	3.52	0.547	0.528	0.013	0	1.00
63	South Saskatchewan River, SK	Saskatchewan	16	4.78	3.10	0.506	0.549	-0.080	0	0.99
64	South Saskatchewan River, SK	Saskatchewan	25	5.00	2.91	0.454	0.400	**0.102**	0	0.96

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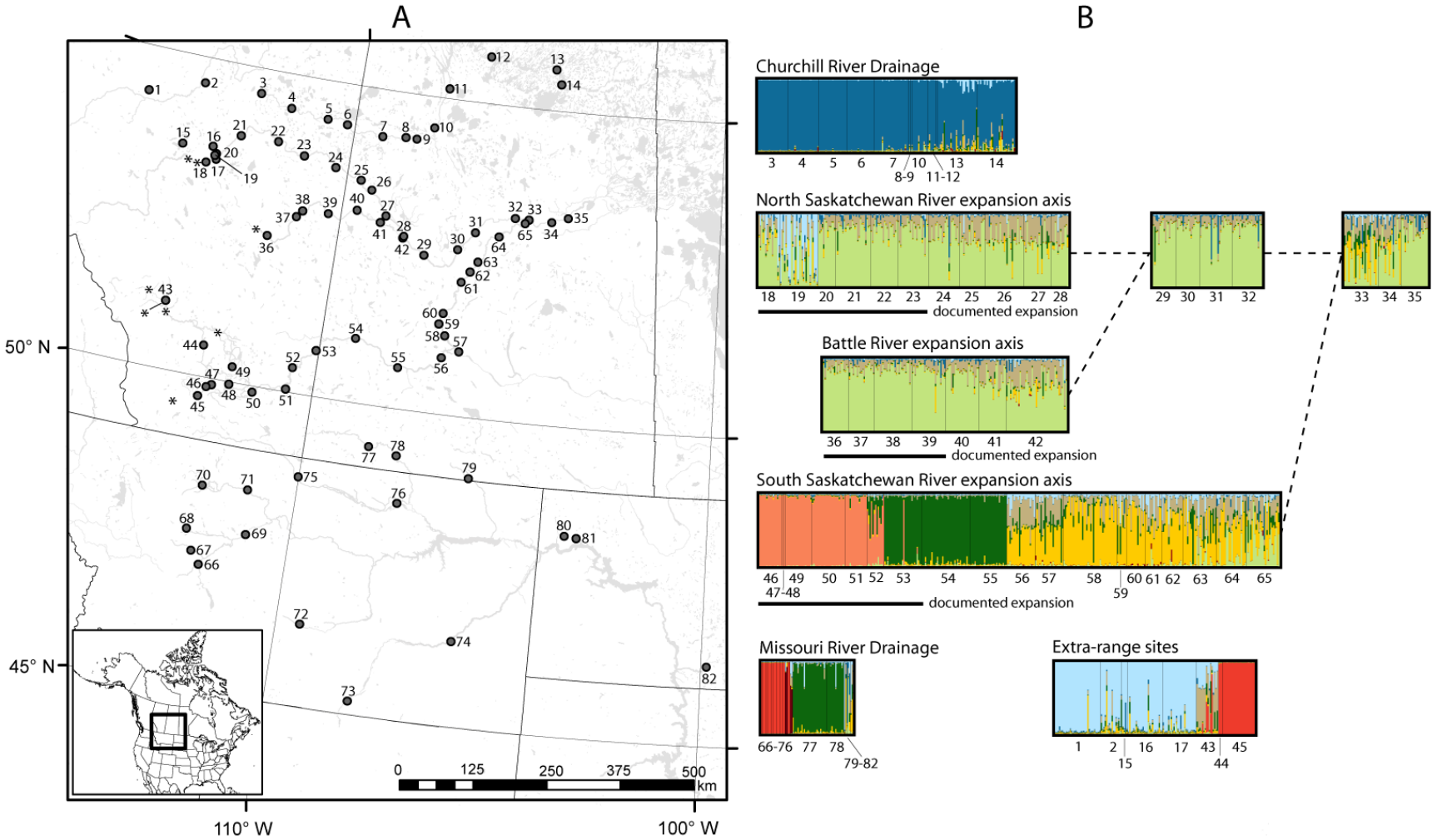
Site	Waterbody/Waterway	Drainage	n	N_A	A_{10}	H_e	H_o	F_{IS}	LD	P_{Wil}
65	South Saskatchewan River, SK	Saskatchewan	22	5.00	3.00	0.482	0.495	-0.025	1	0.99
66	South Saskatchewan River, SK	Saskatchewan	25	4.89	2.70	0.404	0.409	0.042	1	0.99
67	Missouri River, MT	Missouri	3							
68	Dearborn River, MT	Missouri	1							
69	Willow Creek Reservoir, MT	Missouri	3							
70	Missouri River, MT	Missouri	1							
71	Lake Frances, MT	Missouri	3							
72	Tiber Reservoir, MT	Missouri	3							
73	Deadmans Basin, MT	Missouri	2							
74	Big Bull Elk Bay, MT	Missouri	2							
75	Holding pond, Miles City, MT	Missouri	2							
76	Fresno Reservoir, MT	Missouri	2							
77	Nelson Reservoir, MT	Missouri	2							
78	Frenchman River, SK	Missouri	27	2.67	2.17	0.346	0.350	-0.024		0.53
79	Frenchman River, SK	Missouri	14	2.33	2.08	0.353	0.357	-0.023		0.15
80	Poplar River, SK	Missouri	1							
81	Epping-Springbrook Dam, ND	Missouri	1							
82	Kota-Ray Dam, ND	Missouri	3							
83	Beaver Creek, ND	Missouri	2							

Figure 5-1. (following page) (A) Map of sampling locations for the northern crayfish, *Orconectes virilis*, across areas of the central Interior Plains, western Canadian Shield, and the Clark Fork drainage of western Montana. Locations where sampling took place but no crayfish were found are denoted by an asterisk. Numbers correspond to sites in Table 5-1. (B) Population genetic structure of the 1353 individuals sampled from all sites as estimated by the program STRUCTURE. Shown are the results of $K = 7$ plus three additional clusters found during iterative runs. Individual crayfish are each shown as a single vertical coloured line, with length of each coloured segment corresponding to posterior probability of cluster assignment. Black vertical lines separate sites. Black horizontal lines beneath each of the three expansion axes show the extent of known expansion from ~ 1990 – 2005.


A
B
50° N
45° N
110° W
100° W
0 125 250 375 500 km
Churchill River Drainage
3 4 5 6 7 8-9 10 11-12 13 14
North Saskatchewan River expansion axis
18 19 20 21 22 23 24 25 26 27 28
documented expansion
29 30 31 32
33 34 35
Battle River expansion axis
36 37 38 39 40 41 42
documented expansion
South Saskatchewan River expansion axis
46 47-48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
documented expansion
Missouri River Drainage
66-76 77 78 79-82
Extra-range sites
1 2 15 16 17 43 44 45

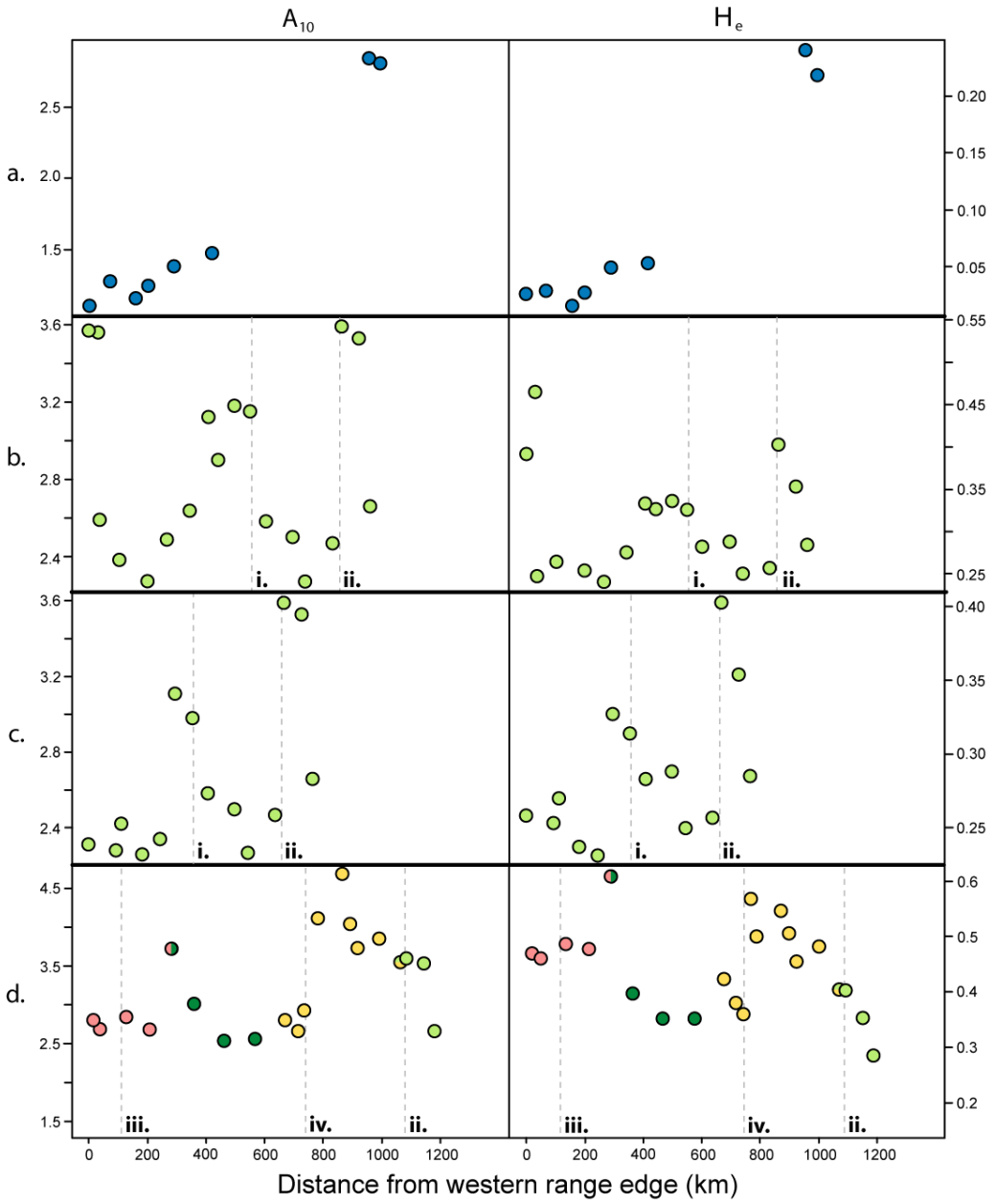


Figure 5-2. Genetic diversity, represented by allelic richness (A_{10}) and expected heterozygosity (H_e), plotted against river distance for the Churchill River, sites 3-14 (a), and expansion axes of the North Saskatchewan (b, sites 18-35), Battle (c, sites 36-42, 29-35), and South Saskatchewan Rivers (d, sites 46, 49-58, 60-65). Colours correspond to majority Structure cluster designation in Figure 5-1. Major river confluences are indicated by a dashed line (i. North Saskatchewan and Battle Rivers; ii. North and South Saskatchewan Rivers; iii. Oldman and Bow Rivers; iv. Qu'Appelle and South Saskatchewan Rivers).

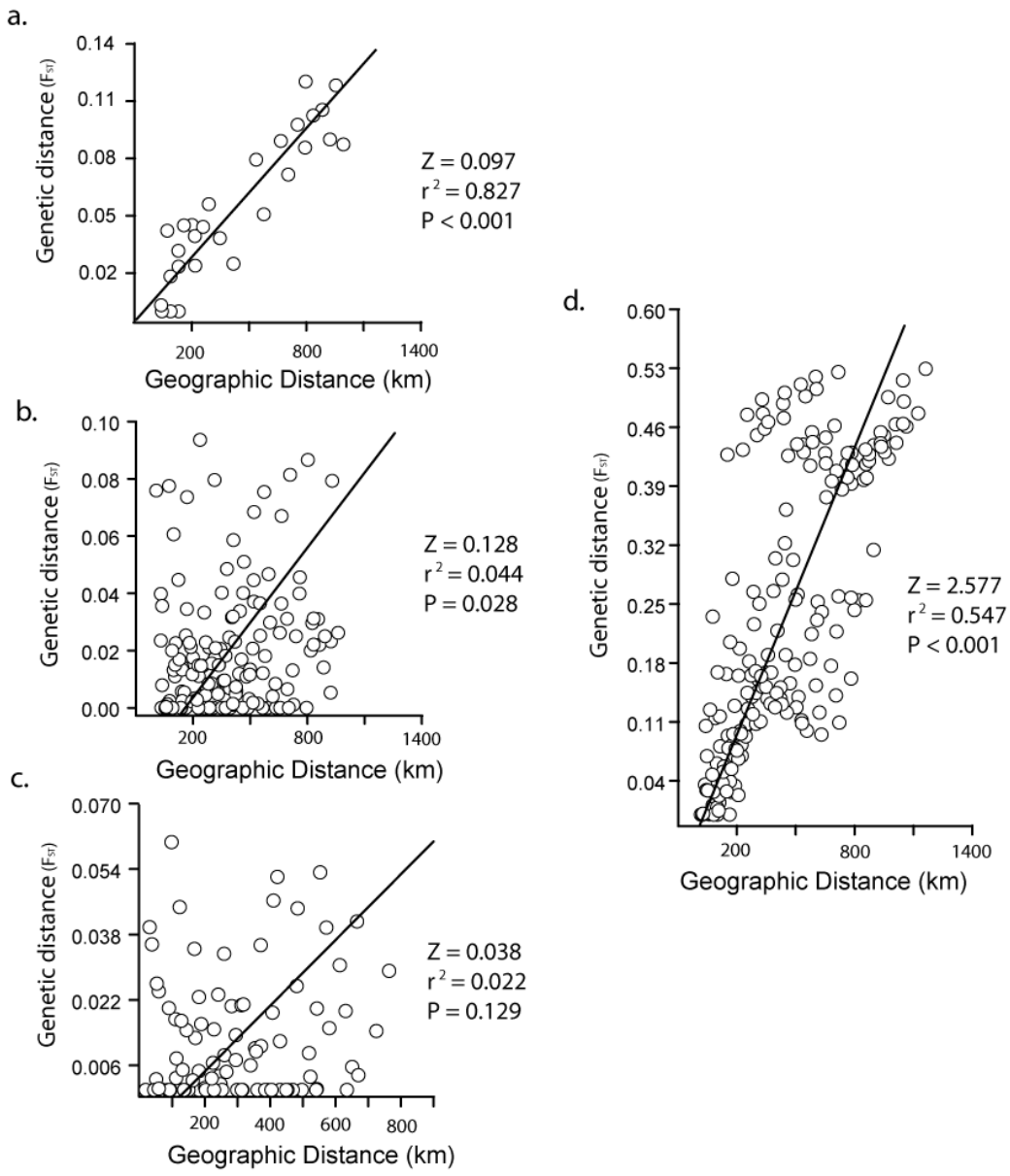


Figure 5-3. Results of Mantel tests to assess isolation by distance across the Churchill River (a) and entirety of each of the three expansion axes (North Saskatchewan River, b; Battle River, c; South Saskatchewan River, d). Values for the Mantel statistic (Z), coefficient of determination (r^2), and significance of the correlation of genetic and geographic distance are provided for each graph. Note that scale differs among graphs.

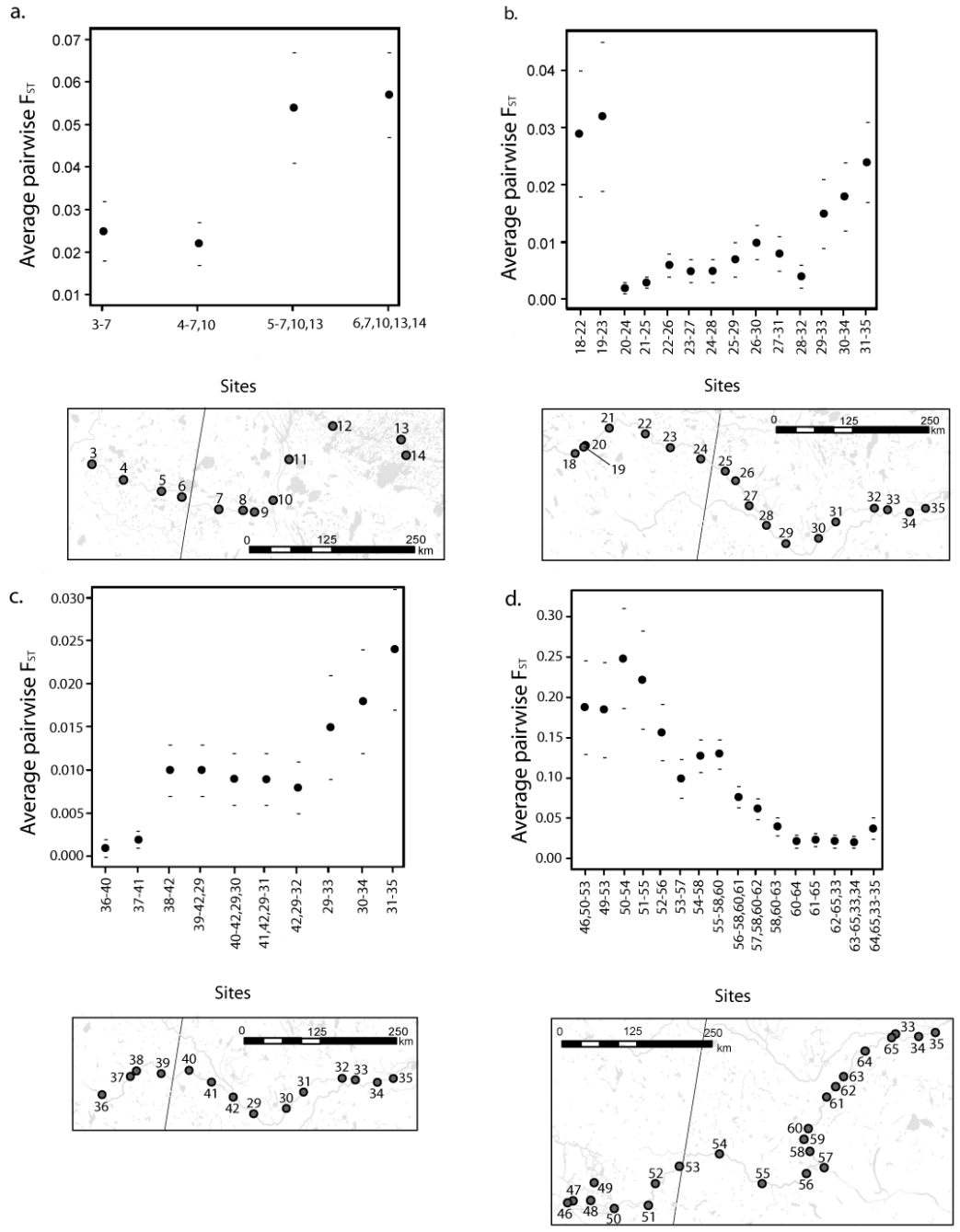


Figure 5-4. Mean F_{ST} values for subsets of 5 sites in a moving-window analysis across the Churchill River Drainage (a) and each of three expansion axes (North Saskatchewan River, b; Battle River, c; South Saskatchewan River, d). Standard errors (+/-) are shown as bars above and below each mean. Sites are listed on each x-axis and correspond to maps below each graph. Site numbers and maps correspond to Figure 5-1.

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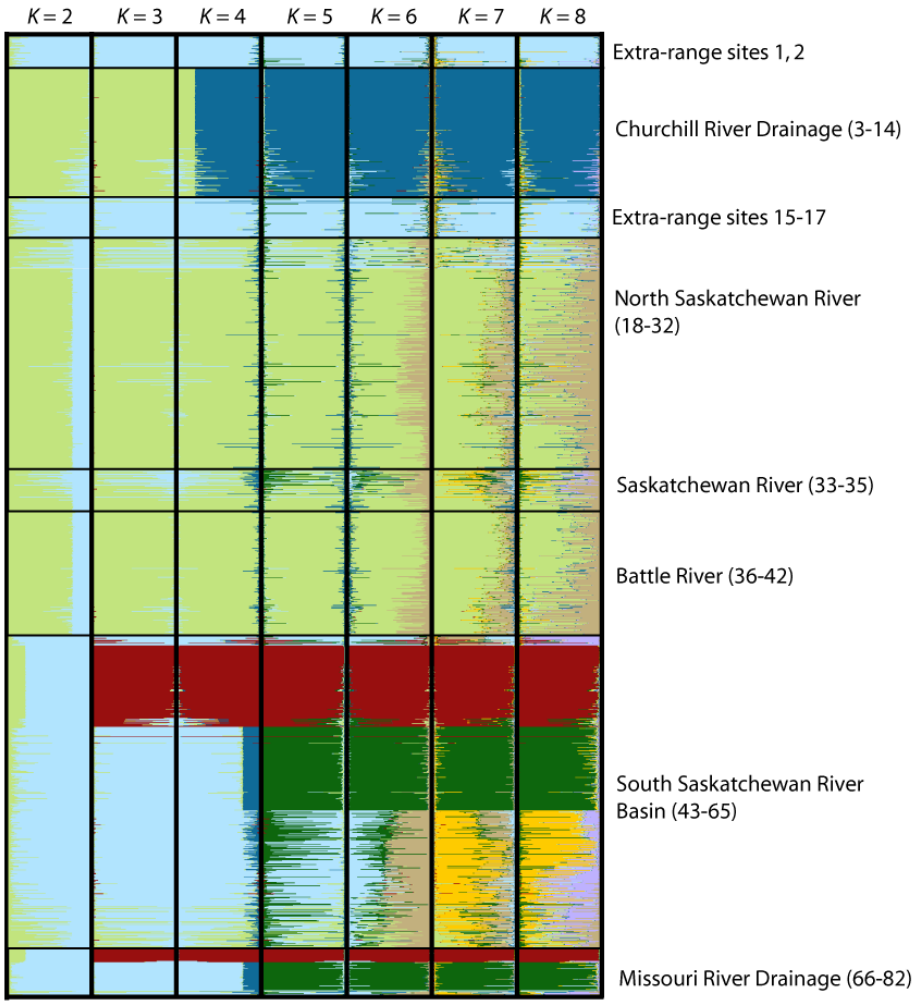
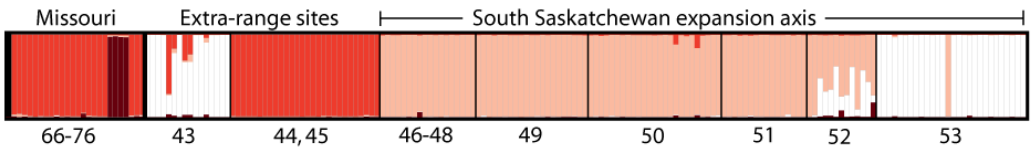


Appendix 5-Ia. Population genetic structure of 1354 individuals from 83 sites estimated by program Structure for successive inferred clusters (K = 2 – 8). Individual crayfish are each shown as a single line, with length of each coloured segment corresponding to posterior probability of cluster assignment. No further clustering was observed beyond K = 7. Black lines separate rivers, labeled to the right of the figure, along with site numbers corresponding to Figure 5-1 and Table 5-1. Sites are ordered numerically from top to bottom within each river division.



Appendix 5-Ib. Additional population genetic structure identified by iterative runs of program Structure. Site numbers correspond to Figure 5-1, Table 5-1, and Appendix 5-Ia.

Appendix 5-IIa. (following page) Pairwise F_{ST} matrix for sites along the North Saskatchewan River expansion axis and including disjunct site comparisons. Significant values are indicated in bold. Negative F_{ST} values have been standardized to 0.000.

	North Saskatchewan River expansion axis																		Disjunct sites					
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	1	2	16	17	44	46
18	—																							
19	0.024	—																						
20	0.008	0.076	—																					
21	0.011	**0.078**	0.000	—																				
22	0.018	**0.074**	0.000	0.000	—																			
23	0.023	**0.094**	0.000	0.000	0.000	—																		
24	0.021	**0.080**	0.000	0.002	0.014	0.002	—																	
25	0.001	0.049	0.000	0.000	0.003	0.006	0.001	—																
26	0.012	**0.059**	0.001	0.006	0.004	0.016	0.013	0.000	—															
27	0.012	0.051	0.000	0.007	0.009	0.011	0.005	0.000	0.000	—														
28	0.037	0.068	0.021	0.034	0.040	0.021	0.001	0.002	0.023	0.001	—													
29	0.030	**0.075**	0.012	0.013	0.032	0.015	0.000	0.002	0.025	0.015	0.000	—												
30	0.007	**0.067**	0.000	0.000	0.003	0.006	0.000	0.000	0.015	0.012	0.013	0.000	—											
31	0.025	**0.081**	0.000	0.000	0.002	0.002	0.000	0.005	0.016	0.015	0.021	0.000	0.000	—										
32	0.031	**0.087**	0.000	0.001	0.008	0.000	0.000	0.005	**0.025**	0.015	0.008	0.000	0.000	0.000	—									
33	0.022	**0.022**	0.030	**0.040**	**0.036**	**0.047**	**0.037**	0.017	**0.023**	0.010	0.017	0.033	**0.035**	**0.045**	**0.040**	—								
34	0.005	0.025	0.014	0.020	0.013	0.026	0.018	0.002	0.011	0.007	0.018	0.021	0.015	0.023	0.020	0.000	—							
35	0.026	**0.079**	0.023	0.031	0.046	0.031	0.004	0.025	**0.045**	0.040	0.032	0.009	0.004	0.003	0.017	**0.061**	0.036	—						
1	**0.173**	**0.114**	**0.264**	**0.274**	**0.276**	**0.287**	**0.274**	**0.229**	**0.247**	**0.234**	**0.254**	**0.268**	**0.259**	**0.284**	**0.283**	**0.198**	**0.208**	**0.260**	—					
2	**0.145**	**0.066**	**0.253**	**0.262**	**0.261**	**0.281**	**0.257**	**0.203**	**0.222**	**0.205**	**0.224**	**0.248**	**0.240**	**0.268**	**0.267**	**0.138**	**0.160**	**0.242**	**0.074**	—				
16	**0.138**	**0.055**	**0.231**	**0.244**	**0.238**	**0.257**	**0.241**	**0.187**	**0.197**	**0.184**	**0.205**	**0.230**	**0.228**	**0.249**	**0.252**	**0.136**	**0.150**	**0.232**	**0.057**	0.024	—			
17	**0.112**	**0.055**	**0.191**	**0.207**	**0.205**	**0.219**	**0.207**	**0.161**	**0.173**	**0.155**	**0.176**	**0.202**	**0.194**	**0.218**	**0.218**	**0.118**	**0.136**	**0.201**	**0.046**	**0.066**	0.024	—		
44	**0.227**	**0.168**	**0.316**	**0.313**	**0.316**	**0.341**	**0.319**	**0.253**	**0.270**	**0.265**	**0.285**	**0.294**	**0.297**	**0.334**	**0.319**	**0.199**	**0.214**	**0.321**	**0.223**	**0.193**	**0.170**	**0.187**	—	
46	**0.705**	**0.642**	**0.779**	**0.754**	**0.766**	**0.770**	**0.749**	**0.726**	**0.712**	**0.722**	**0.746**	**0.754**	**0.751**	**0.764**	**0.761**	**0.683**	**0.722**	**0.749**	**0.614**	**0.699**	**0.642**	**0.612**	**0.680**	—

Appendix 5-IIb. Pairwise F_{ST} matrix for sites along the Battle River expansion axis and including disjunct site comparisons. Significant values are indicated in bold. Negative F_{ST} values have been standardized to 0.000.

	Battle River expansion axis														Disjunct sites					
	36	37	38	39	40	42	43	29	30	31	32	33	34	35	1	2	16	17	44	46
36	—																			
37	0.000	—																		
38	0.000	0.000	—																	
39	0.000	0.000	0.000	—																
40	0.003	0.000	0.005	0.000	—															
42	0.013	0.001	0.005	0.008	0.003	—														
43	**0.010**	0.009	**0.023**	**0.013**	0.017	0.024	—													
29	0.019	0.000	0.007	0.007	0.002	0.003	0.026	—												
30	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.000	—											
31	0.000	0.000	0.000	0.000	0.000	0.001	**0.016**	0.000	0.000	—										
32	0.000	0.000	0.003	0.000	0.000	0.006	**0.021**	0.000	0.000	0.000	—									
33	**0.041**	**0.040**	**0.053**	**0.044**	**0.052**	**0.035**	**0.021**	0.033	**0.035**	**0.045**	**0.040**	—								
34	0.014	0.019	**0.031**	0.020	0.025	0.012	0.011	0.021	0.015	0.023	0.020	0.000	—							
35	0.029	0.004	0.006	0.015	0.009	0.000	**0.046**	0.009	0.004	0.003	0.017	**0.061**	0.036	—						
1	**0.272**	**0.274**	**0.272**	**0.290**	**0.300**	**0.243**	**0.257**	**0.268**	**0.259**	**0.284**	**0.283**	**0.198**	**0.208**	**0.260**	—					
2	**0.258**	**0.257**	**0.262**	**0.278**	**0.288**	**0.220**	**0.228**	**0.248**	**0.240**	**0.268**	**0.267**	**0.138**	**0.160**	**0.242**	**0.074**	—				
16	**0.236**	**0.239**	**0.245**	**0.257**	**0.263**	**0.208**	**0.207**	**0.230**	**0.228**	**0.249**	**0.252**	**0.136**	**0.150**	**0.232**	**0.057**	0.024	—			
17	**0.207**	**0.208**	**0.212**	**0.224**	**0.232**	**0.180**	**0.183**	**0.202**	**0.194**	**0.218**	**0.218**	**0.118**	**0.136**	**0.201**	**0.046**	**0.066**	0.024	—		
44	**0.306**	**0.316**	**0.319**	**0.336**	**0.355**	**0.294**	**0.276**	**0.294**	**0.297**	**0.334**	**0.319**	**0.199**	**0.214**	**0.321**	**0.223**	**0.193**	**0.170**	**0.187**	—	
46	**0.763**	**0.767**	**0.748**	**0.770**	**0.773**	**0.726**	**0.707**	**0.754**	**0.751**	**0.764**	**0.761**	**0.683**	**0.722**	**0.749**	**0.614**	**0.699**	**0.642**	**0.612**	**0.680**	—

Appendix 5-IIc. (following page) Pairwise F_{ST} matrix for sites along the South Saskatchewan River expansion axis including disjunct sites. Comparisons among clusters identified by Structure are provided above the diagonal. Significant values are indicated in bold. Negative F_{ST} values have been standardized to 0.000.

	Upper South Saskatchewan cluster					Mid South Saskatchewan cluster				Lower South Saskatchewan cluster												Disjunct sites					
	47	50	51	52	53	54	55	56	57	58	59	61	62	63	64	65	66	33	34	35	1	2	16	17	44	46
47	—																									
50	0.000	—																								
51	0.014	0.008	—				**0.424**							**0.390**							**0.384**	**0.393**	**0.380**	**0.369**	**0.366**	**0.359**
52	0.034	0.027	0.027	—																						
53	**0.110**	**0.105**	**0.094**	0.047	—																					
54	**0.458**	**0.451**	**0.433**	**0.427**	**0.235**	—																				
55	**0.501**	**0.493**	**0.476**	**0.475**	**0.280**	0.000	—							**0.100**							**0.268**	**0.145**	**0.153**	**0.203**	**0.240**	**0.663**
56	**0.497**	**0.488**	**0.471**	**0.466**	**0.265**	0.023	0.005	—																		
57	**0.448**	**0.439**	**0.430**	**0.426**	**0.265**	**0.160**	**0.165**	**0.169**	—																	
58	**0.462**	**0.454**	**0.442**	**0.440**	**0.279**	**0.138**	**0.145**	**0.167**	0.029	—																
59	**0.525**	**0.520**	**0.505**	**0.511**	**0.362**	**0.169**	**0.165**	**0.205**	**0.125**	**0.105**	—															
61	**0.429**	**0.429**	**0.417**	**0.414**	**0.255**	**0.151**	**0.163**	**0.197**	**0.117**	**0.115**	**0.069**	—														
62	**0.397**	**0.397**	**0.386**	**0.377**	**0.214**	**0.127**	**0.144**	**0.170**	**0.078**	**0.082**	**0.051**	0.000	—													
63	**0.420**	**0.416**	**0.400**	**0.396**	**0.231**	**0.108**	**0.131**	**0.164**	**0.096**	**0.095**	**0.079**	0.022	0.001	—							**0.174**	**0.092**	**0.102**	**0.095**	**0.169**	**0.581**
64	**0.439**	**0.430**	**0.415**	**0.408**	**0.241**	**0.100**	**0.121**	**0.152**	**0.093**	**0.083**	**0.096**	**0.057**	0.029	0.000	—											
65	**0.423**	**0.416**	**0.400**	**0.393**	**0.217**	**0.095**	**0.112**	**0.137**	**0.111**	**0.108**	**0.126**	0.066	0.037	0.008	0.001	—										
66	**0.464**	**0.454**	**0.437**	**0.433**	**0.258**	**0.141**	**0.153**	**0.178**	**0.127**	**0.132**	**0.152**	**0.119**	**0.076**	0.054	0.027	0.007	—									
33	**0.461**	**0.450**	**0.431**	**0.428**	**0.243**	**0.109**	**0.121**	**0.140**	**0.132**	**0.142**	**0.169**	**0.144**	**0.099**	**0.069**	**0.039**	0.011	0.000	—								
34	**0.477**	**0.463**	**0.442**	**0.441**	**0.255**	**0.162**	**0.177**	**0.185**	**0.156**	**0.189**	**0.218**	**0.190**	**0.140**	**0.107**	**0.070**	0.045	0.011	0.000	—							
35	**0.529**	**0.516**	**0.491**	**0.496**	**0.314**	**0.254**	**0.259**	**0.253**	**0.261**	**0.302**	**0.322**	**0.304**	**0.251**	**0.226**	**0.182**	**0.129**	**0.081**	**0.061**	0.036	—						
1	**0.430**	**0.420**	**0.410**	**0.398**	**0.270**	**0.244**	**0.265**	**0.249**	**0.158**	**0.213**	**0.253**	**0.200**	**0.163**	**0.184**	**0.182**	**0.189**	**0.209**	**0.198**	**0.208**	**0.260**	—					
2	**0.454**	**0.447**	**0.432**	**0.425**	**0.256**	**0.137**	**0.147**	**0.143**	**0.107**	**0.142**	**0.165**	**0.129**	**0.097**	**0.125**	**0.111**	**0.123**	**0.150**	**0.138**	**0.160**	**0.242**	**0.074**	—				
16	**0.433**	**0.424**	**0.411**	**0.401**	**0.242**	**0.138**	**0.154**	**0.142**	**0.102**	**0.153**	**0.180**	**0.137**	**0.111**	**0.113**	**0.105**	**0.117**	**0.153**	**0.136**	**0.150**	**0.232**	**0.057**	0.024	—			
17	**0.407**	**0.400**	**0.389**	**0.379**	**0.234**	**0.176**	**0.195**	**0.190**	**0.110**	**0.155**	**0.163**	**0.113**	**0.082**	**0.096**	**0.100**	**0.100**	**0.123**	**0.118**	**0.136**	**0.201**	**0.046**	**0.066**	0.024	—		
44	**0.442**	**0.426**	**0.405**	**0.399**	**0.252**	**0.212**	**0.251**	**0.250**	**0.203**	**0.234**	**0.291**	**0.223**	**0.186**	**0.185**	**0.166**	**0.177**	**0.210**	**0.199**	**0.214**	**0.321**	**0.223**	**0.193**	**0.170**	**0.187**	—	
46	**0.489**	**0.475**	**0.413**	**0.470**	**0.482**	**0.684**	**0.705**	**0.712**	**0.692**	**0.717**	**0.706**	**0.692**	**0.672**	**0.672**	**0.662**	**0.654**	**0.687**	**0.683**	**0.722**	**0.749**	**0.614**	**0.699**	**0.642**	**0.612**	**0.680**	—

Appendix 5-IId. Pairwise F_{ST} matrix for sites along the Churchill River. Disjunct site comparisons are provided in each matrix. Significant values are indicated in bold.

	Churchill River								Disjunct sites					
	3	4	5	6	7	10	13	14	1	2	16	17	44	46
3	—													
4	0.042	—												
5	0.045	0.000	—											
6	0.045	0.000	0.000	—										
7	0.056	0.024	0.023	0.018	—									
10	0.025	0.038	0.044	0.039	0.032	—								
13	**0.087**	**0.090**	0.103	0.086	0.072	0.051	—							
14	**0.118**	**0.106**	**0.120**	0.098	0.089	0.079	0.003	—						
1	**0.456**	**0.460**	**0.462**	**0.448**	**0.454**	**0.397**	**0.326**	**0.311**	—					
2	**0.550**	**0.553**	**0.562**	**0.537**	**0.546**	**0.457**	**0.341**	**0.313**	**0.074**	—				
16	**0.495**	**0.497**	**0.502**	**0.484**	**0.495**	**0.424**	**0.341**	**0.314**	**0.057**	0.024	—			
17	**0.435**	**0.438**	**0.441**	**0.426**	**0.433**	**0.364**	**0.297**	**0.281**	**0.046**	**0.066**	0.024	—		
44	**0.599**	**0.600**	**0.610**	**0.586**	**0.591**	**0.511**	**0.391**	**0.361**	**0.223**	**0.193**	**0.170**	**0.187**	—	
46	**0.893**	**0.891**	**0.896**	**0.888**	**0.883**	**0.861**	**0.780**	**0.762**	**0.614**	**0.699**	**0.642**	**0.612**	**0.680**	—

Appendix 5-IIe. Pairwise F_{ST} matrix for clusters identified by Structure within the Missouri River Drainage. Significance of comparisons hampered by small samples sizes of two clusters (Missouri M, Missouri E)

	Missouri W[a]	Missouri M[b]	Frenchman[c]	Missouri E[d]
Missouri W	—			
Missouri M	0.376	—		
Frenchman	0.614	0.519	—	
Missouri E	0.595	0.338	0.182	—

[a] Missouri W = sites 73-80, 82
[b] Missouri M = sites 81, 83
[c] Frenchman = sites 84, 85
[d] Missouri E = sites 87-89

Appendix 5-III. Results of Mantel tests for isolation by distance (IBD) performed for sites along the Churchill River and the three expansion axes of the North Saskatchewan, Battle, and South Saskatchewan Rivers. Provided are sites included in each set of tests (site numbers correspond to Figure 5-1, Table 5-1), the number of comparisons for each test (n), the Mantel statistic (Z), the slope and standard error of the slope (Slope s.e.), fit of the data (r^2), and significance (P-value) of the IBD relationship. Also shown are the mean F_{ST} value for the sampled sites and standard error of the mean (mean F_{ST} s.e.).

River and sites	n	Z	Slope	Slope s.e.	r^2	P	mean F_{ST}	mean F_{ST} s.e.
Churchill River Drainage								
3-7, 10, 13,14	28	0.0974	1.13	0.092	0.827	< **0.001**	0.055	
3-7	10	0.0045	2.77	0.750	0.413	**0.030**	0.025	0.007
4-7, 10	10	0.0047	1.84	0.412	0.598	**0.027**	0.022	0.005
5-7, 10, 13	10	0.0300	1.34	0.082	0.970	**0.008**	0.054	0.013
6-7, 10, 13, 14	10	0.0338	1.09	0.134	0.880	**0.015**	0.057	0.010
North Saskatchewan River expansion axis								
18-35	153	0.1284	0.87	0.070	0.044	**0.028**	0.019	
18-22	10	0.0024	-5.17	1.808	0.021	0.528	0.029	0.011
19-23	10	0.0041	5.47	1.934	0.000	0.245	0.032	0.013
20-24	10	0.0003	-0.54	0.190	0.002	0.502	0.002	0.001
21-25	10	0.0004	-0.55	0.193	0.000	0.424	0.003	0.001
22-26	10	0.0009	0.88	0.291	0.130	0.143	0.006	0.002
23-27	10	0.0008	0.95	0.231	0.531	**0.037**	0.005	0.002
24-28	10	0.0005	1.39	0.484	0.028	0.293	0.005	0.002
25-29	10	0.0009	1.89	0.601	0.196	0.109	0.007	0.003
26-30	10	0.0016	1.45	0.409	0.365	**0.047**	0.010	0.003
27-31	10	0.0013	1.22	0.286	0.559	**0.020**	0.008	0.003

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River and sites	n	Z	Slope	Slope s.e.	r^2	P	mean F_{ST}	mean F_{ST} s.e.
28-32	10	0.0008	1.00	0.316	0.198	0.170	0.004	0.002
29-33	10	0.0021	2.73	0.960	0.008	0.362	0.015	0.006
30-34	10	0.0022	2.75	0.961	0.020	0.244	0.018	0.006
31-35	10	0.0023	-3.41	1.158	0.077	0.260	0.024	0.007
Battle River expansion axis								
36-42, 29-35	91	0.0381	0.80	0.084	0.022	0.129	0.013	
36-40	10	0.0001	0.28	0.084	0.248	0.136	0.001	0.001
37-41	10	0.0003	0.47	0.160	0.079	0.269	0.002	0.001
38-42	10	0.0014	1.45	0.472	0.152	0.167	0.010	0.003
39-42, 29	10	0.0010	-1.59	0.542	0.076	0.277	0.010	0.003
40-42,29, 30	10	0.0008	-1.55	0.458	0.301	**0.002**	0.009	0.003
41, 42, 29-31	10	0.0009	-1.54	0.510	0.118	**0.034**	0.009	0.003
42, 29-32	10	0.0012	1.37	0.473	0.046	0.347	0.008	0.003
29-33	10	0.0021	2.73	0.960	0.008	0.362	0.015	0.006
30-34	10	0.0022	2.75	0.961	0.020	0.244	0.018	0.006
31-35	10	0.0023	-3.41	1.158	0.077	0.260	0.024	0.007
South Saskatchewan expansion axis								
46, 49-65, 33-35	190	2.5769	5.71	0.280	0.547	**<0.001**	0.229	
47, 50-53	10	0.0397	20.55	6.090	0.298	0.076	0.188	0.058
49-53	10	0.0371	23.00	6.620	0.338	0.057	0.185	0.059
50-54	10	0.0532	22.56	4.670	0.657	**0.005**	0.249	0.062
51-55	10	0.0505	20.11	5.520	0.397	0.07	0.222	0.061
52-56	10	0.0353	10.62	3.390	0.187	0.093	0.157	0.035

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River and sites	n	Z	Slope	Slope s.e.	r^2	P	mean F_{ST}	mean F_{ST} s.e.
53-57	10	0.0218	7.43	2.268	0.255	0.193	0.100	0.024
54-58	10	0.0206	7.72	2.307	0.286	0.066	0.128	0.020
55-58, 60	10	0.0159	9.58	1.774	0.726	**0.014**	0.130	0.018
56-58, 60, 61	10	0.0076	7.62	2.693	0.002	0.462	0.077	0.013
57, 58, 60-62	10	0.0072	7.17	2.401	0.102	0.116	0.062	0.013
58, 60-63	10	0.0051	6.27	1.482	0.553	**0.009**	0.040	0.011
60-64	10	0.0031	4.49	0.888	0.687	**0.017**	0.022	0.008
61-65	10	0.0036	4.28	0.746	0.757	**0.009**	0.024	0.008
62-65, 33	10	0.0035	4.06	0.571	0.842	**0.003**	0.022	0.008
63-65, 33, 34	10	0.0035	3.82	0.397	0.913	**0.012**	0.021	0.007
64, 65, 33-35	10	0.0050	8.25	1.731	0.648	**0.017**	0.038	0.013

Chapter 6. Distribution and first reports of Branchiobdellida (Annelida: Clitellata) on crayfish in the Prairie Provinces of Canada[1]

6.1 ABSTRACT

Orconectes virilis (northern crayfish) were collected from 67 sites in Alberta, Saskatchewan, Manitoba, and far-western Ontario, Canada, and yielded two species of branchiobdellidans, *Cambarincola vitreus* and *C. chirocephalus*. This is the first report of branchiobdellidans in the Prairie Provinces of Canada. *Cambarincola vitreus* was distributed across the study area, but *C. chirocephalus* appeared to be restricted to southeastern Saskatchewan, southern Manitoba, and the site in western Ontario. Neither branchiobdellidan species was observed on crayfish in the Beaver River nor the South Saskatchewan River and associated tributaries upstream (west) of Saskatoon, Saskatchewan, despite multiple sampling at these locations.

KEY WORDS: crayfish worm, *Cambarincola* spp., *Cambarincola vitreus*, *Cambarincola chirocephalus*, *Orconectes virilis*, host-symbiont distribution

6.2 MAIN TEXT

Branchiobdellidans, or crayfish worms, are obligate ectosymbionts primarily of astacoidean crayfishes (Brinkhurst and Gelder 2001). The distribution of branchiobdellidans in North America extends from Costa Rica north to the general area along the Canadian-USA border (Gelder et al. 2002). Reports of branchiobdellidans in Canada are restricted to southern British Columbia (Gelder and Hall 1990), the St Lawrence River drainage extending from Lake Erie and Lake Ontario to the river's mouth (Gelder et al. 2001), and New Brunswick (Gelder et al. 2008). Although branchiobdellidans have not been reported in the intermediate region (western Ontario and the Prairie Provinces) of Canada, presence of potential hosts (*Orconectes* spp.) has been documented in

[1] A version of this chapter has been published. Williams, B.W., S.R. Gelder, and H. Proctor. 2009. Western North American Naturalist 69:119-124. Formatting follows this journal's guidelines.

these areas (Crocker and Barr 1968, Hamr 2002). The dearth of reports of branchiobdellidans from the Prairie Provinces is likely a result of inadequate targeted sampling (Gelder et al. 2002), or of recent westward range expansion of crayfish. Unidentified branchiobdellidans were observed on a newly established population of *Orconectes virilis* (Hagen 1870), the northern crayfish, collected from the North Saskatchewan River in Edmonton, Alberta in 2002 (H. Proctor, pers. obs.). The historical range of *O. virilis* in Alberta extended only along the Beaver River drainage from the province's eastern border with Saskatchewan (Clifford 1991); however, recent collections have demonstrated that *O. virilis* is now present in additional Albertan drainage systems in both the south and central parts of the province (Terry Clayton, Alberta Sustainable Resource Development, pers. comm).

A recent study investigated and documented the occurrence of branchiobdellidans in New Brunswick for the first time (Gelder et al. 2008), establishing the eastern distributional limit of crayfish worms in North America. The current study was initiated to establish the presence and distribution of branchiobdellidans in the Canadian Prairie Provinces with the additional intent of delineating the northern limit of branchiobdellidans in North America.

Northern crayfish were collected by 'kick-netting' for up to 2 hours per site or in deployed Gee minnow traps (Wildlife Supply Company, Buffalo, NY, USA) baited with salmon (*Oncorhynchus* sp.) and left for 12-24 h. The minnow traps were modified to accommodate crayfish by increasing entrance hole size to approximately 60 mm. Sixty-seven sites across Alberta, Saskatchewan, Manitoba, and western Ontario, near the border of Manitoba, were sampled between August 2006 and November 2007 (Fig. 6-1, Appendix 6-I). Each crayfish was separately preserved in a container of 95% ethanol. In the laboratory, the external surface and branchial chambers of each crayfish and debris at the bottom of the collection jar were examined for branchiobdellidans under a dissecting microscope. Worms were then transferred to specimen jars with fresh 95% ethanol and labeled. Stored branchiobdellidans were cleared in methyl salicylate, infiltrated with Canada balsam and individually mounted on glass slides (Brinkhurst and Gelder 2001).

Microscopic examination of branchiobdellidans was performed using a compound microscope with both brightfield and differential interference contrast (DIC) illumination. Species identifications were made using Hoffman (1963).

Northern crayfish were collected from 66 sites across the Prairie Provinces and one site in far-western Ontario (Figure 6-1, Appendix 6-I). Two species of branchiobdellidans were identified from 35 sites: *Cambarincola vitreus* Ellis 1919 and *Cambarincola chirocephalus* Ellis 1919. *Cambarincola vitreus* was distributed widely across the study area, whereas *C. chirocephalus* was observed only in southeastern Saskatchewan, southern Manitoba, and far western Ontario. When *C. vitreus* and *C. chirocephalus* were found on crayfish from the same site, the species usually were cohabitant on a single crayfish. Branchiobdellidans were not detected at 32 sites, including multiple (>5) sampling locations in the Beaver River and in the South Saskatchewan River and its associated tributaries upstream (west) of Saskatoon, SK (Bow River, Oldman River). Additional rivers in Saskatchewan yielded no branchiobdellidans, but were minimally sampled (≤ 2 sites and/or ≤ 5 crayfish).

Branchiobdellidan presence and distribution in the Prairie Provinces and far-western Ontario, Canada, was unknown prior to this study. This study has established that two species of branchiobdellidans, *C. vitreus* and *C. chirocephalus*, inhabit *O. virilis* hosts in the region as a continuous extension of previously known ranges; *C. vitreus* and *C. chirocephalus* both occur widely throughout the Mississippi River drainage (Hoffman 1963, Gelder et al. 2002) and have been described from northern Great Lakes and Great Plains states (Gelder et al. 2002).

The northern crayfish was the only crayfish collected during this study despite records of additional *Orconectes* spp. in Manitoba and Ontario (Hamr 2002). Branchiobdellidans may exhibit a host preference when more than one host species is present (Brown and Creed 2004). Therefore, examinations of different crayfish species in the region may reveal additional branchiobdellidan species.

Non-detection of branchiobdellidans in many sampled waterways does not necessarily indicate true absence. The number of sampling locations and

specimens collected were limited in certain areas. However, the lack of observations in intensively sampled river systems suggests branchiobdellidan distribution may not be entirely coincident with host distribution. No branchiobdellidans were found on 47 crayfish examined from six sites along the Beaver River, the known northern limit of *O. virilis* distribution in Alberta and western Saskatchewan (Clifford 1991, I. Phillips, Saskatchewan Watershed Authority, pers. comm.). Although additional sampling is needed in the Beaver River and its mainstream, the Churchill River, absence of branchiobdellidans might result from an inability of the ectosymbionts to tolerate environmental conditions of northern latitudes (e.g., low temperatures, short ice-free season). In contrast, absence of branchiobdellidan observations in the South Saskatchewan River and associated tributaries upstream of Saskatoon is possibly related to the presence of two dams in central Saskatchewan. *Cambarincola vitreus* occurs on *O. virilis* downstream of the Gardiner Dam in the South Saskatchewan River, C. *chirocephalus* was found on crayfish downstream of the Qu'Appelle River Dam in the Qu'Appelle River, but neither species was detected on any of the 200 crayfish examined from 8 sites upstream of the dams (Figure 6-1).

Distribution patterns of branchiobdellidans can further our understanding of dispersal patterns of crayfish, as branchiobdellidan movement is contingent upon movement of the host. In addition, combined host-symbiont distribution patterns may provide insight into ecological interactions between the associated organisms (Whiteman et al. 2004). The records provided in the present study will form the basis for future analyses of branchiobdellidan-crayfish relationships, including reasons for concomitant versus crayfish-only westward dispersal.

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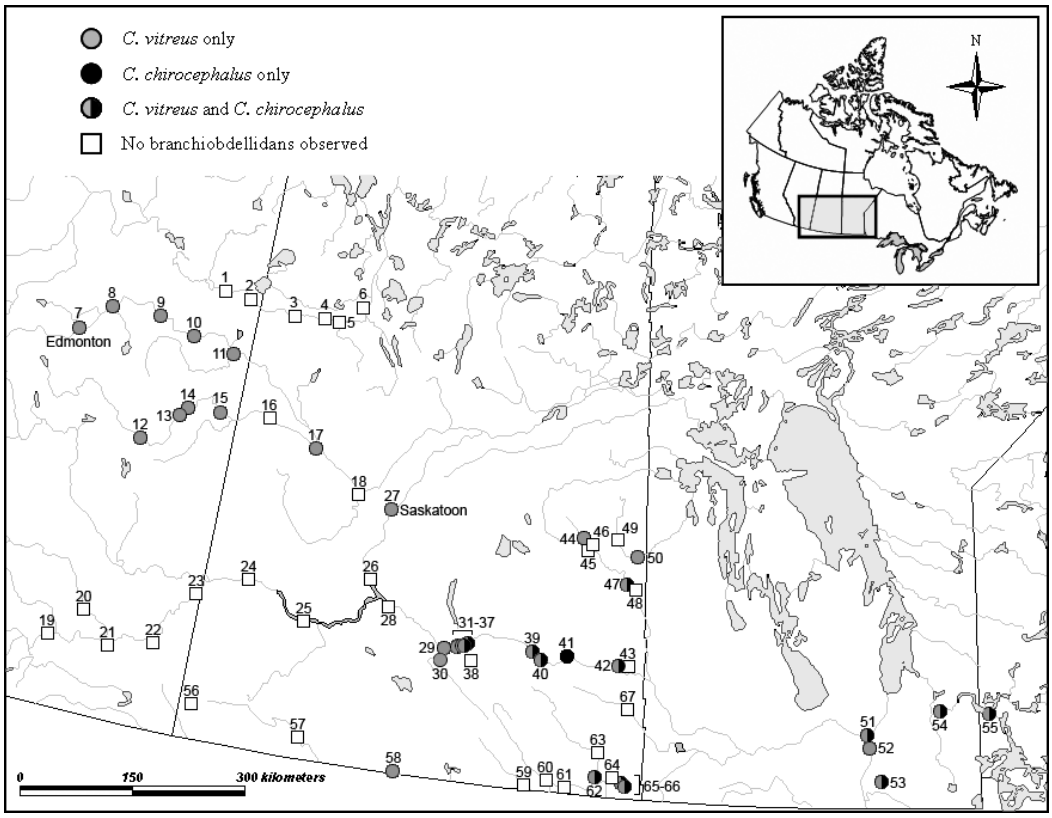

Figure 6-1. Sampling locations of *Orconectes virilis* in Alberta, Saskatchewan, Manitoba, and Ontario showing detection or nondetection of two branchiobdellidan species, *Cambarincola vitreus* and *C. chirocephalus*. Numbers correspond to locations listed in Appendix 6-I.

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APPENDIX 6-I

Detection (+) or non-detection (-) of two branchiobdellidan species, *Cambarincola vitreus* and *C. chirocephalus*, from *O. virilis* collection sites in Alberta, Saskatchewan, Manitoba, and Ontario, Canada, with information on the number of hosts examined from each site and date collected (month and year). Site numbers correspond to locations in Figure 6-1.

Site #	Coordinates	Location	# crayfish examined	Date	*C. vit.*	*C. chir.*
1	54.389°N 110.755°W	Beaver River, Rte. 41, AB	21	Jul-07	-	-
2	54.355°N 110.216°W	Beaver River, Rte. 28, AB	20	Jul-07	-	-
3	54.260°N 109.221°W	Beaver River, Rte. 26, SK	3	Sep-07	-	-
4	54.296°N 108.602°W	Beaver River, Hwy 4, SK	1	Sep-06	-	-
5	54.510°N 107.868°W	Beaver River, Rte. 155, SK	1	Sep-07	-	-
6	54.296°N 108.302°W	Beaver River, Rte. 903, SK	1	Sep-07	-	-
7	53.530°N 113.521°W	North Saskatchewan River, Edmonton, AB	3	Sep-07	+	-
8	53.888°N 112.974°W	North Saskatchewan River, Rte 38, AB	18	Aug-07	+	-
9	53.906°N 111.963°W	North Saskatchewan River, Desjarlais Crossing, AB	16	Aug-07	+	-
10	53.755°N 111.216°W	North Saskatchewan River, Rte. 881, AB	20	Aug-07	+	-
11	53.659°N 110.337°W	North Saskatchewan River, Rte. 897, AB	14	Aug-07	+	-
12	52.409°N 111.810°W	Battle River, Rte. 36, AB	20	Aug-07	+	-
13	52.786°N 111.144°W	Battle River, Wainright, AB	17	Jun-07	+	-
14	52.893°N 111.007°W	Battle River, Fabyan, AB	12	Jun-07	+	-
15	52.918°N 110.334°W	Battle River, Rte. 897, AB	24	Sep-07	+	-

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Site #	Coordinates	Location	# crayfish examined	Date	*C. vit.*	*C. chir.*
16	52.975°N 109.343°W	Battle River, Lilydale, SK	1	Sep-06	-	-
17	52.718°N 108.310°W	Battle River, N.B., SK	5	Sep-06	+	-
18	52.323°N 107.402°W	Eagle Creek, Hwy. 398, SK	2	Jul-06	-	-
19	49.856°N 112.625°W	Oldman River, Rte. 845, AB	17	Aug-07	-	-
20	50.246°N 112.079°W	Bow River, Rte. 36, AB	20	Sep-07	-	-
21	49.904°N 111.476°W	South Saskatchewan River, Rte. 879, AB	25	Aug-07	-	-
22	50.045°N 110.674°W	South Saskatchewan River, Medicine Hat, AB	16	Aug-07	-	-
23	50.732°N 110.075°W	South Saskatchewan River, Rte. 41, AB	29	Aug-07	-	-
24	51.024°N 109.134°W	South Saskatchewan River, Lemsford Ferry, SK	36	Aug-07	-	-
25	50.656°N 107.975°W	South Saskatchewan River, Saskatchewan Landing Provincial Park, SK	27	Oct-07	-	-
26	51.279°N 106.845°W	South Saskatchewan River, Danielson Park, SK	30	Sep-07	-	-
27	52.137°N 106.646°W	South Saskatchewan River, Saskatoon, SK	12	Jul-06	+	-
28	50.985°N 106.416°W	Qu'Appelle River, Hwy 14, SK	3	Jul-06	-	-
29	50.570°N 105.281°W	Qu'Appelle River, Buffalo Pound, SK	12	Aug-06	+	+
30	50.436°N 105.304°W	Moosejaw River, Tosbror, SK	16	Sep-06	+	-
31	50.620°N 105.032°W	Qu'Appelle River, HighHill, SK	8	Sep-06	+	+
32	50.630°N 105.008°W	Qu'Appelle River, Disley, SK	5	Sep-06	+	+
33	50.642°N 104.928°W	Qu'Appelle River, Shotgun, SK	14	Aug-06	+	+
34	50.645°N 104.886°W	Qu'Appelle River, west of Lumsden, SK	1	Jul-06	-	-

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Site #	Coordinates	Location	# crayfish examined	Date	*C. vit.*	*C. chir.*
35	50.645°N 104.886°W	Qu'Appelle River, Wong's Rapids, SK	6	Aug-06	+	+
36	50.651°N 104.874°W	Qu'Appelle River, Lumsden, SK	1	Jun-06	+	-
37	50.675°N 104.831°W	Qu'Appelle River, Market Gardens, SK	14	Aug-06	+	+
38	50.477°N 104.709°W	Wascana Creek, SK	5	Aug-07	-	-
39	50.661°N 103.600°W	Qu'Appelle River, Katepwa, SK	5	Aug-06	+	+
40	50.573°N 103.412°W	Qu'Appelle River, Range Road 105, SK	5	Aug-06	+	+
41	50.641°N 102.912°W	Qu'Appelle River, Hwy 47, SK	7	Aug-06	-	+
42	50.579°N 101.950°W	Kaposvar Creek, Hazel Cliffe, SK	2	Sep-06	+	+
43	50.590°N 101.740°W	Cut Arm Creek, Hwy. 8, SK	1	Sep-06	-	-
44	52.086°N 102.814°W	Assinaboine River, Ketchim, SK	6	Sep-06	+	-
45	51.940°N 102.719°W	Conjouring Creek, Preeceville, SK	3	Sep-06	-	-
46	52.023°N 102.625°W	Lillian River, Lady Lake, SK	7	Sep-06	-	-
47	51.565°N 101.917°W	Assinaboine River, Kamsack, SK	5	Sep-06	+	+
48	51.521°N 101.713°W	Little Boggy Creek, Runnymeade, SK	1	Sep-06	-	-
49	52.115°N 102.139°W	Swan River, Swan Plain, SK	2	Sep-06	-	-
50	51.907°N 101.719°W	Swan River, Arran, SK	2	Sep-06	+	-
51	49.876°N 097.232°W	Assiniboine River, Assiniboine Park, MB	6	Oct-07	+	+
52	49.723°N 097.173°W	LaSalle River, LaBarriere Park, MB	2	Oct-07	+	-
53	49.317°N 096.945°W	Rat River, St. Malo, MB	12	Sep-07	+	+

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Site #	Coordinates	Location	# crayfish examined	Date	*C. vit.*	*C. chir.*
54	50.160°N 095.867°W	Pinawa diversion, Pinawa, MB	1	Sep-07	+	+
55	50.120°N 094.944°W	Winnipeg River, ON	6		+	+
56	49.412°N 109.744°W	Battle Creek, Rd. 615, SK	1	Oct-06	-	-
57	49.250°N 107.717°W	Frenchman River, Val Marie, SK	14	Jun-07	-	-
58	49.030°N 105.894°W	Poplar River, Lacordaire, SK	1	Sep-06	+	-
59	49.063°N 103.499°W	Long Creek, Torquay, SK	1	Sep-06	-	-
60	49.145°N 103.091°W	Souris River, Raferty Dam Outflow, SK	8	Aug-06	-	-
61	49.078°N 102.753°W	Souris River, Roche Percee, SK	5	Aug-06	-	-
62	49.231°N 102.226°W	Moose Mountain Creek, Oxbow, SK	5	Aug-06	+	+
63	49.523°N 102.172°W	Moose Mountain Creek, Carlyle, SK	4	Aug-06	-	-
64	49.236°N 101.904°W	Antler River, SK	1	Jul-07	-	-
65	49.193°N 101.711°W	Antler River, Carnduff, SK	5	Jul-07	+	+
66	49.141°N 101.654°W	Antler River, south of Carnduff, SK	7	Jul-07	+	+
67	50.076°N 101.704°W	Moosomin Reservoir, SK	1	Jul-06	-	-

Chapter 7. Molecular phylogeny of North American Branchiobdellida (Annelida: Clitellata)[1]

7.1 Abstract

Branchiobdellidans, or crayfish worms, are ectosymbiotic clitellate annelids associated primarily with freshwater crayfishes. The main objectives of our study were to infer a molecular phylogeny for the North American Branchiobdellida, examine its congruence with morphology-based hypotheses of relationships at the subfamily and genus level, and use our dataset to assess consistency of GenBank-archived branchiobdellidan sequences. We used nucleotide sequence data from two mtDNA genes (COI and 16S rDNA) and three nuclear genes (28S rDNA, 18S rDNA, and ITS1) to estimate phylogenetic relationships among 47 described and one undescribed species of Branchiobdellida. We recovered a monophyletic branchiobdellidan clade with generally short branch lengths, suggesting that the taxon has likely undergone a recent and rapid radiation in North America. Results from our phylogenetic analyses indicate that current taxonomic groupings are largely unsupported by the molecular data. All four subfamilies are either paraphyletic or polyphyletic, and only three of six sampled non-monotypic genera were monophyletic. We found a high rate (49%) of inconsistency in GenBank-archived sequences, over 70% of which can be attributed to field- or laboratory-based error.

Keywords: branchiobdellidan, crayfish worm, ectosymbiont, evolution, GenBank error

[1] A version of this chapter has been submitted for publication. Williams, B.W., S.R. Gelder, H.C. Proctor, and D.W. Coltman. In review. Molecular Phylogenetics and Evolution.

7.2 Introduction

Branchiobdellidans, or crayfish worms, are a group of small (1-12 mm long) ectosymbiotic clitellate worms associated with freshwater crustaceans, primarily crayfishes (Gelder, 1999). The symbiotic association is obligate, in which branchiobdellidan embryonic development is viable only when cocoons are deposited on live hosts. The Branchiobdellida is unusual within the class Clitellata in that no free-living members are known among the approximately 140 described species (Gelder, 2010). The branchiobdellidan-host symbiosis is common across much of the Holarctic, including North and Central America, East Asia, and the Euro-Mediterranean region (Fard and Gelder, 2011; Gelder, 1999). Despite their widespread distribution, and the ecological and economic importance of their crayfish hosts, many aspects of branchiobdellidan ecology and evolution remain obscure.

Much of our understanding of branchiobdellidan phylogeny has resulted from studies focused on elucidating higher relationships in Clitellata (e.g., Brinkhurst and Gelder, 1989; McHugh, 2000; Martin et al., 2000; Martin, 2001), Annelida (e.g., Moon et al., 1996), and Metazoa (e.g., Kim et al., 1996, Winnepenninckx et al., 1998). In particular, considerable attention has been paid to the relative phylogenetic placement of branchiobdellidans, leeches (Hirudinea), and acanthobdellidans (Acanthobdellida) within the Clitellata (e.g., Brinkhurst, 1994,1999; Brinkhurst and Gelder, 1989; Grube, 1851; Holt, 1965; Holt, 1989; Livanow, 1931; Martin et al., 2000; Martin, 2001; Michaelsen, 1919; Purshke et al., 1993; Siddall et al., 2001). While sister relationships among these three taxa remain unresolved, Branchiobdellida, Hirudinea, and Acanthobdellida are currently considered to be of equivalent ordinal ranking based on molecular data (Erséus and Källersjö, 2004; Kaygorodova and Sherbakov, 2006; Martin, 2001; Rousset et al., 2008; Siddall et al., 2001) and sperm ultrastructural characteristics (Ferraguti and Erséus, 1999).

Despite the interest in clitellate phylogeny and systematics, few studies have examined phylogenetic relationships within the Branchiobdellida, which currently consists of one family, four subfamilies, and 22 genera (Gelder, 2010,

2011). Initial phylogenetic assessments were largely intuitive arrangements reflective of taxonomy (Holt, 1968, 1986). Subsequent attempts at phylogenetic reconstruction within the Branchiobdellida have utilized morphological characters (Gelder and Brinkhurst, 1990), spermatological characters (Cardini and Ferraguti, 2004; Cardini et al., 2000), and molecular markers (Gelder and Siddall, 2001), each approach resulting in relatively low resolution and branch-support among most included taxa. The single-gene phylogenies recovered by Gelder and Siddall (2001) based on 18S rDNA and cytochrome c oxidase subunit I (COI) were each largely phylogenetically uninformative, and relative placement of several taxa differed greatly between trees and from relationships suggested by independent non-molecular phylogenetic datasets (e.g., Cardini and Ferraguti, 2004; Gelder and Brinkhurst, 1990). These discrepancies could be due to independent evolutionary histories of each gene tree (e.g., Maddison, 1997) and/or homoplasy, the latter suggested as an explanation for consistently low support for phylogenetic relationships within the Branchiobdellida (Cardini and Ferraguti, 2004). Alternatively, inconsistencies between the gene trees of Gelder and Siddall (2001) could indicate problems with the sequences themselves.

Public-access repositories for molecular sequences, such as GenBank (Benson et al., 2012), are invaluable resources for ecological and evolutionary research. However, several publications have demonstrated that errors are widespread in both archived raw data and associated annotations (Bridge et al., 2003; Harris, 2003; Nilsson et al., 2006; Valkiūnas et al., 2008). Errors in published sequences can be particularly problematic for understudied taxa where original data from a small number of studies are likely to be re-used in future analyses. In the absence of internal and/or external quality control, mistakes in original sequence data, and thus erroneous inferences resulting from those data, can be perpetuated across numerous studies and over long periods of time. The effect of any given error depends on its nature (e.g., misidentification or mis-archiving of associated information, contamination, methodological, analytical) and the context or questions asked with the erroneous data. Branchiobdellidan sequences have been produced by relatively few studies (i.e., Apakupakul et al.,

1999; Füreder et al., 2009; Gelder and Siddall, 2001; Jamieson et al., 2002; Kim et al., 1996; Moon et al., 1996; Rosenwarne et al., In press; Siddall and Burreson, 1998), and even fewer laboratories, yet have been included in nearly 20 published molecular analyses. Many branchiobdellidan species have patchy or limited distributions. In addition, species identification may be considered challenging based on a general lack of diagnostic external or sclerotized characters. Consequently, collection and identification of branchiobdellidans might not be highly tractable to many researchers interested in including representatives of the taxon in a broader study, and such researchers instead trust the veracity of GenBank identifications. Testing this veracity is rendered difficult because none of the original studies provide evidence that voucher specimens were deposited in publically accessible collections, or whether multiple individuals of the 'same' species were examined for evidence of cryptic species-level diversity (the latter being likely in externally homogenous organisms). The common use of archived branchiobdellidan sequences, the lack of explicitly stated quality control methods in the original studies, and a lack of taxon and sequence replication underscore the need for a critical evaluation of archived molecular data for this group.

In the present study, we focus on the North American branchiobdellidans, which comprise approximately two-thirds of the total number of described species Holarctic-wide and 16 of the 22 genera, including all 10 genera in the most genus-rich subfamily, Cambarincolinae (Gelder, 2010, 2011). Despite this diversity, few North American taxa have been studied beyond documentation of morphological characteristics used in species descriptions. We provide a molecular-based phylogenetic hypothesis for North American branchiobdellidans using the most comprehensive taxon sampling (47 described species representing 13 genera and all four subfamilies) and gene sampling for Branchiobdellida to date. In addition, we assess concordance of our dataset with the existing morphology-based hypotheses of relationships at the subfamily and genus level. A final objective of our study is to evaluate consistency and accuracy in all GenBank-archived North American branchiobdellidan sequences relative to nominal conspecifics in our dataset.

7.3 Material and methods

7.3.1. Sampling, taxon identification, and outgroup selection

Crayfish hosts were collected by hand, kick-net, or seine from several locales across the United States and Canada (Table 7-1). In most cases, live worms were removed from their hosts, identified to species using a wet mount technique under a light microscope (Gelder, 2010), and transferred to 95% ethanol for preservation. Where branchiobdellidans were collected from crayfish already preserved in 70 – 95% ethanol, we removed the posterior 2-3 segments of each worm for DNA analysis. The remainder of each branchiobdellidan was cleared in methyl-salicylate, mounted in Canada balsam, and identified under differential interference contrast (DIC) illumination using a Nikon microscope.

We obtained all sequence data for North American branchiobdellidans available on GenBank, consisting of 39 sequences from 17 species. We collected and sequenced all but one of these species, *Pterodrilus annulatus* Gelder, 1996. Consequently, we included archived sequence data (COI and 18S rDNA) from *P. annulatus* in all of our phylogenetic analyses, but the 37 other GenBank sequences were included only in single-locus analyses to assess concordance of these sequences with our data.

Our study included a total of 47 named and one undescribed species representing all four subfamilies and 13 of the 16 currently recognized North American branchiobdellidan genera (Table 7-1). Five of the 13 sampled genera are monotypic (*Bdellodrilus*, *Cronodrilius*, *Uglukodrilus*, *Magmatodrilus*, *Triannulata*). We collected two or more species from each of seven of the eight remaining genera. We were only able to collect one of the two species of *Ceratodrilus*. Two of the unsampled genera, *Forbesodrilus* and *Tettodrilus*, are monotypic with small endemic ranges (Gelder, 2011; Holt, 1968). The third, *Ellisodrilus*, includes three species, none of which were encountered during this study despite our sampling in the drainage from which type specimens of *Ellisodrilus carronamus* Holt, 1988 were obtained (Holt, 1988).

We included five outgroup taxa, including three leeches, *Erpobdella obscura* (Verrill, 1872) (Erpobdellidae), *Helobdella stagnalis* (Linnaeus, 1758) (Glossiphoniidae), and *Glossiphonia complanata* (Linnaeus, 1758) (Glossiphoniidae), identified using the taxonomic key and descriptions in Klemm (1985), and two members of the Lumbriculidae, *Eremidrilus coyote* Fend & Rodriguez, 2003, and *Lumbriculus variegatus* (Müller, 1774). Voucher specimens of extracted individuals (hologenophores, sensu Pleijel et al., 2008) of six of the nominal branchiobdellidan species and all five outgroup taxa were retained following the protocol for preserved specimen identification described above. Paragenophores, or reference specimens collected at the same site and identified as the same species as those individuals included in this study, were retained for several additional species or identified variants. Hologenophore and paragenophore specimens for most taxa are deposited in the New Brunswick Museum (NBM), Saint John, New Brunswick, Canada (Table 7-2; Catalogue nos. pending).

7.3.2. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted either from a single whole worm or posterior end using the QIAGEN DNeasy kit (QIAGEN Inc., Valencia CA) and standard protocol. We amplified and sequenced regions of two mitochondrial genes, COI and 16S rDNA, and three nuclear genes, 28S rDNA, 18S rDNA, and internal transcribed spacer 1 (ITS1) using primers listed in Table 7-3. All sequences were amplified as single fragments with the exception of 18S rDNA, which was amplified as three overlapping segments using the primer pairs A/L, C/Y, and B/O (Apakupakul et al., 1999; Medlin et al., 1988) for the first, second and third fragments, respectively.

We performed PCR amplification in a total volume of 25 µL consisting of 1x PCR reaction buffer (10 mM Tris pH 8.8, 0.1 % Triton X-100, 50 mM KCl, 0.16 mg/ml BSA), 0.16 µM forward and reverse primers, 0.2 µM dNTPs, optimized volume of $MgCl_2$ (Table 7-3), 25-100 ng template DNA, and 0.3 U *Taq* DNA polymerase. Thermal profile for amplification was 94°C for 1 minute, 3

cycles of 94°C for 30 seconds, primer specific annealing temperature (Table 7-3) for 20 seconds, and 72°C for 5 seconds followed by 33 cycles of 94°C for 15 seconds, annealing temperature for 20 seconds, and 72°C for 1 second, with a final 72°C extension for 30 minutes. Amplified fragments were cleaned using ExoSAP-IT and subsequently sequenced and run on an ABI 3730 DNA Analyzer (Applied Biosystems).

Where possible, at least two individuals per morphospecies were extracted, amplified, and sequenced in separate batches as both a quality control measure and a test of conspecificity of morphospecies. We included a single exemplar individual in subsequent analyses, unless polyphyly of members of one morphospecies was observed in preliminary analyses.

7.3.3. Sequence alignment and partition homogeneity

We assembled and edited sequence chromatograms using SeqMan Pro (DNASTAR Inc., Madison, WI, USA). Nucleotide sequences were aligned for COI, 16S rDNA, 28S rDNA, and 18S rDNA using the L-INS-i strategy in MAFFT v.6 (online version; Katoh, 2005, 2008; Katoh et al., 2002) with a gap opening penalty of 1.53 and 0.1 offset value. Nucleotide sequences for ITS1 were independently aligned using the structural alignment strategy Q-INS-i in MAFFT under default settings. Alignments were manually checked and edited using MEGA v. 5.0 (Tamura et al., 2011).

We tested for substitution saturation of each gene using the information theory entropy index (Xia and Lemey, 2009; Xia et al., 2003) in DAMBE (Xia, 2001; Xia and Xie, 2001) with gaps considered unknown states. We assessed data congruence of the different genes using the incongruence length difference (ILD) test (Farris et al., 1994, 1995). The ILD test was conducted for all 26 gene combinations using a heuristic search with 500 replicates (TBR branch swapping and simple taxon addition). Statistically significant incongruence was evaluated at $P \leq 0.01$ based on Cunningham (1997).

7.3.4. Phylogenetic analyses

Maximum likelihood (ML) phylogenetic analysis was performed with RAxML v7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) via the CIPRES Science Gateway v. 3.1 (Miller et al., 2010). Individual sequences were partitioned and run under the GTRGAMMA model with parameters estimated separately for each partition. Statistical support for branching patterns was estimated concurrently with the tree search using RAxML rapid bootstrapping of 1000 replicates and the GTRCAT model (Stamatakis et al., 2008). We used jModelTest 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) to determine the most appropriate substitution model for each gene based on Akaike's Information criterion (AIC), and estimate parameters for Bayesian inference (BI) phylogenetic analysis. We performed partitioned BI phylogenetic analyses using MrBayes 3.2.1 (Ronquist et al., 2012). Two independent runs were executed, each with 5,000,000 generations sampled every 1000 generations. We assessed convergence of BI runs by tracking average standard deviation of split frequencies between runs and by plotting the log likelihood of sampled trees in Tracer v1.5 (Rambaut and Drummond, 2007). Stationarity was achieved at an average standard deviation of split frequencies < 0.002. We removed the first 25% of sampled trees as burnin and obtained a 50% majority-rule consensus tree from the remaining trees.

7.3.5. Single-gene analyses for data consistency

We included all GenBank-archived sequences for North American branchiobdellidans in single-locus analyses (COI, 18S rDNA, and 28S rDNA) of our data to assess consistency in sequence concordance and thus relative placement in each gene tree. Nucleotide sequences for each locus were aligned using MAFFT (L-INS-i strategy, gap opening penalty 1.53, 0.1 offset value) and visually inspected in MEGA. We calculated uncorrected p-distances among sequences in MEGA. We performed ML phylogenetic analysis for each locus using RAxML as described above for section *2.4.*

7.4 Results

7.4.1. Taxon sampling, alignment

We collected and sequenced a minimum of two individuals for all but two nominal branchiobdellidan species (*Cronodrilus ogygius* Holt, 1968 and *Pterodrilus choritonamus* Holt, 1968). Preliminary analysis showed that most morphospecies were monophyletic, and therefore were subsequently represented by a single exemplar in our phylogenetic analyses. Individuals identified as *Ankyrodrilus koronaeus* Holt, 1965, *Ankyrodrilus legaeus* Holt, 1965, *Sathodrilus inversus* (Ellis, 1919), and *Cambarincola philadelphicus* (Leidy, 1851) were each polyphyletic, and therefore represented by two (*A. koronaeus*, *A. legaeus*, *S. inversus*) or more (four: *C. philadelphicus*) exemplars. Consequently, our phylogenetic analyses included 55 individual crayfish worms and 5 outgroup species for a total of 60 terminal taxa.

We successfully obtained COI sequences for 57 terminal taxa (658 bp unambiguous alignment), 16S rDNA sequences for 59 terminals (501 bp aligned, including gaps), 28S rDNA sequences for 59 terminals (331 bp unambiguous alignment), 18S rDNA sequences for 60 terminals (1816 bp aligned, including gaps), and ITS1 sequences for 47 terminals (1522 bp aligned, including gaps). The total concatenated alignment was 4828 bp, with 2050 variable and 1468 parsimony informative sites. Accession numbers for sequences (GenBank) are provided in Table 7-2.

7.4.2. Phylogenetic analyses

None of the five loci displayed significant sequence saturation based on the information theory entropy index. Similarly, results of the ILD test indicated that no significant ($P \leq 0.01$) incongruence was exhibited by any of the 26 locus combinations (Supplementary Table 7-1). The best fit model of evolution for each gene was GTR + Γ + I (COI, 28S rDNA, 18S rDNA), TIM2 + Γ + I (16S rDNA), and GTR + Γ (ITS1). Our ML (Fig. 7-1, Supplementary Fig. 7-S1) and BI (not shown) trees were nearly identical, with two minor differences: one within the clade containing *Xironodrilus* and *Ankyrodrilus* (*Xironodrilus formosus* Ellis,

1919 (ML) versus *Xironodrilus appalachius* Goodnight, 1943 (BI) as the basal-most branch of the clade), and the other within the clade containing *Pterodrilus* and *Oedipodrilus macbaini* (Holt, 1955) (*O. macbaini* weakly supported as sister taxon to *Pterodrilus alcicornus* Moore, 1895 (ML) versus *O. macbaini* basal to a well-supported monophyletic *Pterodrilus* clade (BI)).

7.4.2.1. Phylogenetic relationships based on the combined dataset

We recovered the North American Branchiobdellida as a well-supported monophyletic group (100/0.99, bootstrap and posterior probability values, respectively) with several well-supported subclades (Fig. 7-1, Supplementary Fig. 7-S1). However, in most cases, subclades are not reflective of morphology-based taxonomic groupings. Based on these sequence data, none of the four subfamilies are monophyletic. Branchiobdellinae and Bdellodrilinae are clearly polyphyletic, and the monogeneric Xironodrilinae is paraphyletic to the branchiobdelline genus *Ankyrodrilus*. Much of the Cambarincolinae forms a strongly supported monophyletic group (96/1.00), with the exception of *Triannulata magna* Goodnight, 1940, located in a more basal position within the ingroup, and the bdellodriline *Cronodrilus ogygius* found within the larger cambarincoline clade. The two genera of the subfamily Branchiobdellinae, *Xironogiton* and *Ankyrodrilus*, are each monophyletic with strong support (100/1.00). In contrast, three of the four sampled non-monotypic genera within the Cambarincolinae are polyphyletic (*Sathodrilus*, *Cambarincola*, and *Oedipodrilus*). The ML tree shows the fourth non-monotypic cambarincoline genus, *Pterodrilus*, as weakly paraphyletic with respect to *Oedipodrilus macbaini* (bootstrap value of 65). However, the BI tree displays *O. macbaini* as the sister group to a strongly supported monophyletic *Pterodrilus* (posterior probability value of 0.99; results not shown).

7.4.2.2. Single-gene phylogenetic analysis and data consistency

We assessed congruence of the 37 GenBank-archived sequences of North American branchiobdellidans using single-locus analyses based on the

combination of p-distance values relative to nominal conspecifics in our dataset and clustering patterns within each gene tree. We found 18 sequences that were inconsistent with our conspecifics, including nine COI and nine 18S rDNA sequences (Table 7-4, Supplementary Fig. 7-S2, 7-S3). No major discrepancies were found with the two published 28S rDNA sequences. Thirteen of the identified inconsistencies can be attributed to either laboratory or field error, such as misidentification or contamination and sequencing error. The remaining five inconsistencies might be indicative of cryptic species or high intraspecific variation. Alternatively, several of these latter inconsistencies, resulting in paraphyletic relationships with our conspecifics, could be due to inter-laboratory differences in sequencing or sequence-editing methods.

7.5 Discussion

7.5.1. Phylogenetic relationships of North American Branchiobdellida

Our analyses included almost 50% of the 105 described North American branchiobdellidan species and all but three of the 16 described North American genera, providing by far the largest taxonomic representation in branchiobdellidan phylogenetic reconstruction to date. Monophyly of Branchiobdellida relative to sampled representatives from the sister taxon, Hirudinea, was strongly supported by analysis of each single gene (data not shown) and the combined data set (Fig. 7-1, Supplementary Fig. 7-S1). Branch lengths within the ingroup are generally short, suggesting that branchiobdellidans have likely undergone a recent and rapid radiation in North America.

Monophyly of branchiobdellidan subfamilies is not supported by our data (Fig. 7-1, Supplementary Fig. 7-S1), which is consistent with results of Gelder and Brinkhurst (1990) and Gelder and Siddall (2001). Further, we show strong support for paraphyly or polyphyly of all four subfamilies. The North American Branchiobdellinae consists of two genera, *Ankyrodrilus* and *Xironogiton*. Although these genera each form a well-supported monophyletic grouping, they are found in two distinctly separate clades within the ingroup. Indeed, *Ankyrodrilus* is grouped, with high support, with the two sampled species of the

subfamily Xironodrilinae. In turn, it appears that Xironodrilinae is paraphyletic, with *Xironodrilus formosus* in a basal position to *Xironodrilus appalachius* and *Ankyrodrilus*, although support for this particular topology is weak (66/0.66). The Xironodrilinae and *Ankyrodrilus* are morphologically very similar, and are indistinguishable based on external characters and host microhabitat; however, the single character used to differentiate the two genera (location of entry of the vasa deferentia into the glandular atrium) currently results in their placement in separate subfamilies. Cambarincolinae is also clearly not monophyletic, with the monotypic *Triannulata magna* separated from a single large clade containing all remaining sampled representatives of the subfamily. The three monotypic genera comprising Bdellodrilinae are scattered throughout the branchiobdellidan ingroup. *Bdellodrilus illuminatus* (Moore, 1894) is one of the basal-most ingroup taxa, whereas *Uglukodrilus hemophagus* (Holt, 1977) is sister to, and *Cronodrilus ogygius* nested in, a large Cambarincolinae-dominated clade.

Only three (*Xironogiton*, *Pterodrilus*, and *Ankyrodrilus*) of the six genera with more than one sampled species are supported as monophyletic groupings by our molecular data (Fig. 7-1, Supplementary Fig. 7-S1), although *Pterodrilus* was identified as such only by the BI analysis (not shown). Monophyly of both *Pterodrilus* and *Xironogiton* have been previously suggested with analyses of sperm ultrastructure (Cardini and Ferraguti, 2004; Cardini et al., 2000), albeit with limited taxon sampling (two species for each genus). Cohesion within each of these two genera is also supported by distinctive morphological similarities, although not synapomorphies, among all representative species. All *Xironogiton* species exhibit a reduced spermatheca, and dorso-ventral flattening of posterior segments forming a flask-shaped body (Gelder and Hall, 1990). All *Pterodrilus* species share small body size (< 2 mm in length), a dorsal ridge across segment 8, and display remarkable consistency in jaw size, jaw shape, and size and shape of the male reproductive system (Gelder, 1996; Williams and Gelder, 2011). Our analyses indicate a close relationship of *Oedipodrilus macbaini* to *Pterodrilus*. Although this association is supported by a few morphological similarities, e.g., jaw structure (Holt 1955, 1967), distinctive morphological differences in male

reproductive structures and female spermathecae (Holt, 1988) have clearly separated the taxa taxonomically. The third monophyletic genus, *Ankyrodrilus* presents a pattern whereby we observe greater genetic divergence between sites than between species (Fig. 7-1, Supplementary Fig. 7-S1). This suggests that the two species of the genus, *A. koronaeus* and *A. legaeus*, may be conspecific. Indeed, these two species are separated solely by small differences in dentition (Holt, 1965), a character that is highly variable within several branchiobdellidan species (Nurminen, 1966).

Our data show that *Cambarincola* and *Sathodrilus*, the two largest North American branchiobdellidan genera with 47 and 18 described species, respectively (Gelder, 2011; Gelder et al., 2002), are clearly polyphyletic, separating into several different well-supported clades based largely on geography. Although lack of phylogenetic cohesion among *Cambarincola* species was observed in previous studies (Cardini & Ferraguti, 2004; Gelder and Siddall, 2001), taxon sampling was too sparse and branch support too weak to reveal clustering patterns. The presence of well-supported geographic rather than taxonomic clustering in our phylogeny suggests that inappropriate emphasis has been given to the morphological characters currently used to define several genera.

7.5.2. Implications for branchiobdellidan taxonomy

Our observation of a general lack of molecular support for branchiobdellidan subfamilies and also for several genera indicates a need for reexamination of the morphological characters used for taxonomy of this group. Uncertainty about morphology-based subfamily designations is not a new concern. Gelder and Brinkhurst (1990) showed that three of the four subfamilies, Branchiobdellinae, Bdellodrilinae, and Xironodrilinae, each lack synapomorphies shared by all member genera. Genera of the remaining subfamily, Cambarincolinae are defined by a single synapomorphy: the ental (= distal) position of the vasa deferentia as they enter the glandular atrium. Although Gelder and Brinkhurst (1990) retained all family (= subfamily in current

taxonomy) distinctions based on morphological similarity, they pointed out the need for detailed examination of systematic relationships. However, our study is the first to provide the taxonomic breadth and analytical support needed to critically evaluate both subfamily and genus designations. Our results suggest new phylogenetic groupings, and provide a starting point to assess the utility of morphological characters currently used for systematic rankings within Branchiobdellida.

7.5.3. Data discrepancies and cryptic diversity

We found that almost half (18/37 = 49%) of the archived sequences for North American branchiobdellidans were inconsistent with nominal conspecifics from our study; over 70% (13/18) of these discrepancies can be attributed to either field- or laboratory-based error (Table 7-4). The majority of these errors appear to be due to misidentification or mislabeling. If made prior to PCR amplification, issues of mistaken identity should be consistent across all genes assayed for the specimen. This is the pattern that we observed for sequences erroneously annotated in GenBank as *Ankyrodrilus legaeus* (GenBank ID: AF310705, AF310688), *Sathodrilus attenuatus* Holt, 1981 (GenBank ID: AF310719, AF310702), and *Cambarincola holti* Hoffman, 1963 (GenBank ID: AF116012, AF115975). In contrast, errors made during the amplification, sequencing, or post-processing stages of molecular analysis are unlikely to affect all assayed genes for a given specimen. As a result, it is important to evaluate consistency in phylogenetic patterns across gene trees. We observed clustering of three species, *Ceratodrilus ophiorhysis* Holt, 1960, *Xironodrilus formosus,* and *Magmatodrilus obscurus* (Goodnight, 1940), all based on COI sequences from Gelder and Siddall (2001), with leech outgroup species rather than within the monophyletic clade of Branchiobdellida (Supplementary Fig. 7-S2). Further, a BLAST (Altschul et al., 1990) search of the GenBank database resulted in high (93 - 98 %) sequence similarity of these three published sequences to those of several leech species. A similar association of *Ceratodrilus ophiorhysis*, *Xironodrilus formosus,* and *Magmatodrilus obscurus* to leeches was not observed

in analysis of 18S rDNA sequences. In fact, 18S rDNA sequences of the former two species were consistent with the nominal conspecifics in our dataset (Table 7-4, Supplementary Fig. 7-S3), suggesting that the original specimens were not misidentified.

Methodological error has also contributed to inconsistencies in archived branchiobdellidan sequences. Martin (2001) identified a 46-bp insertion in the two 18S rDNA sequences (GenBank ID: Z83756, *Xironogiton victoriensis* Gelder and Hall, 1990; GenBank ID: Z83755, *Sathodrilus attenuatus*) of Moon et al. (1996) and Kim et al. (1996) that has since not been observed in 18S rDNA sequences of additional branchiobdellidan species, including our own. Presence of this insertion results in long branches within the 18S rDNA gene tree (Supplementary Fig. 7-S3). If we remove the insertion from Z83756, we find that the sequence is largely consistent with our conspecific *Xironogiton victoriensis*. In contrast, Z83755 is highly inconsistent with all branchiobdellidan species, displaying a 10-fold greater genetic divergence (p-distance) from our conspecific *Sathodrilus attenuatus* than the mean genetic divergence across the entire branchiobdellidan ingroup (Table 7-4). The 18S rDNA sequence representing *Magmatodrilus obscurus* (GenBank ID: AF310699) also results in a long branch within the 18S rDNA gene tree, and shows even greater genetic divergence from its conspecific than Z83755 (Table 7-4, Supplementary Fig. 7-S3). Given these differences, it is likely that the grouping of Z83755 and AF310699 with branchiobdellidan representatives in several studies (see references in Table 7-4) is a result of long-branch attraction rather than phylogenetic similarity.

A few inconsistencies in archived branchiobdellidan sequences are likely due not to laboratory or identification error, but rather may indicate the presence of morphologically cryptic species, or as yet unrecognized intra- and interspecific variation. Branchiobdellidans have presented several taxonomic problems as a result of morphological convergence. For example, *Xironogiton instabilis* (Moore, 1894), found in eastern North America, and *X. victoriensis*, from northwestern North America, are highly morphologically similar, and for many years were not acknowledged to be different species (Holt, 1968, 1974). Sperm ultrastructure

(Cardini et al., 2000), molecular data (present study), and subtle morphological differences (Gelder and Hall, 1990) now support the distinctiveness of *X. instabilis* and *X. victoriensis*. Other species have proven to be equally taxonomically challenging. For example, *Cambarincola philadelphicus* is considered among the most problematic branchiobdellidan taxa, as it is widely distributed across much of central and eastern USA and includes several described morphological variants (Gelder et al., 2002; Hoffman, 1963). One variant of *C. philadelphicus*, found primarily in parts of Tennessee and Kentucky, closely resembles *Cambarincola chirocephalus* Ellis, 1919 (Hoffman, 1963). The latter species is also common across central North America (Gelder et al., 2002; Hoffman, 1963), although the degree of overlap between *C. philadelphicus* and *C. chirocephalus* is unknown. Our molecular data suggest that *C. philadelphicus* sensu lato comprises at least four cryptic and unrelated taxa (Fig. 7-1, Supplementary Fig. 7-S1, 7-S2). Likewise, *C. philadelphicus* sequences from Gelder and Siddall (2001) (GenBank ID: AF310713, AF310696) may represent genetic variation within a complex that includes *C. chirocephalus* and several *C. philadelphicus* variants.

The high error rate (35%) in GenBank-archived North American branchiobdellidans is greater than similar errors reported for the taxonomically problematic fungi (~ 20%; Bridge et al., 2003; Nilsson et al., 2006). These findings are concerning, particularly as several of the erroneous branchiobdellidan sequences have been subsequently used in published studies (Table 7-4). The implications of using erroneous sequence data depend on the nature of the error and the context in which the data are used, but can result in dramatic misinterpretations of taxonomic and phylogenetic relationships (e.g., Groenenberg et al., 2011), or evolutionary patterns (e.g., lineage-specific mutation rates; Martin et al., 2000). Mistaken identity between two branchiobdellidan species would likely not affect inferences made at higher systematic rankings (e.g., Kaygorodova and Sherbakov, 2006; Martin, 2001), but would bias interpretation of relationships within the Branchiobdellida. For example, the strongly supported relationship of *Sathodrilus attenuatus* and *Xironogiton victoriensis* displayed in

Figure 2 of Gelder and Siddall (2001) is not a result of hybridization and introgression, as suggested, but is an artifact of their misidentification of *Sathodrilus attenuatus* (= *Xironogiton victoriensis*). The high support of a close relationship between *Cambarincola holti* and *Cambarincola philadelphicus* sensu lato in Gelder and Siddall (2001) can likewise be explained by the misidentification of *C. holti* (= *Cambarincola philadelphicus*).

In contrast, mistaken identity between two distinctly different groups of organisms (e.g., branchiobdellidans and leeches) would affect inferences made at all systematic rankings. Monophyly of the Branchiobdellida has never been debated in a phylogenetic context, although various assumed synapomorphies have hampered attempts at inferring relationships among branchiobdellidans, leeches, acanthobdellidans, and lumbriculids based on morphological characters (review in Brinkhurst, 1999). Morphological and molecular data clearly distinguish branchiobdellidans from other clitellate annelid groups (e.g., Brinkhurst, 1994, 1999; Martin, 2001; Erséus and Källersjö, 2004). Despite this, when we incorporated GenBank-archived COI sequences into a single-gene analysis with our samples, results indicated that *Ceratodrilus ophiorhysis*, *Xironodrilus formosus*, and *Magmatodrilus obscurus* were more closely related to leeches than to other branchiobdellidans (Table 7-4, Supplementary Fig. 7-S2). In the absence of our own data as external quality control, the COI gene tree constructed using GenBank data brings into question monophyly of Branchiobdellida.

Despite efforts of several researchers to catalog the taxonomic diversity of the North American Branchiobdellida (e.g., M.M. Ellis, C.J. Goodnight, P.C. Holt, J.P. Moore), our understanding of the diversity, distribution, and ecology of these organisms remains limited. Molecular data offer much potential in the study of these and other taxonomically challenging groups, but the numerous errors in identification described above and potential for cryptic species being hidden under single names underscore the importance of stringent quality control and deposition of vouchers or properly archived reference samples in accessible collections.

7.6 Conclusions

This study provides the most complete and well-supported phylogeny for the Branchiobdellida to date. Phylogenetic patterns based on our molecular data are not consistent with current taxonomy. It is clear that more work is needed to identify additional taxonomically relevant characters (e.g., cuticle chemistry, Smith et al., 1986) and to characterize intraspecific morphological and genetic variation in the Branchiobdellida. The detection of several erroneous North American branchiobdellidan sequences in GenBank highlights the importance of quality control and voucher or reference samples, particularly in little-studied organisms.

7.7 Acknowledgements

The authors thank Steve Fend for providing specimens of *Eremidrilus coyote* and *Lumbriculus variegatus*. We also thank those who assisted with crayfish-branchiobdellidan sampling: Eric Larson and Casey Dunn (University of Tennessee, Knoxville), Julian Olden (University of Washington, Seattle), Christopher Pearl (USGS), David Withers (Tennessee Natural Heritage Program), Hannah Withers, and Carl Williams and Rick Bivens (Tennessee Wildlife Resources Agency). Logical assistance from several others made much of our sampling possible including Ralph and Kay Brinkhurst, R. Deedee Kathman (Tennessee Department of Transportation), Keith Langdon, Becky Nichols, Paul Super, Matt Kulp, and Adriean Major (National Park Service), Chuck Cooper and Chuck Parker (Discover Life in America), David Parrish and Bill Horton (Idaho Fish and Game). This study was funded by an Alberta Conservation Association Biodiversity Grant (BWW), Discover Life in America Grant (BWW, SRG), and NSERC Discovery Grants (to each of DWC and HCP).

Table 7-1. List of the 47 nominal and one unidentified branchiobdellidan species and five outgroup taxa included in the present study, indicating collection locality and host species from which removed (where known and if applicable).

Taxon	Collection locality	Host species
Branchiobdellinae Goodnight, 1940		
Ankyrodrilus koronaeus Holt, 1965	Indian Creek, TN; Flat Creek, TN	*Cambarus angularis*; *Cambarus tenebrosus*
Ankyroldrilus legaeus Holt, 1965	Indian Creek, TN: Flat Creek, TN	*Cambarus angularis*; *Cambarus tenebrosus*
Xironogiton instabilis (Moore, 1894)	Naples Creek, NY	*Cambarus bartoni*
Xironogiton kittitasi Holt, 1974	Teanaway River, WA	*Pacifastacus leniusculus*
Xironogiton occidentalis Ellis, 1919	Olalla Creek, OR	*Pacifastacus leniusculus*
Xironogiton victoriensis Gelder & Hall, 1990	Yakima River, WA	*Pacifastacus leniusculus*
Bdellodrilinae Brinkhurst & Gelder, 2001		
Bdellodrilus illuminatus (Moore, 1894)	Kennedy Brook, ME	*Cambarus bartoni*
Cronodrilus ogygius Holt, 1968 [b]	Tallapoosa River, GA	*Procambarus spiculifer*
Uglukodrilus hemophagus (Holt, 1977)	Boise River, ID	*Pacifastacus leniusculus*
Cambarincolinae Goodnight, 1940		
Cambarincola bobbi Holt, 1988	Tributary to Middle Fork Cedar Creek, TN	*Cambarus tenebrosus*
Cambarincola chirocephalus Ellis, 1919	Lake of the Woods, MN	*Orconectes virilis*
Cambarincola fallax Hoffman, 1963	Twentymile Creek, NC	*Cambarus bartonii*
Cambarincola floridanus Goodnight, 1941	Tallapoosa River, GA	*Procambarus spiculifer*
Cambarincola gracilis Robinson, 1954	Bear Creek, OR	*Pacifastacus leniusculus*
Cambarincola heterognathus Hoffman, 1963	Byrd Creek, TN	*Cambarus* sp.
Cambarincola holostomus Hoffman, 1963	Sams Creek, TN	*Cambarus bartonii*
Cambarincola holti Hoffman, 1963	Flat Creek, TN	*Cambarus graysonii*
Cambarincola ingens Hoffman, 1963	Big Creek, NC	Cataloochee morph *Cambarus*
Cambarincola macrodontus Ellis, 1912	Granite River, MN	*Orconectes virilis*
Cambarincola mesochoreus Hoffman, 1963	Berkshires area, MA	*Orconectes virilis*
Cambarincola meyeri Goodnight, 1942	Oconaluftee River, NC	*Cambarus bartonii*
Cambarincola okadai Yamaguchi, 1933	Olalla Creek, OR	*Pacifastacus leniusculus*
Cambarincola osceola Hoffman, 1963	Berkshires area, MA	*Orconectes virilis*

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Taxon	Collection locality	Host species
Cambarincola pamelae Holt, 1984	Eugene, OR	*Procambarus clarkii*
Cambarincola philadelphicus (Leidy, 1851)	Coon Hollow Creek, TN; Tallapoosa River, GA; Carnton Creek, TN; Mill Creek, TN	*Cambarus* sp.; *Procambarus spiculifer*; *Orconectes durelli*; *Cambarus tenebrosus*
Cambarincola serratus Holt, 1981	Malad River, ID	*Pacifastacus connectens*
Cambarincola shoshone Hoffman, 1963	Hagerman Fish Hatchery, ID	*Pacifastacus connectens*
Cambarincola vitreus Ellis, 1919	Battle River, AB	*Orconectes virilis*
Unidentified *Cambarincola* sp.	Flat Creek, TN	*Cambarus graysoni*
Ceratodrilus ophiorhysis Holt, 1960	Malad River, ID	*Pacifastacus connectens*
Magmatodrilus obscurus (Goodnight, 1940)	Crystal Lake., CA	*Pacifastacus fortis*
Oedipodrilus anisognathus Holt, 1988	Abrams Creek, TN	*Orconectes forceps*
Oedipodrilus macbaini (Holt, 1955)	Tributary to Locke Branch, TN	*Orconectes compressus*
Pterodrilus alcicornus Moore, 1895	LeConte Creek, TN	*Cambarus bartonii*
Pterodrilus annulatus Gelder, 1996 [a]	-	-
Pterodrilus cedrus Holt, 1968	Mill Creek, TN	*Orconectes placidus*
Pterodrilus choritonamus Holt, 1968 [b]	Mill Creek, TN	*Cambarus tenebrosus*
Pterodrilus distichus Moore, 1895	Canandaigua Outlet, NY	*Cambarus bartonii*
Pterodrilus hobbsi Holt, 1968	Cosby Creek, TN; Flat Creek, TN	*Cambarus longirostris*; *Cambarus tenebrosus*
Pterodrilus robinae Williams & Gelder, 2011	Sevenmile Creek, TN	*Orconectes durelli*
Sathodrilus attenuatus Holt, 1981	Kalama River, WA	*Pacifastacus leniusculus*
Sathodrilus chehalisae Holt, 1981	Chehalis River, WA	*Pacifastacus leniusculus*
Sathodrilus inversus (Ellis, 1919)	Teanaway River, WA; Yakima River, WA	*Pacifastacus leniusculus*
Sathodrilus lobatus Holt, 1977	Olalla Creek, OR	*Pacifastacus leniusculus*
Sathodrilus norbyi Holt, 1977	Little Klickitat River, WA	*Pacifastacus leniusculus*
Triannulata magna Goodnight, 1940	Bear Creek, OR	*Pacifastacus leniusculus*
Xironodrilinae Brinkhurst & Gelder, 2001		
Xironodrilus appalachius Goodnight, 1943	Cosby Creek, TN	*Cambarus bartonii*
Xironodrilus formosus Ellis, 1919	Morgan Creek, TN	*Orconectes placidus*

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Taxon	Collection locality	Host species
Outgroups		
Helobdella stagnalis (Linnaeus, 1758)	Mantle Lake, ME`	-
Glossiphonia complanata (Linnaeus, 1758)	Mantle Lake, ME	-
Erpobdella obscura (Verrill, 1872)	Arnold Brook Lake, ME	-
Eremidrilus coyote Fend & Rodriguez, 2003	Coyote Creek, CA	-
Lumbriculus variegatus (Müller, 1774)	Culture, Aquatic Foods, Fresno, CA	-

[a] Taxon represented solely by sequences from Gelder and Siddall (2001)
[b] Only one individual representing the species was extracted, amplified and sequenced.

Table 7-2. Alphabetical list of branchiobdellidan species used in the full phylogenetic analysis. Outgroup taxa are at the bottom of the table. Availability of slide-mounted reference specimens is indicated (P = paragenophore, H = hologenophore) (NBM catalogue numbers pending). GenBank accession numbers are given for sequences representing all five loci: cytochrome c oxidase subunit I (COI), 16S rDNA, 28S rDNA, 18S rDNA, and internal transcribed spacer 1 (ITS1).

Taxon	Reference	COI	16S rDNA	28S rDNA	18S rDNA	ITS1
Ankyrodrilus koronaeus						
Flat Creek	**P, H**	JQ821586	JQ821409	JQ821527	JQ821468	-
Indian Creek		JQ821588	JQ821411	JQ821529	JQ821470	-
Ankyrodrilus legaeus						
Flat Creek	**P, H**	JQ821587	JQ821410	JQ821528	JQ821469	-
Indian Creek		JQ821589	JQ821412	JQ821530	JQ821471	-
Bdellodrilus illuminatus	**P**	JQ821634	JQ821459	JQ821577	JQ821518	JQ821683
Cambarincola bobbi	**P**	JQ821620	JQ821445	JQ821563	JQ821504	JQ821670
Cambarincola chirocephalus	**P**	JQ821603	JQ821428	JQ821546	JQ821487	JQ821654
Cambarincola fallax	**P**	JQ821621	JQ821446	JQ821564	JQ821505	JQ821671
Cambarincola floridanus	**P**	JQ821617	JQ821442	JQ821560	JQ821501	JQ821667
Cambarincola gracilis	**P**	JQ821591	JQ821414	JQ821532	JQ821473	JQ821641
Cambarincola heterognathus	**P**	JQ821625	JQ821450	JQ821568	JQ821509	JQ821674
Cambarincola holostomus	**P**	JQ821639	JQ821465	JQ821583	JQ821524	JQ821686
Cambarincola holti	**P**	JQ821623	JQ821448	JQ821566	JQ821507	-
Cambarincola ingens	**P**	JQ821626	JQ821451	JQ821569	JQ821510	JQ821675
Cambarincola macrodontus	**P**	JQ821618	JQ821443	JQ821561	JQ821502	JQ821668
Cambarincola mesochoreus	**P**	JQ821619	JQ821444	JQ821562	JQ821503	JQ821669
Cambarincola meyeri	**P**	JQ821601	JQ821426	JQ821544	JQ821485	JQ821652
Cambarincola okadai	**P**	JQ821595	JQ821420	JQ821538	JQ821479	JQ821647
Cambarincola osceola	**P**	JQ821616	JQ821441	JQ821559	JQ821500	JQ821666
Cambarincola pamelae	**P**	JQ821614	JQ821439	JQ821557	JQ821498	JQ821664

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Taxon	Reference	COI	16S rDNA	28S rDNA	18S rDNA	ITS1
Cambarincola philadelphicus						
Lineage 1 (Tallapoosa)	**P**	JQ821627	JQ821452	JQ821570	JQ821511	JQ821676
Lineage 2 (Mill)		JQ821640	JQ821466	JQ821584	JQ821525	JQ821687
Lineage 3 (Coon Hollow)	**-**	JQ821604	JQ821429	JQ821547	JQ821488	JQ821655
Lineage 4 (Carnton)		JQ821602	JQ821427	JQ821545	JQ821486	JQ821653
Cambarincola serratus	**P**	JQ821594	JQ821419	JQ821537	JQ821478	JQ821646
Cambarincola shoshone	**P**	**-**	JQ821416	JQ821534	JQ821475	JQ821643
Cambarincola vitreus	**P**	JQ821615	JQ821440	JQ821558	JQ821499	JQ821665
Unidentified *Cambarincola* sp.	**P**	JQ821622	JQ821447	JQ821565	JQ821506	JQ821672
Ceratodrilus ophiorhysis	**P**	JQ821599	JQ821424	JQ821542	JQ821483	**-**
Cronodrilus ogygius	**-**	JQ821613	JQ821438	JQ821556	JQ821497	JQ821663
Magmatodrilus obscurus	**P**	JQ821600	JQ821425	JQ821543	JQ821484	JQ821651
Oedipodrilus anisognathus	**-**	JQ821624	JQ821449	JQ821567	JQ821508	JQ821673
Oedipodrilus macbaini	**P**	JQ821605	JQ821430	JQ821548	JQ821489	**-**
Pterodrilus alcicornus	**P**	JQ821606	JQ821431	JQ821549	JQ821490	JQ821656
Pterodrilus annulatus	-	AF310718 [a]	-	-	AF310701 [a]	-
Pterodrilus cedrus	**P**	JQ821609	JQ821434	JQ821552	JQ821493	JQ821659
Pterodrilus choritonamus	**-**	JQ821608	JQ821433	JQ821551	JQ821492	JQ821658
Pterodrilus distichus	**P**	JQ821611	JQ821436	JQ821554	JQ821495	JQ821661
Pterodrilus hobbsi						
Lineage 1 (Cosby)	**P**	JQ821612	JQ821437	JQ821555	JQ821496	JQ821662
Lineage 2 (Flat)	**H**	JQ821607	JQ821432	JQ821550	JQ821491	JQ821657
Pterodrilus robinae	**P**	JQ821610	JQ821435	JQ821553	JQ821494	JQ821660
Sathodrilus attenuatus	**H**	JQ821592	JQ821417	JQ821535	JQ821476	JQ821644
Sathodrilus chehalisae	**P**	JQ821596	JQ821421	JQ821539	JQ821480	JQ821648
Sathodrilus inversus						
Lineage 1 (Teanaway)	**P, H**	JQ821598	JQ821423	JQ821541	JQ821482	JQ821650
Lineage 2 (Yakima)	**P, H**	**-**	JQ821415	JQ821533	JQ821474	JQ821642
Sathodrilus lobatus	**P**	JQ821597	JQ821422	JQ821540	JQ821481	JQ821649
Sathodrilus norbyi	**P**	JQ821593	JQ821418	JQ821536	JQ821477	JQ821645
Triannulata magna	**P**	JQ821629	JQ821454	JQ821572	JQ821513	JQ821678

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Taxon	Reference	COI	16S rDNA	28S rDNA	18S rDNA	ITS1
Uglukodrilus hemophagus	**P**	JQ821628	JQ821453	JQ821571	JQ821512	JQ821677
Xironodrilus appalachius	**P**	JQ821585	JQ821408	JQ821526	JQ821467	-
Xironodrilus formosus	**P**	JQ821590	JQ821413	JQ821531	JQ821472	-
Xironogiton instabilis	**P**	JQ821630	JQ821455	JQ821573	JQ821514	JQ821679
Xironogiton kittitasi	**P**	JQ821632	JQ821457	JQ821575	JQ821516	JQ821681
Xironogiton occidentalis	**P**	JQ821633	JQ821458	JQ821576	JQ821517	JQ821682
Xironogiton victoriensis	**P**	JQ821631	JQ821456	JQ821574	JQ821515	JQ821680
Helobdella stagnalis	**H**	-	JQ821461	JQ821579	JQ821520	-
Glossiphonia complanata	**H**	JQ821635	JQ821460	JQ821578	JQ821519	-
Erpobdella obscura	**H**	JQ821638	JQ821464	JQ821582	JQ821523	-
Eremidrilus coyote	**H**	JQ821636	JQ821462	JQ821580	JQ821521	JQ821684
Lumbriculus variegatus	**H**	JQ821637	JQ821463	JQ821581	JQ821522	JQ821685

[a] Sequences from Gelder and Siddall (2001) obtained from Genbank

Table 7-3. Primers, primer references, annealing temperature, and $MgCl_2$ concentration (mM) for each locus used in phylogenetic analyses.

Locus	Primer	Primer sequence 5' – 3'	Reference	T_a (°C)	$MgCl_2$
Nuclear					
18S rDNA					
	A	AACCTGGTTGATCCTGCCAGT	Medlin et al. (1988)	55 [a]	2
	L	CCAACTACGAGCTTTTTAACTG	Apakupakul et al. (1999)		
	C	CGGTAATTCCAGCTCCAATAG	Apakupakul et al. (1999)	55 [a]	2
	Y	CAGACAAATCGCTCCACCAAC	Apakupakul et al. (1999)		
	B	TGATCCTTCCGCAGGTTCACCT	Medlin et al. (1988)	60 [a]	2
	O	AAGGGCACCACCAGGAGTGGAG	Apakupakul et al. (1999)		
28S rDNA					
	C1'	ACCCGCTGAATTTAAGCAT	Lê et al. (1993)	55 [a]	1.5
	C2'	TGAACTCTCTCTTCAAAGTTCTTTTC	Lê et al. (1993)		
ITS1	IST1A	CACACCGCCCGTCGCTACTACCG	Kerans et al. (2004)	52	2
	ITS1B	GTGCGTTCGAAGTGTCGATGATCAA	Kerans et al. (2004)		
Mitochondrial					
CO-I					
	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	48	2.5
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)		
16S rDNA					
	ArL	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)	50	3
	BrH	CCGGTCTGACTCAGATCACGT	Palumbi et al. (1991)		

[a] Touchdown procedure used, dropping annealing temperatures from 55 – 51° C (18S rDNA A/L and C/Y, 28S rDNA) and 60 – 52° C (18S rDNA B/O).

Table 7-4. Results of single-gene tests for data congruence of branchiobdellidan species obtained from GenBank. Provided are the species name, locus, GenBank accession number, studies that have used the sequence, uncorrected p-distance, relationship to conspecific from the current study (Rel.; mono = monophyletic, poly = polyphyletic, para = paraphyletic), and a comment on if the sequence is consistent with our conspecifics, or if not, how or why the difference. Abnormally high p-distance values for the Branchiobdellida are in bold. Single-gene phylogenetic representations of COI and 18S rDNA based on these data are found in Supplementary Figs. 7-S2 and 7-S3.

Species	locus	Accession	Application [a]	p-dist (%)	Rel.	Comment on data congruence
Ankyrodrilus legaeus	COI	AF310705	I,J	**16.38**	poly	Misidentified *Xironodrilus formosus*
	18S	AF310688	I,J,M	**0.47**	poly	Misidentified *Xironodrilus formosus*
Bdellodrilus illuminatus	COI	AF310706	I,J	0.86	mono	Consistent
	18S	AF310689	I,J,M	0.17	mono	Consistent
Cambarincola gracilis	COI	AF310709	I,J	12.07	mono	Consistent
	18S	AF310692	I,J,M	0.00	mono	Consistent
Cambarincola holti	COI	AF116012	E	**17.53**	poly	Misidentification; similar to AF310713 (*C. philadelphicus*)
	COI	AF003263	D	**16.95**	poly	Archiving error; consistent with *Cambarincola fallax* [b]
	18S	AF115975	E,H	**0.17**	poly	Misidentification; similar to AF310713 (*Cambarincola philadelphicus*)
Cambarincola mesochoreus	COI	AF310710	I,J	7.76	mono	Consistent
	18S	AF310693	I,J,M	0.00	mono	Consistent
Cambarincola okadai [c]	COI	AF310711	I,J	7.76	mono	Consistent
	18S	AF310694	I,J,M	0.00	para	Possible intraspecific variation
Cambarincola pamelae	COI	AF310712	I,J,N	0.29	mono	Consistent
	28S	AF406601	K	1.74	mono	Consistent
	18S	AF310695	I,J,L,N	0.00	mono	Consistent
Cambarincola philadelphicus	COI	AF310713	I	**14.1-18.7**	poly	Possible cryptic variation
	18S	AF310696	I,M	0.00-**0.30**	poly	Possible cryptic variation

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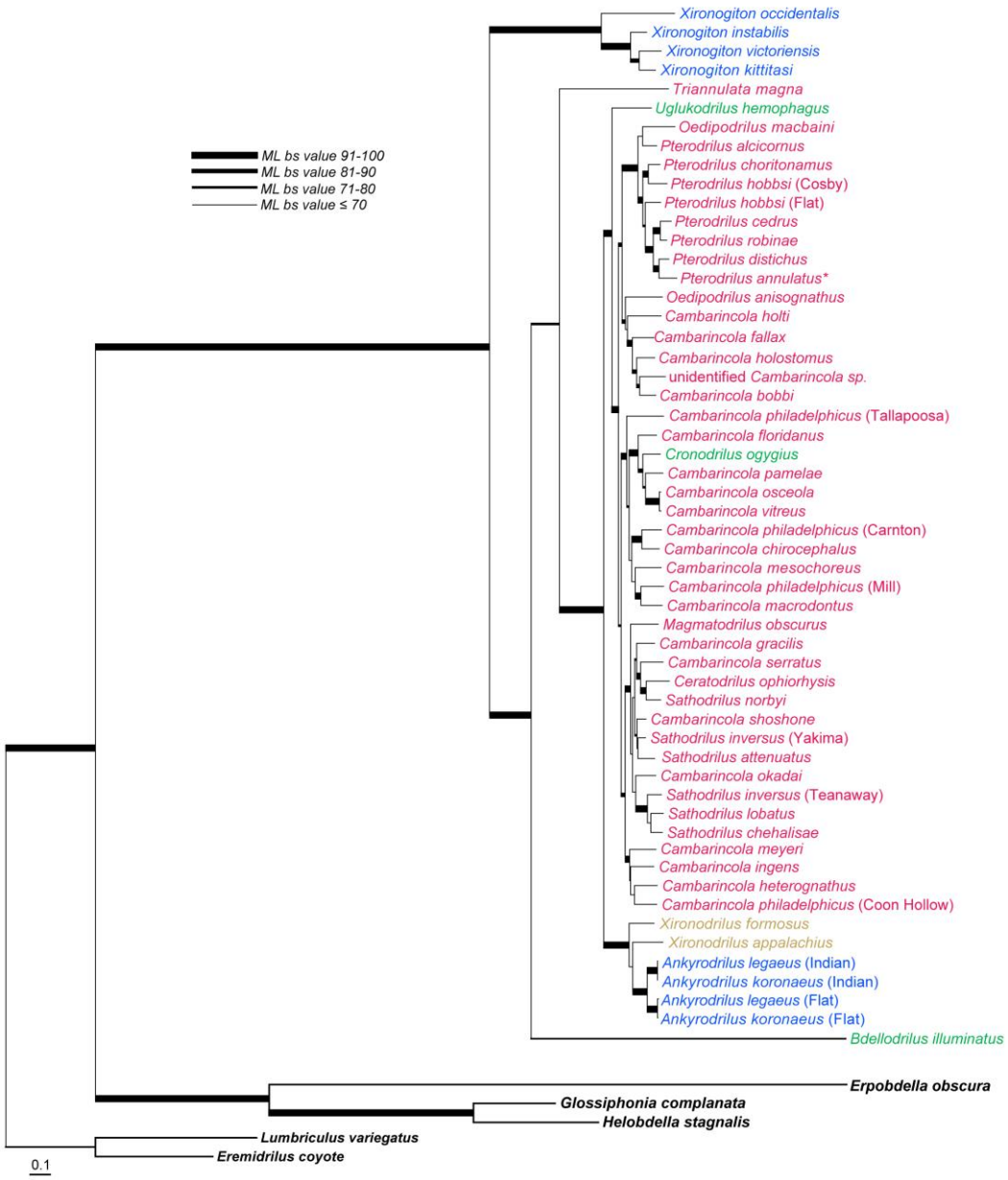
Species	locus	Accession	Application [a]	p-dist (%)	Rel.	Comment on data congruence
Ceratodrilus ophiorhysis	COI	AF310714	I,J	**18.97**	poly	Contamination with or misidentification of leech sp.; species name misspelled in Genbank
	18S	AF310697	I,J,M	0.51	para	Consistent; species name misspelled in Genbank
Cronodrilus ogygius	COI	AF116013	E,I,J	0.00	mono	Consistent
	18S	AF115976	E,H,I,J	0.00	para	Consistent
Magmatodrilus obscurus	COI	AF310716	I	**24.71**	poly	Contamination with or misidentification of leech sp.
	18S	AF310699	I	**11.54**	poly	Long branch; unknown lab error
Oedipodrilus macbaini	COI	AF310717	I,J	14.94	para	Possible intraspecific variation
	18S	AF310700	I,J,M	0.25	para	Possible intraspecific variation
Sathodrilus attenuatus	COI	AF310719	I,J	**20.11**	poly	Misidentified *X. victoriensis*
	18S	AF310702	I,J,M	**1.44**	poly	Misidentified *X. victoriensis*
	18S	Z83755	B,C,F,G,H	**8.99**	poly	Long branch, but not similar to our conspecific representative; 46 bp insertion; lab error
Triannulata magna	COI	AF310720	I,J	1.15	mono	Consistent
	18S	AF310703	I,J,M	0.25	mono	Consistent
Xironodrilus formosus	COI	AF310721	I,J	**23.28**	poly	Contamination with or misidentification of leech sp.; genus name misspelled in Genbank
	28S	AF406600	K	2.43	mono	Consistent
	18S	AF310704	I,J,M	0.08	mono	Consistent; genus name misspelled in Genbank
Xironogiton victoriensis	COI	AF116014	E,I,J	7.18	mono	Consistent
	18S	AF115977	E,H,I,J	0.00	mono	Consistent
	18S	Z83756	A,B,C,F,G,H,P	**2.21**	mono	Long branch; 46 bp insertion; unknown lab error

[a] Studies that have incorporated these sequence into published analyses or reviews: A) Moon et al., 1996; B) Kim et al., 1996; C) Winnepenninckx et al., 1998; D) Siddall and Burreson, 1998; E) Apakupakul et al., 1999; F) McHugh, 2000; G) Martin et al., 2000; H) Martin, 2001; I) Gelder and Siddall, 2001; J) Siddall et al. 2001; K) Jamieson et al. 2002; L) Erséus and Källersjö, 2004, M) Kaygorodova and Sherbakov, 2006; N) Colgan et al., 2006; O) Rousset et al., 2008; P) Marotta et al., 2008.

[b] This sequence was published as *Cambarincola fallax* (Siddall and Burreson 1998), but is archived in GenBank as *C. holti*.

[c] *Cambarincola okadai* (= *Cambarincola montanus*) as per Gelder and Ohtaka (2000), although still listed in GenBank as *Cambarincola montanus.*

Figure 7-1. Maximum likelihood tree of the combined 5 gene dataset (COI + 16S rDNA + 18S rDNA + 28S rDNA + ITS1). Branchiobdellidan terminals are colour-coded by subfamily (Cambarincolinae, red; Branchiobdellinae, blue; Bdellodrilinae, green; Xironodrilinae, tan). Branch thickness denotes bootstrap support. When more than one representative of a species was included, location is provided in parentheses as provided in Table 7-1. The one taxon represented only by sequence data from Gelder and Siddall (2001) is denoted by *. Supplementary Fig. 7-S1 presents a cladogram of this phylogeny to better show branching patterns with support values.


ML bs value 91-100
ML bs value 81-90
ML bs value 71-80
ML bs value ≤ 70
Xironogiton occidentalis
Xironogiton instabilis
Xironogiton victoriensis
Xironogiton kittitasi
Triannulata magna
Uglukodrilus hemophagus
Oedipodrilus macbaini
Pterodrilus alcicornus
Pterodrilus choritonamus
Pterodrilus hobbsi (Cosby)
Pterodrilus hobbsi (Flat)
Pterodrilus cedrus
Pterodrilus robinae
Pterodrilus distichus
Pterodrilus annulatus*
Oedipodrilus anisognathus
Cambarincola holti
Cambarincola fallax
Cambarincola holostomus
unidentified Cambarincola sp.
Cambarincola bobbi
Cambarincola philadelphicus (Tallapoosa)
Cambarincola floridanus
Cronodrilus ogygius
Cambarincola pamelae
Cambarincola osceola
Cambarincola vitreus
Cambarincola philadelphicus (Carnton)
Cambarincola chirocephalus
Cambarincola mesochoreus
Cambarincola philadelphicus (Mill)
Cambarincola macrodontus
Magmatodrilus obscurus
Cambarincola gracilis
Cambarincola serratus
Ceratodrilus ophiorhysis
Sathodrilus norbyi
Cambarincola shoshone
Sathodrilus inversus (Yakima)
Sathodrilus attenuatus
Cambarincola okadai
Sathodrilus inversus (Teanaway)
Sathodrilus lobatus
Sathodrilus chehalisae
Cambarincola meyeri
Cambarincola ingens
Cambarincola heterognathus
Cambarincola philadelphicus (Coon Hollow)
Xironodrilus formosus
Xironodrilus appalachius
Ankyrodrilus legaeus (Indian)
Ankyrodrilus koronaeus (Indian)
Ankyrodrilus legaeus (Flat)
Ankyrodrilus koronaeus (Flat)
Bdellodrilus illuminatus
Erpobdella obscura
Glossiphonia complanata
Helobdella stagnalis
Lumbriculus variegatus
Eremidrilus coyote
0.1

7.8 References

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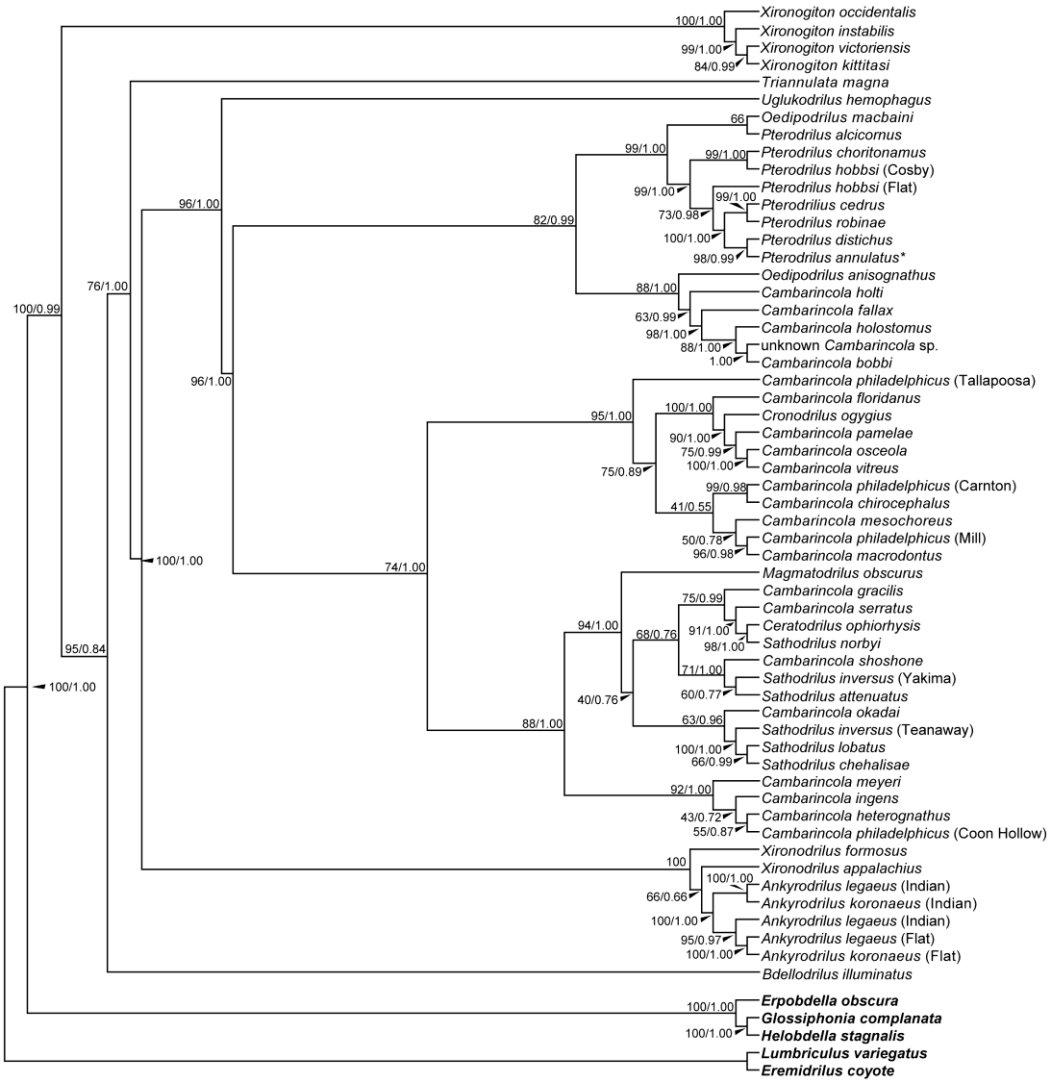
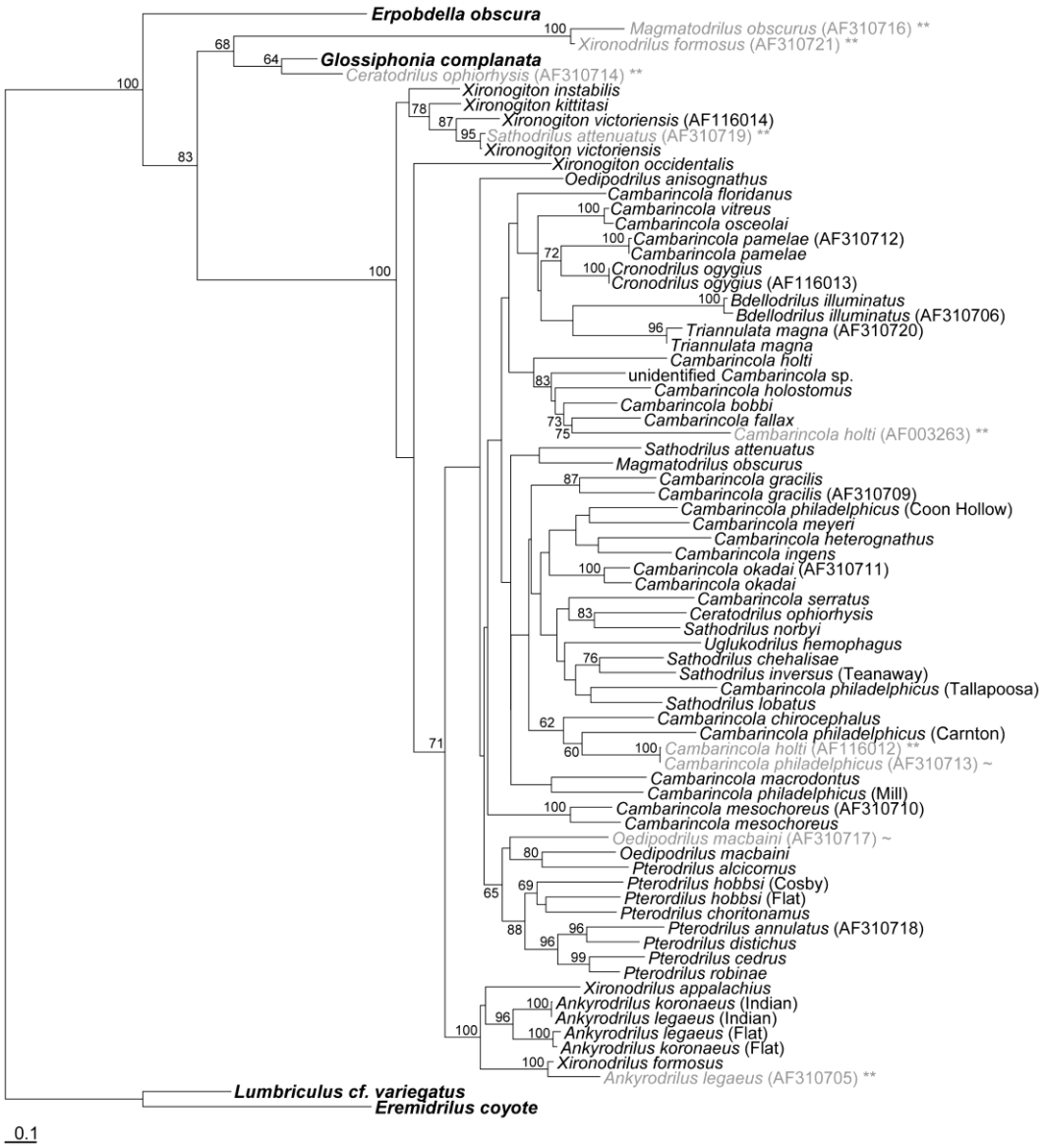
Supplementary Table 7-S1. Results of ILD tests of data congruence for all locus combinations. Included are number of characters (No. char.) included for each comparison. No comparisons were significant at $P \leq 0.010$.

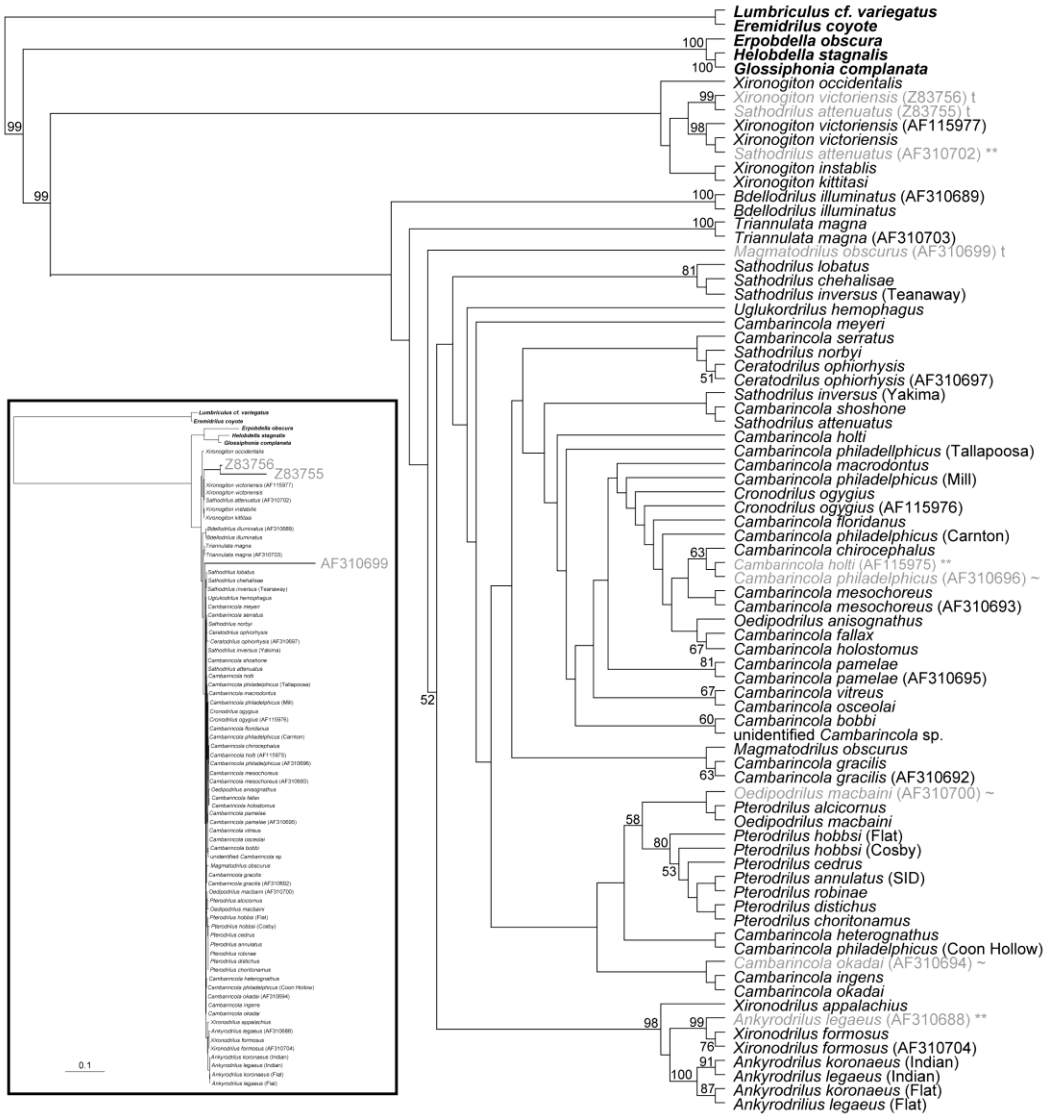
Genes	*P* value	No. char.
COI/16S	0.810	545
COI/28S	1.000	420
COI/18S	1.000	500
COI/ITS1	0.110	1148
16S/28S	0.996	297
16S/18S	0.996	377
16S/ITS1	0.034	1025
28S/18S	0.648	252
28S/ITS1	0.996	900
18S/ITS1	1.000	980
COI/16S/28S	1.000	631
COI/16S/18S	0.996	711
COI/28S/18S	1.000	586
COI/16S/ITS1	0.018	1359
COI/28S/ITS1	0.814	1234
COI/18S/ITS1	0.714	1314
16S/28S/18S	1.000	463
16S/28S/ITS1	0.204	1111
16S/18S/ITS1	0.252	1191
28S/18S/ITS1	1.000	1066
COI/16S/28S/18S	1.000	797
COI/16S/28S/ITS1	0.512	1445
COI/16S/18S/ITS1	0.316	1525
COI/28S/18S/ITS1	0.998	1400
16S/28S/18S/ITS1	0.642	1277
COI/16S/28S/18S/ITS1	0.908	1611



Supplementary Figure 7-S1. Cladogram representation of the maximum likelihood tree resulting from the combined 5 gene dataset (COI + 16S rDNA + 18S rDNA + 28S rDNA + ITS1).



Supplementary Figure 7-S2. Maximum likelihood-based phylogram inferred from the COI dataset including our new data and all published sequences of North American branchiobdellidans. This figure supplements data in Table 7-4. Bootstrap support values > 50 are provided. Outgroup taxa are in bold, whereas suspect taxa from previous studies, labeled with GenBank accession numbers, are shown in red and annotated as misidentified (**) or displaying potential cryptic or intraspecific variation (~).



Supplementary Figure 7-S3. Maximum likelihood-based cladogram inferred from the 18S rDNA dataset including our new data and all published sequences of North American branchiobdellidans. This figure supplements data in Table 7-4. Bootstrap support values > 50 are provided. Outgroup taxa are in bold, and suspect taxa from previous studies, labeled with GenBank accession numbers, are annotated as misidentified (**), unknown lab error or contamination (t), or displaying potential cryptic interspecific or intraspecific variation (~). The corresponding phylogram for the 18S rDNA gene tree is presented in the inset to highlight long branches displayed by three branchiobdellidan taxa (large text).

Chapter 8. Distribution of Entocytheridae (Crustacea: Ostracoda) in the northern prairies of North America and reports of opportunistic clitellate annelids on crayfish hosts[1]

8.1 ABSTRACT

Northern crayfish, *Orconectes virilis*, were collected from 89 sites across Alberta, Saskatchewan, Manitoba, Montana, North Dakota, and Minnesota. The entocytherid ostracod *Thermastrocythere riojai* (Hoff, 1943) was found on *O. virilis* at 45 of the 89 sites, distributed primarily in the eastern and southern portion of the study area. These observations of *T. riojai* greatly extend the known range of the species. The widespread distribution of the *T. riojai* suggests the dearth of entocytherid records from other parts of Canada is a result of non-targeted sampling rather than true absence. In addition, we report on observations of three noteworthy associations of oligochaetes with the crayfish hosts.

KEY WORDS: entocytherid, ostracod, *Orconectes virilis*, *Thermastrocythere riojai*, host-symbiont distribution, enchytraeid, *Chaetogaster*, *Nais*

8.2 MAIN TEXT

The Entocytheridae is a family of ostracods that are obligate ectosymbionts of other crustaceans ranging across southern-central Europe, southern India, Australasia, and North America (Hart and Hart 1974). Two entocytherid subfamilies have been described from North America: Entocytherinae, commensal on freshwater crayfishes of the families Astacidae and Cambaridae and one species of freshwater crab, *Pseudothelphusa veracruzana* Rodriquez and Smalley, 1970, and; Sphaeromicolinae, commensal on freshwater isopods of the family Cirolanidae (Hart and Hart 1974). The known distribution of entocytherids in North America extends from Cuba and Mexico north to a boundary approximate to the United States-Canada border (reviewed in

[1] A version of this chapter has been published. Williams, B.W., K.L. Williams, S.R. Gelder, and H.C. Proctor. Western North American Naturalist 71:276-282. Formatting follows journal's guidelines.

Hart and Hart 1974). Despite widespread occurrence of several potential host crayfish species (e.g., Crocker and Barr 1968, Taylor et al. 1996, Hamr 1998, Hamr 2002, Williams et al. in review) few published records of entocytherids exist in Canada. The description of *Entocythere insignipes* (Sars, 1926) was the sole report from the country until 1970, when *Thermastrocythere riojai* (Hoff, 1943) was found on crayfish collected in the Swan River, near the town of Swan River, western Manitoba (Delorme 1970d, Hart and Hart 1974). No further contributions to the knowledge of entocytherid ostracods in Canada have been made since.

Reports of entocytherids are similarly lacking from large areas of the northern Great Plains of the United States, including Montana and North Dakota. Representatives of another group of crayfish ectosymbionts, branchiobdellidans or crayfish worms, were recently discovered in the Prairie Provinces of Canada (Williams et al. 2009) and in Montana and North Dakota (B. Williams, unpub. obs.). The wide distribution of these worms suggests that the prior dearth of reports was due to lack of targeted sampling. Therefore, it is likely that other crayfish-associated organisms, including entocytherid ostracods, have also gone unnoticed across the region.

The aim of the current study is to document the distribution of entocytherid ostracods associated with crayfish hosts in the Prairie Provinces of Canada, with additional reports from Montana, North Dakota, and Minnesota. We also provide information on incidental, yet notable, observations of three additional taxa associated with crayfish hosts.

Northern crayfish, *Orconectes virilis* (Hagen 1870), were collected as part of an ongoing population genetics study. A subset of specimens previously examined for branchiobdellidans (Williams et al. 2009; Appendix 8-I) were re-examined for entocytherid ostracods. Additional crayfish were collected by hand, kick-netting, or in Gee minnow traps (Wildlife Supply Company, Buffalo, NY, USA) deployed for up to 24 hours. The size of entrance holes in the minnow traps was increased to ~60mm to allow access by adult crayfish. Crayfish were individually preserved in containers of 95% ethanol.

We examined a total of 1376 crayfish collected from 89 sites across Alberta, Saskatchewan, Manitoba, Montana, North Dakota, and Minnesota between August 2006 and October 2010 (Fig. 8-1, Appendix 8-I). The study area includes portions of the Arctic, Hudson Bay, and Gulf of Mexico ocean watersheds. We examined the external surface and branchial chambers of each crayfish and debris at the bottom of sampling containers for presence of entocytherid ostracods using a dissecting microscope. Entocytherids were transferred to separate containers of 95% ethanol for preservation and storage. Representative entocytherids were dehydrated in 100% ethanol, cleared in methyl salicylate, infiltrated with Canada balsam, and mounted on glass slides to museum standards. Species identification was made using the keys and information in Hart and Hart (1974). Slide mounts were deposited in the New Brunswick Museum, Saint John, NB, Canada as catalogue numbers NBM-007156 – 007160.

Entocytherid ostracods were found at 50.1 % (45 of 89) of surveyed sites (Fig. 8-1, Appendix 8-I) in Saskatchewan, Manitoba, Montana, North Dakota, and Minnesota. Entocytherids were attached to setae along the abdomen, around legs, maxillipeds, base of antennae, chelae, and under the rostrum of their crayfish hosts. *Thermastrocythere riojai* was the only species of entocytherid ostracod found.

During our study, we observed additional organisms associated with *O. virilis*. The findings of three of these organisms are noteworthy as all represent previously unreported associations. An enchytraeid oligochaete was found in the gill chamber of an *O. virilis* specimen collected from Nose Creek in Calgary, Alberta (site 32, Fig. 8-1). Although enchytraeids are usually free-living, *Lumbricillus catanensis* (Drago, 1887) has been reported in the branchial chambers of the freshwater crab, *Potamon fluviatilis* (Herbst, 1785) (= *Telphusa fluviatilis*) in Italy (Gelder 1980). We also noted specimens of the oligochaete *Chaetogaster limnaei* Baer, 1827 on the carapace of three *O. virilis* collected in Long Creek, southern Saskatchewan (site 60; Fig. 8-1). Although *C. limnaei* is known world-wide as an ectocommensal of freshwater molluscs, primarily in the gill chamber of gastropods (Gelder 1980), it has not previously been reported in

association with crayfish. Lastly, we recovered additional small oligochaetes from the carapace of *O. virilis* from Long Creek. Although many of the small oligochaetes fragmented during preservation, we identified a subset as *Nais* sp., either *N. alpine* Sperber, 1948 or *N. simplex* Piguet, 1906. *Nais* sp. and other naidids have been observed on freshwater ectoprocts, which Sperber (1948) considered part of their normal foraging habits and is consistent with our observations on crayfish. Both *Nais* species and *C. limnaei* have been previously reported from Canada (Brinkhurst 1986). As each of the above associations was observed only once, presence of these oligochaetes on *O. virilis* in our collection is unlikely to reflect a stable symbiosis. Conversely, our findings are likely to be a result of natural, random wanderings of host and oligochaetes over the substratum.

Prior to this study, *T. riojai* was known in Canada only from a single site in western Manitoba (Delorme 1970d, Hart and Hart 1974). Our survey greatly extends the reported distribution of *T. riojai* westward into central and western Saskatchewan. We also provide new records of the species in areas of Montana, North Dakota, eastern Manitoba, and northern Minnesota. The species is now known to range from eastern Texas and Louisiana north through the northern Great Plains and western Great Lakes States, and into the eastern and central Prairie Provinces (Hart and Hart 1974). Detection of *T. riojai* at sites on the Winnipeg River in Manitoba (sites 67, 68) and Lake of the Woods in Minnesota (site 88) indicate that the species may also be present in adjoining waterways of southwestern Ontario.

It is important to note that the crayfish from Swan River harboring *T. riojai* had been identified as the rusty crayfish, *Orconectes rusticus* (Girard, 1852) (Delorme 1970d). Originating from areas of north-central North America (Tennessee, Kentucky, Indiana, Ohio, Michigan, and southern Ontario; Hobbs 1974), *O. rusticus* is thought to have been only recently introduced to southeastern Manitoba through its use as live bait; the first confirmed report of the species in the province was in 2007 from Falcon Lake (Lowdon 2009). As we were unable to verify species identification of the original collection due to the

absence of known voucher specimens, we suggest that the crayfish collected from Swan River were likely *O. virilis*. *Orconectes virilis* is the only species found throughout much of the Prairie Provinces (Williams et al. in press), including a sampling site in the current study (site 57 – Swan River, Saskatchewan) approximately 50 km upstream from the town of Swan River, Manitoba. The calico crayfish, *Orconectes immunis* (Hagen, 1870), unsampled in this study, is also found in Manitoba, but is restricted to the southeastern portion of the province in the Red River drainage and two isolated areas in the Winnipeg River near the Ontario border.

Although we did not sample in western Manitoba, the distribution of *T. riojai* appears to be contiguous along the rivers of Saskatchewan and Manitoba (Fig. 8-1). Based on our collections and previous records summarized in Hart and Hart (1974) the general northern distribution likely includes the Red and Assiniboine River systems flowing into southern Hudson Bay, the Upper Great Lakes region draining to the Atlantic Ocean, and the Missouri and Upper Mississippi River systems flowing south towards the Gulf of Mexico. The western edge of the observed *T. riojai* distribution was inconsistent across sampled waterways relative to longitude. These differing observed range limits could be a result of incomplete sampling, such as a lack of collections made along the Missouri River in eastern Montana and western North Dakota. However, particularly in rivers that were systematically sampled in this study (e.g., the North Saskatchewan and South Saskatchewan Rivers), variation in range boundaries suggests differing barriers, such as physical (e.g., dams) or environmental barriers (e.g., tolerance limits).

The westernmost collection of *T. riojai* was from Fresno Reservoir on the Milk River in northern Montana (site 76, 48.685°N 100.008°W; Fig. 8-1). However, this collection appears to be isolated and distant from the nearest collections of *T. riojai* to the north and east. No entocytherids were found elsewhere in Montana, including a second site on the Milk River (site 77; Fig. 8-1) or at locations sampled in southern Saskatchewan in streams or rivers that drain into the Milk River (e.g., sites 78-80; Fig. 8-1). An identical distribution pattern

was observed for the branchiobdellidan *Cambarincola vitreus* Ellis, 1919, which was found on *O. virilis* in Fresno Reservoir, but on no other crayfish examined from Montana (B. Williams, unpub. obs.). Although non-detection does not necessarily correspond to true absence, the isolated observations of *T. riojai* and *C. vitreus* in Fresno Reservoir suggest an introduction of the *O. virilis* host from an area where both symbionts are sympatric (for distributions see Hart and Hart 1974, Gelder et al. 2002, Williams et al. 2009).

Orconectes virilis has been expanding its range westward in Montana and in the Prairie Provinces of Canada due to what appears to be a combination of natural dispersal and human-mediated introduction (Williams et al. in press). In some cases, such as with *O. virilis* from the Fresno Reservoir, symbiont presence might be used to indicate likely crayfish introductions. A second host introduction might explain an apparent isolated collection of *T. riojai* in Swift Current Creek, Saskatchewan (site 39, Fig. 8-1). Two major dams, the Qu'Appelle River Dam and the Gardiner Dam, separate the Swift Current Creek collection from both nearest observed collections of *T. riojai* (site 43 on the South Saskatchewan River and site 49 on the Qu'Appelle River).

At the majority of sites where *T. riojai* was observed, the species was common and found on the majority of crayfish examined. In contrast, entocytherids were rare at the western edge of the observed range, suggestive of environmental tolerances or few founders at a leading edge of a range expansion. For example, a total of five *T. riojai* were found among 21 crayfish examined from site 18 (Fig. 8-1). In contrast, *T. riojai* were numerous (>5 per crayfish) in the two isolated populations observed in Fresno Reservoir (site 76) and Swift Current Creek (site 39), despite the examination of only two *O. virilis* hosts from the former site.

Delorme's (1970a, b, c, d) four-part review of freshwater ostracods of Canada included 84 species from 8 families, but only a single species of entocytherid, *T. riojai*, as aforementioned, from Swan River, Manitoba. The earliest recorded entocytherid from Canada, *Cytherites insignipes* Sars, 1926, was a new species description based on examination of three female specimens;

however, collector, locality (other than "Canada"), date of collection, host, and male morphology were all unknown. Hoff (1944) reassigned *C. insignipes* to the genus *Entocythere*, but due to the lack of taxonomically distinguishing male morphological characters, the species is currently considered *incertae sedis* (Hart 1962).

Several entocytherid species are reported from areas immediately south of the USA-Canada border, including *Uncinocythere occidentalis* (Kozloff and Whitman, 1954) and *Uncinocythere columbia* (Dobbin, 1941) in northern Washington, *Uncinocythere stubbsi* Hobbs and Walton, 1966 and *T. riojai* in the Upper Great Lakes States, and *Donnaldsoncythere scalis* Hobbs and Walton, 1963 in upper New England (ranges summarized in Hart and Hart 1974). Several potential crayfish hosts are found throughout southern Canada as extensions of known distributions in the USA. Therefore, it is inevitable that additional records and range extensions of entocytherid ostracods will appear as researchers begin targeted examination of crayfishes in Canada.

Author contributions: BWW made the majority of the field collections across Alberta and Saskatchewan, Canada, identified the crayfish and entocytherid species, and prepared the manuscript; KLW assisted with field collection and laboratory sampling of the entocytherids; SRG identified the additional oligochaetes and assisted with manuscript preparation; HCP provided laboratory space and resources.

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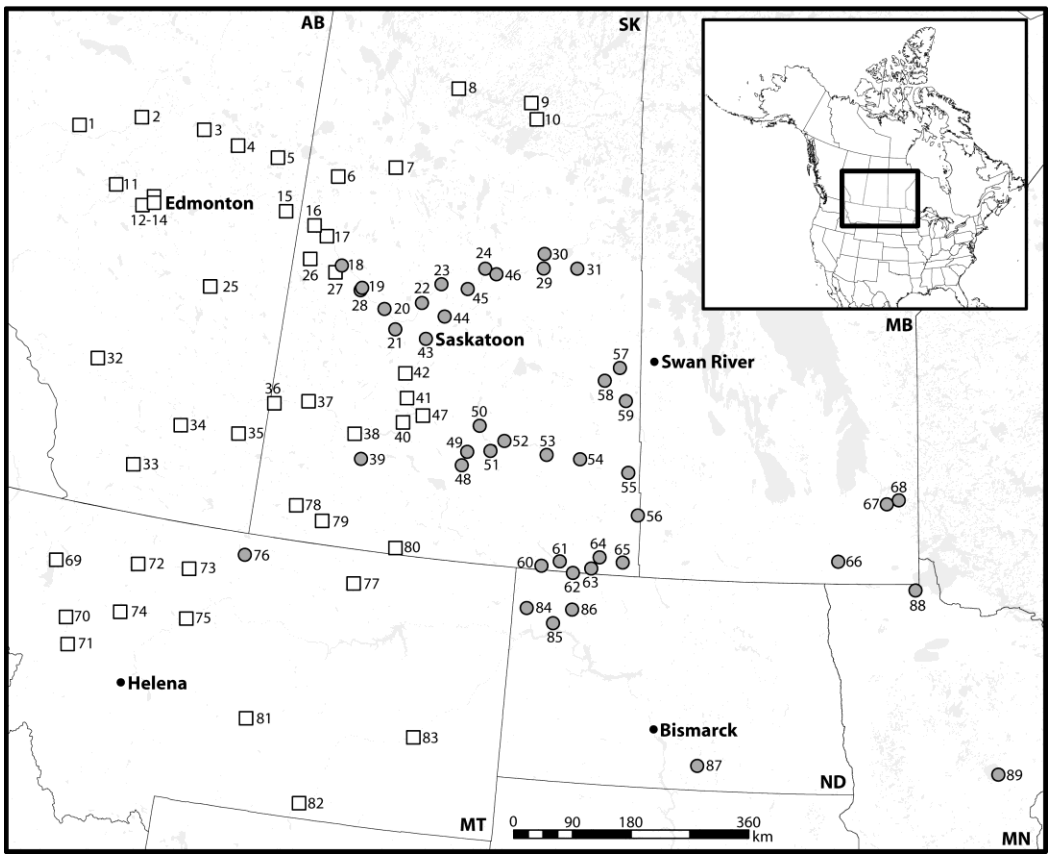

Figure 8-1. *Orconectes virilis* (Hagen 1870) sampling sites in the northern prairies of North America (Alberta, AB; Saskatchewan, SK; Manitoba, MB; Montana, MT; North Dakota, ND; Minnesota, MN) showing detection (shaded circles) and non-detection (open squares) of the entocytherid ostracod *Thermastrocythere riojai* (Hoff, 1943). Inset map delineates the study area in North America. Site numbers correspond to information provided in the appendix.

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APPENDIX 8-I. Detection (+) or non-detection (-) of the entocytherid ostracod *Thermastrocythere riojai* (Hoff, 1943) on *Orconectes virilis* (Hagen 1870) collected across the northern prairies region, with information on sample location (latitude, Lat. and longitude, Long.; NAD 83) and number of hosts examined (*N* hosts). Site numbers correspond to locations in Figure 8-1.

Site	Waterbody	Lat. (°N)	Long. (°W)	*T. riojai*	*N* hosts
1	McLeod Lake	54.293	115.651	-	29
2	Beyette Lake	54.592	114.199	-	15
3	Amisk Lake	54.604	112.647	-	22
4	Amisk River	54.461	111.772	-	23
5	Beaver River	54.389	110.755	-	21
6	Beaver River	54.260	109.221	-	26
7	Beaver River	54.510	107.868	-	12
8	Churchill River	55.733	106.565	-	1
9	Churchill River	55.643	104.734	-	30
10	Churchill River	55.418	104.561	-	28
11	East Pit Lake	53.584	114.464	-	5
12	North Saskatchewan River	53.370	113.751	-	16
13	Bearspaw Lake	53.443	113.505	-	26
14	North Saskatchewan River	53.502	113.561	-	28
15	North Saskatchewan River	53.659	110.337	-	6*
16	North Saskatchewan River	53.523	109.618	-	19
17	North Saskatchewan River	53.396	109.293	-	29
18	North Saskatchewan River	53.245	105.433	+	20
19	North Saskatchewan River	52.743	108.284	+	13

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Site	Waterbody	Lat. (°N)	Long. (°W)	*T. riojai*	*N* hosts
20	North Saskatchewan River	52.491	107.699	+	19
21	Eagle Creek	52.232	107.380	+	26
22	North Saskatchewan River	52.646	106.842	+	18
23	North Saskatchewan River	52.946	106.435	+	24
24	North Saskatchewan River	53.182	105.162	+	22
25	Battle River (36)	52.409	111.810	-	20*
26	Battle River	53.046	109.601	-	25
27	Battle River	52.907	108.949	-	20
28	Battle River	52.717	108.310	+	21
29	Saskatchewan River	53.237	104.464	+	17
30	Torch River	53.539	104.069	+	19
31	Carrot River	53.366	103.264	+	26
32	Nose Creek	51.086	114.047	-	16
33	Henderson Lake	49.688	112.790	-	24
34	Lake Newell Reservoir	50.379	111.911	-	20
35	South Saskatchewan River	50.399	110.589	-	13
36	South Saskatchewan River	50.913	109.890	-	4
37	South Saskatchewan River	51.024	109.134	-	36*
38	South Saskatchewan River	50.656	107.975	-	27*
39	Swift Current Creek	50.308	107.769	+	31
40	South Saskatchewan River	50.905	106.917	-	22
41	South Saskatchewan River	51.259	106.896	-	10

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Site	Waterbody	Lat. (°N)	Long. (°W)	*T. riojai*	*N* hosts
42	South Saskatchewan River	51.613	107.008	-	14
43	South Saskatchewan River	52.137	106.646	+	12*
44	South Saskatchewan River	52.491	106.283	+	25
45	South Saskatchewan River	52.923	105.805	+	22
46	South Saskatchewan River	53.182	105.162	+	25
47	South Saskatchewan River	51.034	106.494	-	21
48	Moose Jaw Creek	50.394	105.497	+	24
49	Qu'Appelle River	50.595	105.411	+	22
50	Last Mountain Lake	50.992	105.180	+	21
51	Qu'Appelle River	50.630	105.007	+	12
52	Qu'Appelle River	50.804	104.581	+	20
53	Qu'Appelle River	50.662	103.603	+	20
54	Qu'Appelle River	50.642	102.847	+	21
55	Qu'Appelle River	50.499	101.728	+	10
56	Pipestone Creek	49.886	101.449	+	28
57	Swan River	51.998	102.075	+	21
58	Assiniboine River	51.792	102.408	+	15
59	Assiniboine River	51.533	101.877	+	20
60	Long Creek	49.062	103.498	+	4
61	Rafferty Dam Reservoir	49.145	103.098	+	25
62	Short Creek Dam	48.992	102.784	+	12
63	Souris River	49.079	102.399	+	17

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Site	Waterbody	Lat. (°N)	Long. (°W)	*T. riojai*	*N* hosts
64	Moose Mountain Creek	49.261	102.239	+	21
65	Antler River	49.193	101.711	+	6
66	Rat River	49.317	96.945	+	2
67	Winnipeg River	50.160	95.867	+	1
68	Winnipeg River	50.210	95.588	+	13
69	Echo Lake	48.121	114.036	-	3
70	Lake Alva	47.316	113.582	-	3
71	Clearwater River	46.945	113.431	-	3
72	Lake Frances	48.285	112.264	-	3
73	Tiber Reservoir	48.342	111.163	-	3
74	Willow Creek Reservoir	47.557	112.443	-	3
75	Missouri River	47.627	111.035	-	4
76	Fresno Reservoir	48.685	110.008	+	2
77	Nelson Reservoir	48.495	107.546	-	2
78	Conglomerate Creek	49.507	109.047	-	4
79	Frenchman River	49.335	108.417	-	27
80	Weatherall Creek	49.093	106.738	-	1
81	Deadmans Basin	46.339	109.426	-	2
82	Yellowtail Dam	45.228	108.072	-	5
83	Settling pond, Miles City	46.387	105.867	-	2
84	Blacktail Dam	48.433	103.735	+	3
85	Kota-Ray Dam	48.239	103.143	+	3

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Site	Waterbody	Lat. (°N)	Long. (°W)	*T. riojai*	*N* hosts
86	White Earth Dam	48.456	102.744	+	3
87	Beaver Creek	46.297	99.866	+	7
88	Lake of the Woods	48.899	95.240	+	3
89	Mille Lacs	46.211	93.528	+	7

* Crayfish collected for Williams et al. (2009) re-examined for presence of entocytherids.

Chapter 9. Synthesis and future directions

9.1 Main text

Prior to starting this research, we had limited understanding of the distribution and colonization history of the northern crayfish in the North American Interior Plains. Perhaps the most comprehensive investigations of *Orconectes virilis* in this region were those of Aiken (1967, 1968a, b, c, 1969a, b). Much of Aiken's research focused on physiological aspects of *O. virilis* (e.g., cold tolerance, 1968c; moulting, 1968b, 1969b; egg deposition, 1969a). However, it was an unpublished survey of *O. virilis* (Aiken 1967) that provided historical context to the recent westward range expansion of the species documented in my thesis. Aiken's survey included all major and several minor waterways throughout Alberta, including several sites along the three river systems (expansion axes) examined in Chapter 5 (Aiken 1967; Williams et al. 2011). At the time of Aiken's survey, the northern crayfish was found in Alberta only in the Beaver and Amisk Rivers (upper reaches of the Churchill River Drainage) (Aiken 1967, 1968a; Williams et al. 2011). The species appeared to be geographically restricted to this area of northern Alberta, as *O. virilis* had not been reported from any downstream locations in the Churchill River Drainage in Saskatchewan or Manitoba. Thus, Aiken suggested that the crayfish may have been introduced into this area by humans. The idea that *O.virilis* was introduced into the Beaver and Amisk Rivers was furthered by Hanson et al. (1990), with forethought to the possibility for additional introduction of the species into novel areas. As such, the work of Hanson et al. (1990) and Chambers et al. (1990, 1991) focused on the impacts of *O. virilis* on various components of Albertan aquatic systems. These authors were seemingly unaware of the report of Sawchyn (1986) documenting presence of the species in middle reaches of the Churchill River, and therefore suggestive that the northern crayfish was not restricted to a small western portion of the drainage, but may indeed be widely distributed across the drainage. This latter notion is supported by my collections (Williams et al. 2011). Indeed, my molecular data

indicate that *O. virilis* underwent historical colonization of the Churchill River Drainage in an east to west (upstream) direction, with no patterns suggestive of human-mediated introduction (Chapter 5).

Aiken (1967) deemed the large rivers of Alberta (e.g., North Saskatchewan, Battle, and South Saskatchewan Rivers), which appeared devoid of *O. virilis* at the time of his survey, to be unsuitable for the northern crayfish. He considered the substrate to be too sandy and lacking the structure necessary for the establishment and persistence of *O. virilis*. The current distribution of the species along these rivers suggests that Aiken's assessment was incorrect; although several water characteristics have changed since the 1960s (e.g., flow, dissolved oxygen; Environment Canada, unpubl.; Alberta Environment, unpubl.), the substrate has largely remained constant, although use of riprap around bridge supports may have provided vacant habitat available to long-distance dispersers. Indeed, my observations during sampling suggest that crayfish habitat might be widely and inconsistently spaced across large stretches of sandy substrate.

My thesis research has focused largely on patterns of distribution and genetic diversity and structure to determine how *O. virilis* has colonized the Interior Plains of North America, both historically (post-glacial range expansion, Chapter 3) and contemporary (recent, rapid range expansion, Chapter 5). Although these data elucidate some aspects of the pathways underlying expansion of the species, the mechanism allowing for rapid dispersal and establishment remain unknown. I have amassed a large, albeit disjointed dataset of several water characteristics (e.g., temperature, flow, dissolved oxygen, pH) for rivers across the Prairies to look for trends that correlate with the rough timing of expansion, and with current range limits. Although the actual rate of expansion is not known, we can identify several candidate variables to test further under controlled laboratory scenarios. Although the northern crayfish is now a permanent resident across much of Alberta, such information can useful in predicting future spread. In addition, we might be able to relate these data to known tolerances of potentially devastating invasive species, not least of which might be the rusty

crayfish, *Orconectes rusticus* (Girard), now present in areas of Manitoba (e.g., Lowdon 2009).

When I began my degree we knew little about *O. virilis* in the Interior Plains. As a result, I found myself doing much more exploratory research than expected. However, the substantial collections and background work have led to several questions and projects. What is presented in this thesis is a subset of the data collected over the last six years. I have sampled over 2500 crayfish across North America, including approximately 2300 *O. virilis*. I have genotyped approximately 2100 *O. virilis* from across the Interior Plains and neighbouring areas in the Pacific and Great Lakes Drainages using my newly developed microsatellite markers (Chapter 4; Williams et al. 2010). Northern crayfish are recent inhabitants of the northern prairies, having colonized much of the region within the last 1500 years. The retreat of the Laurentide ice sheet resulted in several temporary pro-glacial lakes along its margin, forming direct connections between distinct drainages. As walking is the primary dispersal mechanism of crayfish and the species appears to be limited by low temperatures (Aiken 1968c), we might expect that *O. virilis* was not able to take advantage of these temporary connections. We can use genotypic data to test hypotheses of likely post-glacial colonization routes of *O. virilis*, both with and without access via pro-glacial lakes. We can also examine the effects of landscape on population connectivity, as interpreted by genetic structure and diversity of *O. virilis*, with a particular focus on river confluences and barriers (e.g., weirs, dams, waterfalls). Barriers have been shown to influence the genetics of several aquatic organisms (e.g. crayfish, Kerby et al. 2005; bull trout, Costello et al. 2003; cutthroat trout, Neville et al. 2006); however, the potential for limited overland dispersal by crayfish might mitigate population discontinuities based on barrier construction. The ability of crayfish to disperse around such barriers is likely correlated with physical aspects of the structures, such as height, discharge characteristics, presence of vegetated edges, etc.

My work on branchiobdellidans has likewise resulted in numerous interesting questions regarding the ecology and evolution of these organisms. My molecular phylogeny (Chapter 7; Williams et al. in review) provides the basis for several ongoing analyses, including assessments of the biogeography of North American branchiobdellidans and evolutionary trends in taxonomically informative characters. In addition, I continue to examine biodiversity within the Branchiobdellida using a combination of data types (e.g., morphology, e.g., Williams and Gelder 2011; molecular, Williams et al. in review). The entocytherid ostracods offer similar potential for additional exploration, with questions ranging from species composition and distribution across northern North America, including Canada, to basic ecology and phylogenetic relationships among entocyterid species.

There is great research potential for *O. virilis* and for branchiobdellidans and entocytherids, both in the Prairies region and beyond. The products of my doctoral research provide a solid foundation for future studies of these organisms, alone and as a community of interacting symbionts.

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