

**Phylogenetics and molecular identification of *Ochlerotatus communis* and
Oc. punctor complexes (Diptera: Culicidae)**

by

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Abstract

Accurate identification of pathogens and vectors is essential in epidemiological studies of mosquito-borne pathogens. However, the members of the *communis* and *punctor* complexes are difficult to distinguish because they are highly cryptic species, with little to no species-specific morphological characters. The objective of this thesis is to develop molecular tools, including RFLP and DNA barcoding using cytochrome oxidase I (COI), internal transcribed spacer 2 (ITS2) and the intron of ribosomal protein S12 (RPS12) to facilitate identification of the members of these two complexes in Manitoba. A distinct interspecific distance for COI was found between the members of the *communis* complex included here, and diagnostic RFLP profiles were developed for *Oc. communis* and *Oc. churchillensis*. Relatively low average interspecific genetic distances using COI, ITS2 and RPS12 were observed between the members of the *punctor* complex, indicates no discernable boundaries between these species based on DNA barcoding.

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Chapter 1: General Introduction

1.1 Taxonomic history of *Ochlerotatus*

The taxonomy of mosquitoes (Diptera: Culicidae) began with the publishing of the modern classification system “*Systema Naturae*” by Linnaeus (1758) in the 18th century. In his book he classified two mosquito species in the genus *Culex*. *Aedes* and *Anopheles* were later described by Meigen (1818) in German and Latin. *Ochlerotatus* was first described by Lynch Arribálzaga (1891) which was later given subgeneric status under *Aedes* by Edwards (1917). Subsequently, Edwards (1932) published the most commonly recognized taxonomic system of Culicidae including 1400 species in 30 genera with 89 subgenera. However, subsequent taxonomic studies by Lunt and Nielsen (1971a, b) and Harbach and Kitching (1998) resulted in different classifications that were inconsistent with those suggested by Edwards (1932). Reinert (2000) elevated *Ochlerotatus* to generic rank based on larval, pupal, and genitalic characters, though the recognition of *Ochlerotatus* as a distinct genus has been controversial (Black 2004; Savage and Strickman 2004; Edman 2005; Savage 2005). *Ochlerotatus*, as one of the largest genera of Culicidae, now includes 21 subgenera and 276 species (Reinert *et al.* 2009; Rattanarithikul *et al.* 2010). There are 45 *Ochlerotatus* species distributed in Canada (Wood *et al.* 1979; Thielman and Hunter 2007).

1.2 Black-legged *Ochlerotatus* species

Ochlerotatus species can be divided into two main divisions with respect to morphology of the hind legs: black-legged and banded-legged *Ochlerotatus* species. Edwards (1932) characterized the black-legged *Ochlerotatus* species with respect to the absence of tarsal bands on their hind legs. This division includes groups F (*scapularis*-group), G

(*communis*-group) and H (*rusticus*-group) in his classification. Members of group F are distinct from the other two groups by lacking lower mesepimeral setae. Members of group H can be identified from members of group G in the larval stage and only based on the presence of numerous setae on the siphon (Edwards 1932). The black-legged *Ochlerotatus* species show much uniformity in adult morphological characters causing numerous taxonomic difficulties (Rempel 1950; Beckel 1954; Steward 1968).

Accurate and rapid identification of pathogens and vectors is vital in epidemiological studies of mosquito-borne pathogens. However, most morphological characters of *Ochlerotatus* mosquitoes are not species specific. Additionally, hairs and scales important for identification can easily be destroyed through the collection process such as trapping, transporting, and even identification. Thus, morphology-based identification is not always sufficient for the accurate identification of mosquito specimens. Molecular tools can be applied for species identification in all developmental stages as well as for damaged specimens, and therefore can greatly improve the reliability of species identification.

1.3 DNA barcoding and its application for mosquito identification

DNA barcoding is a fairly novel system for accurate and rapid species identification by assessing the diversity of a short standardized gene sequence such as cytochrome c oxidase subunit I (COI) (Hebert *et al.* 2003a). After COI amplification, the similarity of unidentified COI sequences can be related to identified and sequenced reference taxa using a tree-building method such as a Neighbour-Joining (NJ) analysis. The efficiency of this DNA-based identification method depends on intraspecific divergence (levels of DNA variation within a species) and interspecific divergence (levels of DNA variation between species). In general,

lower intraspecific divergence and higher interspecific divergence are required for diagnosable boundaries for species identification using this technique. Although COI is one of the highly conserved genes of the mitochondrial genome, it is composed of some rapidly evolving sites and shows a high incidence of base substitutions at third codon positions (Bernaconi *et al.* 2000). The COI sequences can easily be amplified using universal primers, and be analyzed after performing a simple alignment process. These features indicate the distinct advantage of this gene as a standard identification tag in DNA barcoding, and consequently increase the accessibility of this molecular method for species identification in a wide range of organisms (Hebert *et al.* 2003a). The genetic distance in COI between most studied insect species has been reported as more than 2-3%, and this level of sequence divergence is suggested as a possible threshold for diagnosing species (Hebert *et al.* 2003b). Interspecific genetic distances using COI have been reported as roughly 20 times higher than intraspecific genetic distances in mosquito species (Cywinska *et al.* 2006), highlighting the utility of DNA barcoding as a tool for species identification in this family.

1.4 RFLP and its application as a DNA-based identification tool

Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) is a DNA fingerprinting technique used to detect the nuclear variation of DNA sequences. This molecular method was developed in 1980 for species of bacteria (Botstein *et al.* 1980). After amplification of a target gene, the PCR product will be digested by one or several restriction enzymes. Consequently, based on the specific restriction recognition sites of the enzymes, a number of small DNA fragments will be produced (Hoy 2003). The digested DNA fragments are called restriction fragment length polymorphisms, or RFLPs, which are then resolved according to their size through gel electrophoresis (Roundy *et al.*

1995). In general, the smaller fragments migrate faster than the longer fragments through the gel (Hoy 2003). The RFLP patterns depend on the type of restriction enzyme used, and the sequence variation present in the target DNA at the restriction recognition sites of these enzymes. More than 1400 restriction enzymes have been recorded, most of which recognize restriction sites composed of four to six base pairs on the target DNA (Hoy 2003). Molecular changes such as substitutions or insertions/deletions (indels) in the restriction recognition sites across different specimens may result in the production of different RFLP patterns for these organisms. The molecular changes between the individuals of the same species may also result in variable RFLP patterns for these specimens which may limit repeatability of the RFLP method. Generally, analyzing several populations of a species will reveal any intraspecific variability in RFLP patterns. RFLP has been successfully used as an effective molecular tool for the identification of two cryptic *Ochlerotatus* species: *Oc. triseriatus* Say and *Oc. hendersoni* Cockerell (Reno *et al.* 2000). As sequencing is not required for identification, RFLP may be a cost-effective and rapid alternative to DNA barcoding for species identification.

1.5 Thesis objectives

This thesis consists of two separate studies (Chapters 2 and 3) that each begin with a statement of the problems and objectives followed by materials and methods, results, and discussion for the study. Chapter 4 presents an overall summary and a discussion of future directions based on the results and discussions provided in earlier chapters. Many black-legged *Ochlerotatus* species, particularly the members of *communis* and *punctor* complexes, are difficult to distinguish due to overlapping, quantitative morphological characters across species. Molecular methods offer many advantages for species identification and especially to

distinguish cryptic species accurately for monitoring mosquito-borne pathogens. The overall objective of this thesis is to develop molecular tools, including DNA barcoding and RFLP, to facilitate identification of the members of the *communis* and *punctor* complexes in Manitoba.

Chapter 2: DNA barcoding and PCR-RFLP identification of the *communis* complex (Diptera: Culicidae)

2.1 Introduction

According to Ellis and Brust (1973), the *communis* complex consists of four morphologically cryptic species in North America, including: *Oc. communis* (De Geer 1776), *Oc. churchillensis* (Ellis and Brust 1973), *Oc. nevadensis* (Chapman and Barr 1964), and *Oc. tahoensis* (Dyar 1928). The morphological characteristics for identifying these species are only diagnostic for one species within the complex or for a specific developmental stage or sex, causing the morphology-based identification to be extremely difficult. For example, the shape of the larval comb scales is only diagnostic for the identification of *Oc. nevadensis* in the larval stage (Ellis and Brust 1973). In the adult stage, although males of the complex may be distinguished by the size of the genital gonocoxites, females are much more difficult to identify (Ellis and Brust 1973). The shape of the hind tarsal claw is only diagnostic for the identification of *Oc. communis* from the other members of this complex, but this character is not always reliable (Brust and Munstermann 1992).

The members of the *communis* complex have interesting distributions suggesting that both allopatric and sympatric speciation have played a role in their evolution. *Oc. communis* has a Holarctic distribution and has been collected throughout much of North America (Wood *et al.* 1979). *Oc. nevadensis* has been reported from mountainous regions of southeastern British Columbia and the northwestern United States, suggesting that this species might be reproductively isolated from other species within the complex (Belton 1982, 1983; Schutz and Eldridge 1993). *Oc. tahoensis* has a restricted range of distribution from the Sierra Nevadas to

the southern Cascades and Klamath mountains in California and mainly has been collected at high elevations (1500-3000 m) in these areas (Schutz and Eldridge 1993). *Oc. tahoensis* can be distinguished from *Oc. nevadensis* based on larval morphological characters and from all the other members of this complex with respect to its collection locality (Darsie 1995).

Ochlerotatus communis and *Oc. churchillensis* have a sympatric distribution in northeastern and southeastern Manitoba and western Alberta (Ellis and Brust 1973; Wood *et al.* 1979). These two species are similar in terms of their biology and larval habitats, as both species can be found in snow-melt pools within or at the margins of deciduous and coniferous forests (Ellis and Brust 1973). However, *Oc. churchillensis* females have smaller salivary glands than *Oc. communis* due to their autogenous ovarian development. This character may be useful to distinguish populations of *Oc. churchillensis* from all other members of the complex (Ellis and Brust 1973). Morphometric characteristics of the hind tarsal claws are the only diagnostic features used for the identification of adult females, although the measurements are not discrete with considerable overlap across species (Brust and Munstermann 1992). Slight morphological differences for the males of *Oc. communis* and *Oc. churchillensis* and their reproductive differences originally led Ellis and Brust (1973) to describe *Oc. churchillensis* as a distinct species from *Oc. communis*. Brust and Munstermann (1992) suggested isozyme analysis as a method for distinguishing members of the *communis* complex. However, this method lacks consistency and requires very fresh specimens, thereby limiting the utility of this technique as a routine tool for species identification (Schutz and Eldridge 1993). Currently, there is no reliable method for quickly and accurately identifying *Oc. communis* from *Oc. churchillensis*.

Accurate and rapid identification of pathogens and vectors is essential in monitoring mosquito-borne pathogens. *Oc. communis* is a vector of Jamestown Canyon Virus (JCV) and Snowshoe Hare Virus (SHV) in North America (McLean *et al.* 1981; Heard *et al.* 1990). However, the clinical significance of *Oc. churchillensis* has not yet been studied. Several boreal *Aedes* (Meigen, 1818) and *Ochlerotatus* (Lynch Arribálzaga, 1891) species are the primary vectors for transmission of JCV and SHV across temperate areas of North America (Grimstad 1988; Andreadis *et al.* 2008; Bennett *et al.* 2012). Although antibodies of JCV have been detected in a wide range of animals such as moose, elk, bison, mule deer, domestic bovines and equines, white-tailed deer is the most important amplifying host in the natural transmission cycle of the virus (Grimstad 1988; Rust *et al.* 1999). The common symptoms of JCV infection in humans ranges from nonspecific, mild febrile illnesses, to acute central nervous system infection and respiratory system involvement (Grimstad 1988, 2001). Although SHV was isolated for the first time from Snowshoe Hare (*Lepus americanus* Erxleben) in Montana, USA in 1959 (Burgdorfer *et al.* 1961), its antibodies were later detected in several domestic animals such as equines, cattle, dog and chicken (Bennett *et al.* 2012). The symptoms of SHV infection in humans include fever, headache, vomiting, meningitis and encephalitis (Fauvel *et al.* 1980).

Developing molecular markers could improve the reliability of identification of the cryptic species in the *communis* complex and greatly facilitate mosquito vector surveillance. Although DNA barcoding has been utilized for the identification of several species of *Aedes* and *Ochlerotatus* in Canada by Cywinska *et al.* (2006), *Oc. churchillensis* was not included in their analysis. The major aim of this study was to evaluate the efficacy of DNA barcoding for species identification of the members of the *communis* complex in North America (excluding

Oc. nevadensis). The second objective of the present study was to develop diagnostic RFLP patterns for *Oc. churchillensis* and *Oc. communis* as a rapid species identification technique for use in vector surveillance program in Manitoba.

2.2 Materials and Methods

2.2.1 Mosquito collection

Mosquito specimens were collected from multiple localities in Manitoba using aspirators from May to July 2011 (Table 2.1). The specimens of *Oc. churchillensis* collected by R.A. Brust were obtained from the Wallis-Roughley Museum of Entomology (University of Manitoba). Additional specimens of *Oc. churchillensis* were also obtained from the collected materials of a project entitled “Insect biodiversity in the Churchill region and Wapusk National Park”, which were previously collected using a malaise trap in July 2010. Although using malaise traps is not an appropriate method for trapping mosquito specimens, these specimens were adequately preserved to make putative morphological identifications of *Oc. churchillensis* and allowed for increased sampling of this taxon. Collected female specimens were identified using the morphological keys of Ellis and Brust (1973), Wood *et al.* (1979), and Thielman and Hunter (2007) and deposited in the Wallis-Roughley Museum of Entomology at the University of Manitoba. The specimen locality, voucher, and GenBank numbers for the analyzed specimens is provided in Table 2.1.

2.2.2 DNA extraction and COI mtDNA amplification

Genomic DNA was extracted from the legs of newly collected specimens. However, in order to obtain the highest quality of genomic DNA from previously mounted museum specimens, DNA extraction was carried out from the whole body of these specimens. DNA

extraction was performed using the Qiagen DNeasy extraction kit (Qiagen Inc. Valencia, CA, USA) following the manufacturer's instructions.

Table 2.1. Specimen locality, voucher, and GenBank numbers for *Oc. communis* and *Oc. churchillensis* specimens collected in this study.

Species	Voucher No.	Accession No.	Label information
<i>Oc. churchillensis</i>	0189652	KC713599	Canada, MB, Churchill, near Stephens' Lake 56° N. 94° W. 22-vi-1983, RA Brust
	0192649	KC713607	
	0192040	KC713604	Canada, MB, Churchill, SE of Churchill harbor, 58° N. 94° W. 18-vi-1985, RA Brust
	0355353	KC713602	
	0355354	KC713601	Canada, MB, Churchill, Herchmer, 57°13' N. 94° 19' W. (11-22)-vii-2010, TS Woodcock and PG Kevan
	0355355	KC713603	
<i>Oc. communis</i>	0355356	KC713606	Canada, MB, Winnipeg, Sandilands Prov. Forest 49° 30' N. 96° 0' W. 27-v-2011, HH Namin
	0355357	KC713598	Canada, MB, Whiteshell Prov. Forest, Pine Point Rapids Trail 50° 10' N. 95° 60' W. 03-vi-2011, 10-vi-2011, 17-vi-2011, HH Namin
	0355358	KC713596	
	0355359	KC713600	
	0355361	KC713605	Canada, MB, Spruce Woods Prov. Forest, Marsh's lake, 49° 40' N. 99° 16' W. 24-vi-2011, HH Namin
	0355360	KC713597	Canada, MB, Winnipeg, Birds Hill Prov. Pk. West Pine Ridge, 50° 00' N. 96° 90' W. 30-vi-2011, HH Namin

Universal standard barcoding primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994) were used to amplify an approximately 660bp fragment of COI. The PCR reaction mixtures contained 1x Standard *Taq* Reaction Buffer (10 mM Tris-HCl; 50 mM KCl; 1.5 mM MgCl₂ pH 8.3, New England Biolabs, Ipswich, MA, USA), 200 µM dNTP (Invitrogen, Carlsbad, CA, USA), 0.4 µM of each primer, 1 unit *Taq* DNA polymerase (New England Biolabs), approximately 1-2 µg of genomic DNA, and purified water to a final volume of 50 µL. The PCR amplification protocol consisted of an initial denaturation at 95°C for 60 s, followed by 30 cycles of 60s at

94°C, 60 s at 50°C, 60 s at 72°C, and a final extension of 72°C for 7 min. The PCR products were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions, but using 25% of the recommended reagent amount. Sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), with reaction products analyzed on an Applied Biosystems 3730xl DNA Analyzer at Eurofins MWG Operon (Huntsville, AL, USA).

2.2.3 Data analysis

Sequences were assembled, trimmed and edited using Geneious version 5.4.5 (Drummond *et al.* 2011) and deposited in GenBank (see Table 2.1 for Accession numbers). Additional analyzed COI sequences of *Ochlerotatus* species were obtained from the published sequence data of Mitchell *et al.* (2002), Morlais and Severson (2002), Cywinska *et al.* (2006), and Gibson *et al.* (2012), and are listed in Table 2.2. Multiple sequence alignments were performed using ClustalX v. 2.0.11 (Larkin *et al.* 2007) with default settings and checked manually to find the correct COI open reading frame. Inter- and intra-species genetic uncorrected p-distances were calculated using MEGA 5 (Tamura *et al.* 2011).

The general time-reversible model, with a parameter for invariant sites and rate heterogeneity modeled under a gamma distribution (GTR+I+G), was determined as the best fitting model of nucleotide substitution for the analyzed data matrix using the Akaike Information Criterion (AIC). The model selection test was carried out using PAUP beta v. 4.0 b10 (Swofford 2000), ModelTest v.3.7 (Posada and Crandall 1998), and the ModelTest Server (Posada 2006).

Table 2.2. List of additional analyzed sequences with their GenBank accession numbers and respective references.

Species	Accession No.	Reference	Species	Accession No.	Reference	Species	Accession No.	Reference	
<i>Ae. cinereus</i>	GU907869	Cywinska <i>et al.</i> 2006	<i>Oc. canadensis</i>	GU907863	Cywinska <i>et al.</i> 2006	<i>Oc. grossbecki</i>	GU907901	Cywinska <i>et al.</i> 2006	
	GU907870			GU907864			GU907902		
	GU907871			GU907865			GU907903		
	GU907872			GU907866			GU907904		
<i>Ae. aegypti</i>	AF425846	Mitchell <i>et al.</i> 2002	<i>Oc. cantator</i>	GU907867		<i>Oc. implicatus</i>	GU907905		
	AF380835	Morlais and Severson 2002		GU907868			GU907906		
	AF390097			GU907873			GU907907		
	AY056597			GU907874			GU907908		
<i>Ae. vexans</i>	AY056598	Cywinska <i>et al.</i> 2006	<i>Oc. communis</i>	GU907875	Gibson <i>et al.</i> 2012	<i>Oc. intrudens</i>	GU907909	Cywinska <i>et al.</i> 2006	
	GU907989			GU907876			GU907910		
	GU907990			GU907877			GU907911		
	GU907991			NEONW119-11			GU907912		
	GU907992			NEONW120-11			GU907913		
	GU907993			NEONW121-11			GU907914		
	GU907994			NEONW122-11		<i>Oc. japonicus</i>	GU907915		
	GU907995			NEONW123-11			GU907916		
	GU907996			NEONW124-11			GU907917		
	GU907997			NEONW125-11			GU907918		
	GU907998			NEONW126-11			GU907919		
	GU907999			NEONW127-11			GU907920		
	GU908000			NEONW128-11			GU907921		
	GU908001			NEONW129-11			GU907922		
<i>Cu. territans</i>	GU908103	Cywinska <i>et al.</i> 2006	<i>Oc. communis</i>	NEONW130-11	Gibson <i>et al.</i> 2012	<i>Oc. provocans</i>	GU907923	Cywinska <i>et al.</i> 2006	
	GU908104			NEONW131-11			GU907924		
	GU908105			NEONW132-11			GU907925		
<i>Oc. abserratus</i>	GU907830	Gibson <i>et al.</i> 2012		NEONW133-11			GU907926		
	GU907831			NEONW135-11		<i>Oc. riparius</i>	GU907927		
	GU907832			NEONW136-11			GU907928		
	GU907833			NEONW137-11			GU907929		
	GU907834			NEONW138-11		<i>Oc. sollicitans</i>	GU907930		
	GU907835			NEONW139-11			GU907931		
	GU907836			NEONW140-11			GU907932		
	GU907837			NEONW142-11			GU907933		
	GU907838			NEONT214-10			GU907934		
	GU907845			NEONT216-10			GU907935		
<i>Oc. atropalpus</i>	GU907846	Gibson <i>et al.</i> 2012		NEONT217-10			GU907936		
	GU907847	<i>Oc. dorsalis</i>	NEONT224-10	<i>Oc. sticticus</i>		GU907937			
	GU907848		NEONT225-10			GU907938			
	GU907849		NEONT226-10			GU907939			
	GU907850	<i>Oc. euedes</i>	GU907878	Cywinska <i>et al.</i> 2006	<i>Oc. stimulans</i>	GU907940	Cywinska <i>et al.</i> 2006		
<i>Oc. aurifer</i>	JX259520		Gibson <i>et al.</i> 2012			GU907879		GU907941	
	JX259521					GU907880		GU907942	
	JX259522					GU907881		GU907943	
	JX259523					GU907882		GU907944	
	JX259524					GU907883		GU907945	
	JX259525					GU907884		GU907946	
	JX259526					GU907885		GU907947	
	JX259527					GU907886		GU907948	
	JX259528					GU907887		GU907949	
	JX259529					GU907888		GU907950	
<i>Oc. canadensis</i>	GU907851	Cywinska <i>et al.</i> 2006	<i>Oc. excrucians</i>	GU907889	Cywinska <i>et al.</i> 2006	<i>Oc. stimulans</i>	GU907951	Cywinska <i>et al.</i> 2006	
	GU907852			GU907890			GU907952		
	GU907853			GU907891			GU907953		
	GU907854			GU907892			GU907954		
	GU907855			GU907893			GU907955		
	GU907856			GU907894			GU907956		
	GU907857		<i>Oc. fitchii</i>	GU907895		<i>Oc. stimulans</i>	GU907957		
	GU907858			GU907896			GU907958		
	GU907859			GU907897			GU907959		
	GU907860			GU907898			GU907960		
	GU907861			GU907899			GU907961		
	GU907862			GU907900			GU907962		
							GU907963		

Table 2.2 Continued. List of additional analyzed sequences with their GenBank accession numbers and respective references.

Species	Accession No.	Reference
<i>Oc. stimulans</i>	GU907964	Cywinska <i>et al.</i> 2006
	GU907965	
	GU907966	
	GU907967	
	GU907968	
<i>Oc. tahoensis</i>	JX259678	Gibson <i>et al.</i> 2012
	JX259677	
<i>Oc. triseriatus</i>	GU907969	Cywinska <i>et al.</i> 2006
	GU907970	
	GU907971	
	GU907972	
	GU907973	
	GU907974	
	GU907975	
	GU907976	
	GU907977	
	GU907978	
<i>Oc. trivittatus</i>	GU907979	
	GU907980	
	GU907981	
	GU907982	
	GU907983	
	GU907984	

Bayesian phylogenetic inference was performed using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with two independent searches with four chains each, default priors, and run for 5,000,000 generations, sampling every 1000th generation. Similar to phylogenetic analysis of Cywinska *et al.* (2006), *Culex territans* (Walker, 1848) was chosen as the outgroup for the Bayesian analysis. Convergence of runs and suitable mixing was ascertained by examining the likelihood plots for each run and when the average standard deviation of split frequencies was less than 0.02 (Huelsenbeck and

Ronquist 2001; Ronquist and Huelsenbeck 2003). Trees were summarized from the two independent searches using majority rule consensus after discarding 25% of the samples for burn-in.

2.2.4 Restriction digestion reaction

AluI was determined as the best restriction enzyme among 360 commercially restriction enzymes available on NEB Cutter 2.0 (Vincze *et al.* 2003) for producing diagnostic RFLP pattern for each species. The digestion mixtures consisted of 5-7 µL PCR products (approximately 50-150 ng), 5 units of *AluI* (Invitrogen), 1x incubation buffer (1.5 mM Tris-HCl, pH 7.5; 1.5 mM MgCl₂; 0.15 mM Dithiothreitol) and purified water to a final volume of 15 µL. The digested mixture was incubated following the manufacturer's digestion enzyme instructions (37 °C for two hours), and the digested fragments were resolved on a 1.5% agarose gel, stained with Gel Red (Biotium Inc. California, USA). The restricted DNA patterns were photographed on a Gel Doc EZ imager (BioRAD Inc, USA) and the length of each restriction fragment was estimated using a 50-base DNA ladder (Novagen, USA).

2.3 Results

2.3.1 Sequence analysis

The final alignment was 658bp in length of which 236 (35.9%) sites were variable and 224 (34%) were parsimony informative. The COI sequences, as previously reported by Cywinska *et al.* (2006), showed a strong A-T base composition bias ranging from 65.4% *Oc. implicatus* (Vockeroth, 1954a) to 70.6% *Aedes vexans* (Meigen, 1830), with an average of 68% for all taxa. No stop codons or insertion/deletion events were observed among the studied sequences. Intraspecific genetic variation within the *communis* complex ranged from

0% for *Oc. tahoensis* to 0.94% for *Oc. churchillensis* (Table 2.3). However, only two sequences of *Oc. tahoensis* were included in this analysis and no reference sequence for *Oc. nevadensis* was available for this study. Among the members of the *communis* complex (excluding *Oc. nevadensis*), the highest interspecific genetic variation was observed between *Oc. churchillensis* and *Oc. communis* (5.1%) while the lowest value was detected between *Oc. churchillensis* and *Oc. tahoensis* (3.0% - Table 2.3).

Table 2.3. Average inter- and intraspecific p-distances among the members of the *communis* complex based on a 658bp sequence of cytochrome oxidase 1 and the number of analyzed specimens for each species.

	Taxon	Average interspecific p-distance		Number of specimens	Average intraspecific p-distance
		1	2		
1	<i>Oc. churchillensis</i>			6	0.0094
2	<i>Oc. communis</i>	0.051		39	0.0084
3	<i>Oc. tahoensis</i>	0.030	0.045	2	0

2.3.2 Bayesian analysis of *Aedes* and *Ochlerotatus*

Based on Bayesian inference of phylogenetic relationships, *Aedes* and *Ochlerotatus* were recovered as well supported monophyletic sister taxa (Fig. 2.1). Within the members of the *communis* complex included in this study, *Oc. churchillensis* was recovered as sister to *Oc. tahoensis* (Fig. 2.1). However, one sequence of *Oc. aurifer* (GU907850) was recovered within the *Oc. churchillensis* clade. Since all the other sequences of *Oc. aurifer* were recovered as a well supported monophyletic clade (posterior probability 1), and sister to *Oc. intrudens*, this sequence (GU907850) was likely contaminated or the specimen was misidentified prior to sequencing (Fig. 2.1).

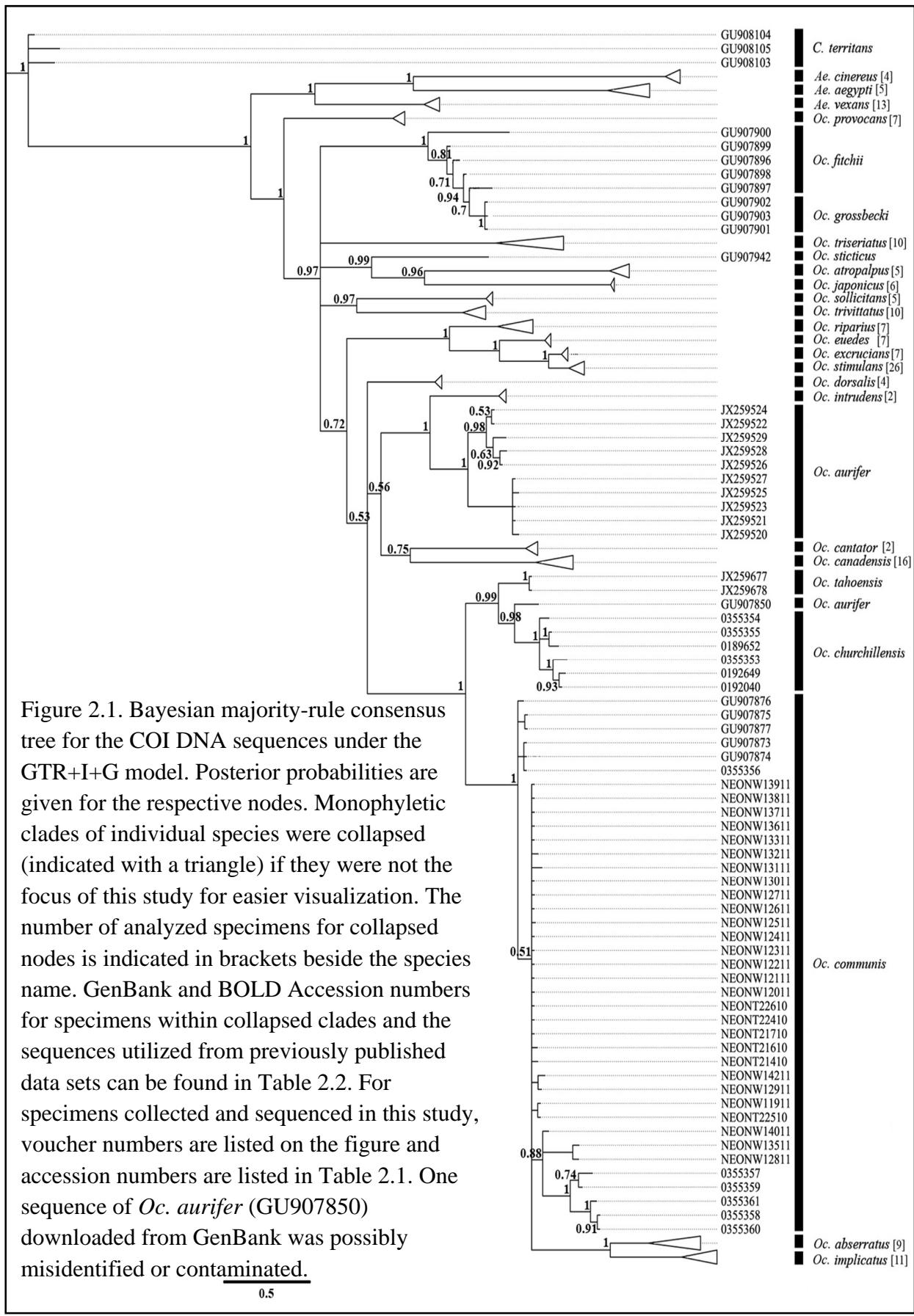


Figure 2.1. Bayesian majority-rule consensus tree for the COI DNA sequences under the GTR+I+G model. Posterior probabilities are given for the respective nodes. Monophyletic clades of individual species were collapsed (indicated with a triangle) if they were not the focus of this study for easier visualization. The number of analyzed specimens for collapsed nodes is indicated in brackets beside the species name. GenBank and BOLD Accession numbers for specimens within collapsed clades and the sequences utilized from previously published data sets can be found in Table 2.2. For specimens collected and sequenced in this study, voucher numbers are listed on the figure and accession numbers are listed in Table 2.1. One sequence of *Oc. aurifer* (GU907850) downloaded from GenBank was possibly misidentified or contaminated.

This sequence of *Oc. aurifer* (GU907850) was downloaded from GenBank and has not been published in a peer reviewed journal, highlighting the risks associated with using GenBank sequences, particularly from unpublished data. The *communis* complex was recovered as paraphyletic with respect to *Oc. abserratus* + *Oc. implicatus* (Fig. 2.1).

2.3.3 PCR-RFLP analysis of *Oc. churchillensis* and *Oc. communis*

The PCR-RFLP diagnostic patterns were developed for the identification of *Oc. churchillensis* and *Oc. communis* according to the species-specific restriction sites of *AluI* (5'-AGCT-3') in COI sequences. The developed RFLP patterns were consistent with the predicted digestion patterns for the sequences of *Oc. communis* and *Oc. churchillensis* obtained from GenBank, BOLD, and the specimens collected in this study using NEB Cutter 2.0 (Vincze *et al.* 2003). Corresponding to the observed clades for *Oc. churchillensis* in the Bayesian analysis, three different RFLP patterns were detected for the individuals of this species after digestion with *AluI*. The first RFLP pattern was characterized by fragments of approximately 250, 200, 160, and 120bp and was observed in three specimens of *Oc. churchillensis* including 0355353, 0192649, and 0192040 (Fig. 2.2: lanes 1, 5, and 6, respectively). The second RFLP profile was typified by fragments of approximately 320, 250, 120, and 50bp, and was observed in two specimens of *Oc. churchillensis* including 0355355 and 0189652 (Fig. 2.2: lanes 3 and 4, respectively). The third RFLP pattern included fragments of approximately 250, two fragments of 150, and one fragment at 120 and 50bp. This pattern was only observed in one specimen of *Oc. churchillensis* (Fig. 2.2: 0355354 in lane 2). Variation at the third codon position of amino acids 139 and 175 cause the three different RFLP patterns observed in *Oc. churchillensis*. Alternatively, digestion with *AluI* produced one consistent diagnostic RFLP pattern for all specimens of *Oc. communis* tested in

this study. The *Oc. communis* RFLP profile was characterized by fragments of approximately 400, 150, 120 and 50bp (Fig. 2.2: 0355356-0355361 shown in lanes 7-12, respectively). This RFLP pattern was also consistent with the predicted RFLP pattern for all the sequences of *Oc. communis* downloaded from GenBank and BOLD using NEB Cutter 2.0 (Vincze *et al.* 2003).

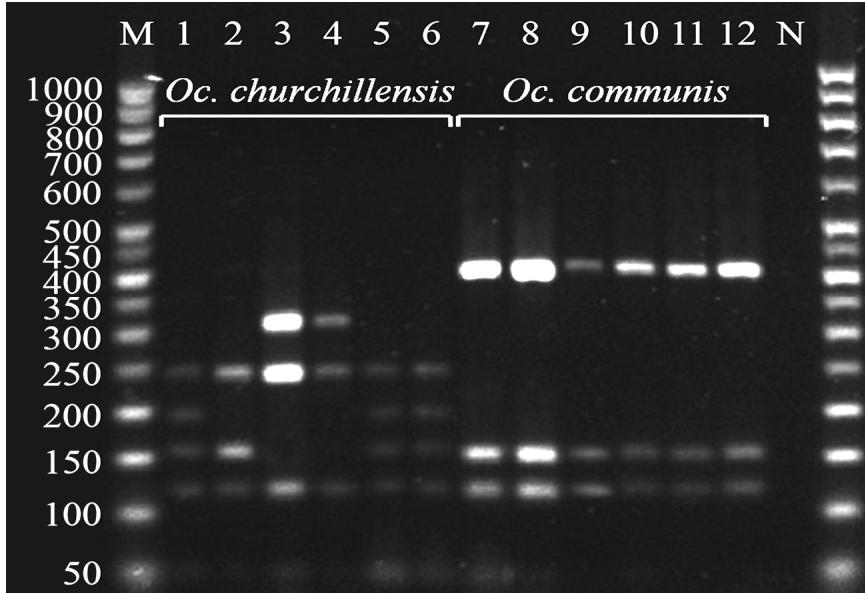


Figure 2.2. Digestion patterns of COI PCR products with *AluI* restriction enzyme for *Oc. communis* and *Oc. churchillensis*. Gel lanes are labeled across the top as follows: M: 50bp DNA ladder, 1-6: *Oc. churchillensis* (0355353-5, 0189652, 0192649, 0192040), 7-12: *Oc. communis* (0355356-61) and N: Negative control. According to the RFLP patterns, a 250bp band was detected in all specimens of *Oc. churchillensis* (never observed in *Oc. communis*), and a 400bp band was observed for all specimens of *Oc. communis* (never observed in *Oc. churchillensis*).

Although the analyzed specimens of *Oc. communis* and *Oc. churchillensis* were not collected from the sympatric areas, the RFLP pattern for *Oc. communis* (with the largest fragment at 400bp) was distinct from all RFLP profiles for *Oc. churchillensis* (with no bands larger than 320bp) after digestion using *AluI*. These distinctive profiles were created because

the 73rd and 74th amino acids (based on the complete CO1 gene from *Aedes aegypti* L. NC_010241.1) are conserved Glycine and Alanine residues, respectively in the *Oc. communis* complex. In all members of the complex analyzed here, the codon for the Glycine residue is always GGA. However, the downstream Alanine residue is different in *Oc. churchillensis* (GCT) and *Oc. communis* (GCC), causing an additional cut at this site for *Oc. churchillensis*. Thus, this mutation confers the diagnostic RFLP patterns for these species. Based on the two downloaded sequences analyzed using NEB Cutter 2.0 (Vincze *et al.* 2003), it is predicted that *Oc. tahoensis* would not be cut by *AluI* at this site, as the Alanine residue is encoded by GCA. Therefore, this species would exhibit the same 400bp fragment as observed in *Oc. communis* after digestion by *AluI* (Fig. 2.2).

2.4 Discussion

A sequence divergence of less than 2% has been reported by Cywinska *et al.* (2006) for species delineation of *Aedes* and *Ochlerotatus* species through DNA barcoding. The low intraspecific variability (average 0.6%) and high interspecific distances (average 4.2%) between *Oc. churchillensis*, *Oc. communis* and *Oc. tahoensis* indicate that these species can be delineated using COI barcodes (Table 2.2). These distinct barcodes support the conclusions of Ellis and Brust (1973) that *Oc. churchillensis* is a distinct species from *Oc. communis*. The distinct DNA barcode for *Oc. tahoensis* also confirms the previous results by Ellis and Brust (1973) and Brust and Munstermann (1992) based on morphological and allozyme evidence for resurrecting *Oc. tahoensis* as a valid, distinct species from *Oc. communis*.

Aedes and *Ochlerotatus* were recovered as well supported monophyletic sister taxa in our Bayesian analysis (Fig. 2.1). These results support the proposed classification of Culicidae

by Reinert *et al.* (2000) and also are congruent with the results of Shepard *et al.* (2006) based on 18s rDNA, indicting *Aedes* and *Ochlerotatus* as distinct valid genera. Although the recognition of *Ochlerotatus* as a distinct genus from *Aedes* has been controversial (Black 2004; Savage and Strickman 2004; Edman 2005; Savage 2005), the current study provides additional evidence to confirm Reinert *et al.*'s (2000) recognition of *Ochlerotatus*. In spite of distinct morphological differences that separate *Oc. abserratus* and *Oc. implicatus* from members of the *communis* complex (Carpenter and LaCasse 1955; Wood *et al.* 1979), the phylogenetic relationships recovered here suggest that the *communis* complex may include additional species (Fig. 2.1). Since not all the taxa within the *communis* complex were included in this study and one gene phylogenies are typically inadequate for resolving phylogenetic relationships (Rokas *et al.* 2003; Rubinoff and Holland 2005; Sharanowski *et al.* 2011), additional genes and taxon sampling will be necessary to establish the limits of the members of this complex.

Although efforts were made to collect *Oc. churchillensis* from the known sympatric localities in southern Manitoba, all the analyzed specimens of this species were collected in northern Manitoba, near Churchill. This indicates that currently this species probably has a more restricted distribution, similar to *Oc. tahoensis* and *Oc. nevadensis*, which can facilitate identification of all species within the *communis* complex according to their locality. The PCR-RFLP method can be used to reduce taxonomic uncertainty and can facilitate the efficient identification of these cryptic species in all life stages. Additionally, as sequencing is not required for identification, this method allows relatively inexpensive molecular identification compared with DNA-barcoding for distinguishing *Oc. churchillensis* from *Oc. communis*. This technique is quick and requires approximately six hours to perform, including

15 minutes for DNA extraction (using fast DNA extraction kits), two hours for DNA amplification, one hour for checking the integrity of PCR products, two hours for restriction digestion, and one hour to visualize the RFLP banding patterns.

Chapter 3: Phylogenetic analysis and molecular identification tools for identifying the *Ochlerotatus punctor* species complex (Diptera: Culicidae) in Manitoba

3.1 Introduction

Predicting the transmission patterns of mosquito-borne pathogens relies on species specific identification of the vectors and pathogens. Rapid and accurate identification of mosquito species is especially important to determine potential vectors with numerous emerging (or re-emerging) arboviruses (Gould and Higgs, 2009). Within the black-legged mosquitoes of the genus *Ochlerotatus* Lynch Arribálzaga, there are several morphologically cryptic species groups that are exceedingly difficult to identify using morphology. This can limit the efficiency and efficacy of mosquito surveillance programs, especially where morphologically cryptic species occur in sympatry. In Manitoba, where mosquito surveillance and control are important public issues, there are several cryptic *Ochlerotatus* species groups that occur in sympatry, including Edwards' (1932) *punctor* subgroup (group G).

The *punctor* subgroup was originally placed by Edwards (1932) in the subgenus *Ochlerotatus* of genus *Aedes* Meigen (1818) and subsequently, Reinert (2000) elevated *Ochlerotatus* to generic rank. Although this classification has been controversial (Black 2004; Savage and Strickman 2004; Edman 2005; Savage 2005), it has been supported by molecular phylogenetic analyses (Shepard *et al.* 2006; Cywinska *et al.* 2006; Namin *et al.* 2013). In North America, the *punctor* subgroup (also referred to here as the *punctor* complex) consists of five cryptic species including *Oc. aboriginis* (Dyar, 1917), *Oc. abserratus* (Felt and Young, 1904), *Oc. hexodontus* (Dyar, 1916), *Oc. punctodes* (Dyar 1919a), and *Oc. punctor* (Kirby, 1837). *Oc. punctodes* is predominant in salt marsh pools from the coastal region of Alaska

(Frohne 1953; Knight 1948). *Oc. aboriginis* is distributed in temperate forests of the northern Pacific, western slopes of northern Idaho, high elevation regions of the Oregon and Washington Cascades, and mountainous areas of coastal British Columbia (Gjullin and Eddy 1972; Wood *et al.* 1979; Furnell 1986). *Oc. abserratus* has a geographically separated distribution from *Oc. aboriginis* and *Oc. punctodes* (Carpenter and LaCasse 1955). *Oc. abserratus* larvae can be found in early spring pools in eastern Canada and the northeastern United States with its range extending west to Manitoba, south to Maryland and north to Hudson Bay (Carpenter and LaCasse 1955; Gimnig 2000). *Oc. puncotor* and *Oc. hexodontus* are Holarctic species and widely distributed throughout most of Canada and the northern United States (Wood *et al.* 1979). *Oc. puncotor* has been primarily reported from boreal forests, whereas *Oc. hexodontus* has been more frequently associated with tundra, or high elevations in North America (Jenkins and Knight 1950; Wood *et al.* 1979; Furnell 1986). However, the extent of the distribution of these species is not well documented. *Oc. abserratus*, *Oc. hexodontus* and *Oc. puncotor* are morphologically cryptic and have a sympatric distribution in Manitoba, and thus pose particular difficulties for mosquito identification and surveillance programs in this region.

Larval and adult female characteristics are typically utilized to identify members of the cryptic *puncotor* complex; however, the characters are not diagnostic for every species. For example, the number and size of seta 7-II of the second abdominal segment and the length of seta 1-X of the saddle are the main larval characters used for the identification of *Oc. aboriginis* from the other members of this complex (Carpenter and LaCasse 1955; Wood *et al.* 1979). *Oc. punctodes* can be distinguished from the other four species based on the size and shape of the anal papillae and saddle, and by its geographical distribution and biology (Knight

1951; Frohne 1953; Carpenter and LaCasse 1955). The number and size of caudal setae (2-X and 3-X) of the saddle (Fig. 3.1) and length and the number of comb scales on the last abdominal segment (Fig. 3.2) are used for distinguishing larvae of *Oc. abserratus*, *Oc. punctor* and *Oc. hexodontus* from each other (Carpenter and LaCasse 1955; Wood *et al.* 1979). However, these three species exhibit morphological variation in these characters which overlap across species (Knight 1951; Vockeroth 1954b; Wood *et al.* 1979; Furnell 1986).

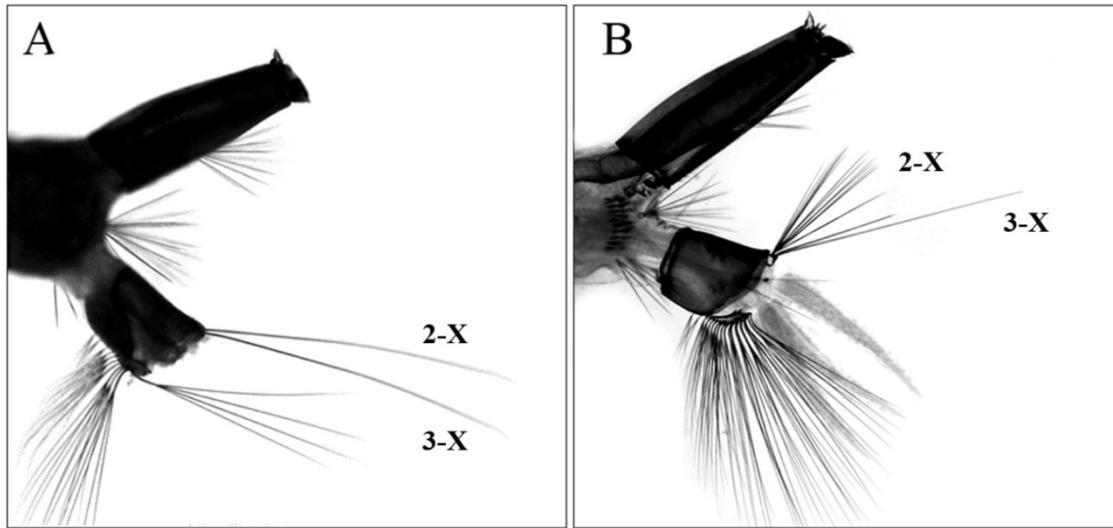


Fig. 3.1. Terminal segment for larva of *Oc. abserratus* (A), and *Oc. hexodontus* (B). Caudal setae (2-X and 3-X) are unbranched and of equal length in *Oc. abserratus* (A), while they are branched and of variable length in both *Oc. hexodontus* and *Oc. punctor* (B) (Carpenter and LaCasse 1955; Wood *et al.* 1979).

The number of scales on the probasisternum (Fig. 3.3) has been reported as the most reliable feature for identifying adult females of *Oc. abserratus*, *Oc. punctor* and *Oc. hexodontus* from each other by Gimnig (2000). However, there are no diagnostic morphological characters for distinguishing adult males (Knight 1951; Wood *et al.* 1979).

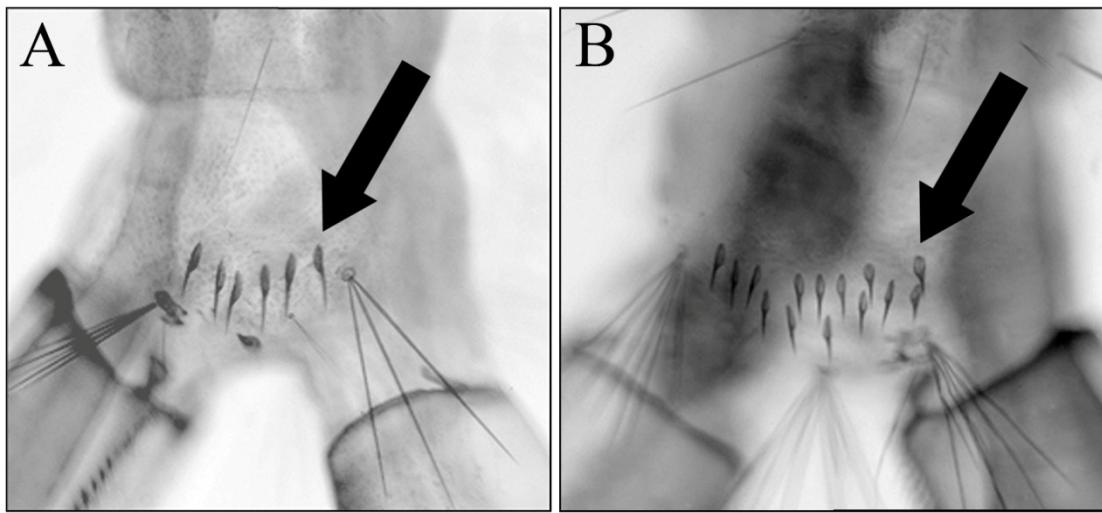


Fig. 3.2. The comb scales on the last abdominal segment are indicated by arrows for larva of *Oc. hexodontus* (A) and *Oc. punctor* (B). There are 4-12 comb scales (usually fewer than 10) in *Oc. abserratus* and *Oc. hexodontus* (A), and 5-25 (usually more than 10) in *Oc. punctor* (B) (Carpenter and LaCasse 1955; Wood *et al.* 1979).

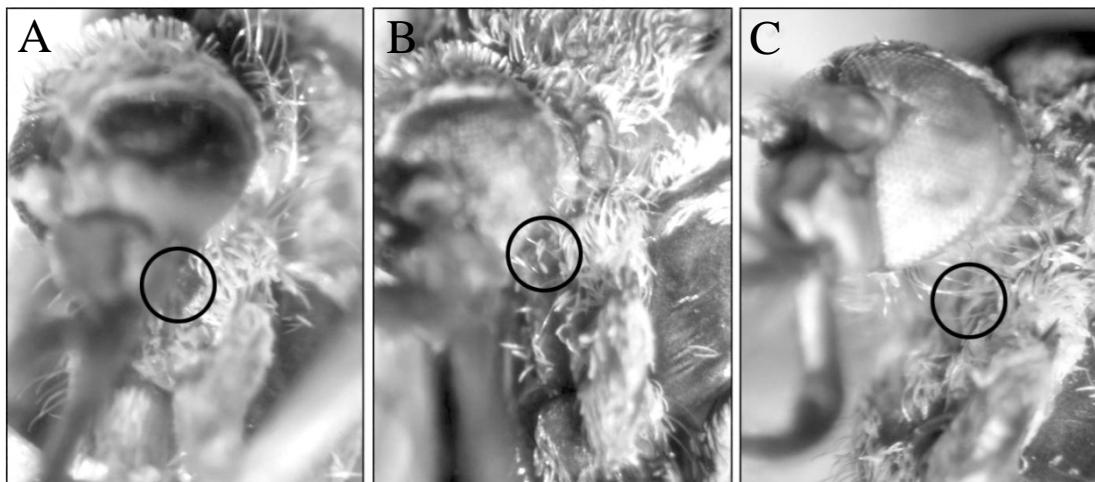


Fig. 3.3. The probasisternum areas for members of the *punctor* complex are indicated by circles. This area is bare in *Oc. abserratus* (A), while *Oc. punctor* has few scales ($n < 10$) on the probasisternum (B), and this area is heavily scaled ($n > 10$) in *Oc. hexodontus* (C) (Gimnig 2000).

All members of the *punctor* complex are univoltine and their eggs hatch after passing through an obligatory diapause (Furnell 1986). *Oc. abserratus*, *Oc. hexodontus*, and *Oc. punctor* opportunistically feed on a variety of hosts including humans, domestic animals and

small mammals and are vectors of Jamestown Canyon and Snowshoe Hare viruses (Belloncik *et al.* 1983; Heard *et al.* 1990; Campbell *et al.* 1991). However, lack of sufficient diagnostic characters in all life stages greatly increases the risk of misidentification for members of the *punctor* complex, especially for *Oc. abserratus*, *Oc. hexodontus*, and *Oc. punctor*.

Gimnig (2000) used isoenzyme analysis to identify *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor*, however, isoenzyme analysis requires very fresh specimens and the lack of consistency of this method can limit the application of this technique for routine species identification, particularly for disease vector monitoring programs (Schutz and Eldridge 1993). The application of DNA barcoding as a molecular tool for species identification has been shown in a wide range of organisms, including several species of *Aedes* and *Ochlerotatus* (Hebert *et al.* 2003a, 2003b; Cywinska *et al.* 2006; Namin *et al.* 2013). Additionally, Reno *et al.* (2000) demonstrated the usefulness of PCR-RFLP to identify cryptic *Ochlerotatus* species including *Oc. triseriatus* (Say, 1823) and *Oc. hendersoni* (Cockerell, 1918). However, the efficiency of these methods to distinguish the members of the *punctor* complex has not yet been evaluated.

The purpose of this study was to develop rapid molecular identification tools, including DNA barcoding and PCR-RFLP, for accurate identification of the members of the *punctor* complex (excluding *Oc. aboriginis* and *Oc. punctodes*) to facilitate vector-borne surveillance programs in Manitoba. In a previous study based on one gene (Namin *et al.* 2013), members of the *communis* complex were recovered as paraphyletic with respect to *Oc. abserratus* and *Oc. implicatus* (Vockeroth, 1954a). Thus, the other objective of this study was to examine the phylogenetic relationships between the *punctor* and *communis* complexes and several other black-legged *Ochlerotatus* species using additional genetic sampling.

3.2 Materials and Methods

3.2.1 Mosquito collection

Female mosquito specimens belonging to 12 *Ochlerotatus* species were collected in 2011 from southern Manitoba using sweep nets and aspirators. Some specimens of *Oc. churchillensis* were obtained from the Wallis-Roughley Museum of Entomology (University of Manitoba). Additional mosquito specimens collected from 2008 to 2010 were obtained from Tom Woodcock as part of the “Insect biodiversity in the Churchill Region and Wapusk National Park Project”. Collection data for sequenced voucher specimens are shown in Fig. 3.4 and Table 3.1. Adult female specimens were identified morphologically using the keys of Carpenter and LaCasse (1955), Wood *et al.* (1979), and Thielman and Hunter (2007), and deposited in the Wallis-Roughley Museum of Entomology at the University of Manitoba.

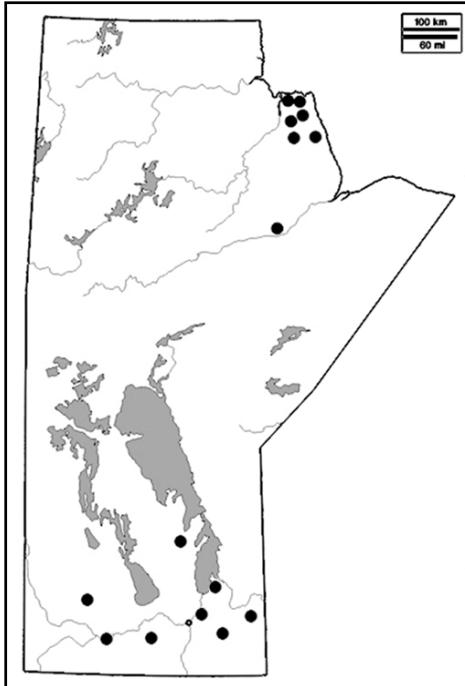


Figure 3.4. Map of Manitoba with the localities of the collected mosquito specimens.

Table 3.1. Specimen locality and voucher numbers for the collected mosquito specimens used in this study.

species	Voucher No.	Label information
<i>Oc. abserratus</i>	0355504	Canada: MB, Sandilands Prov. Forest 49° 30' N. 96° 0' W. 01-vi-2011, HH Namin
	0355505	Canada: MB, Whiteshell Prov. Forest, Pine Point Rapids Trail 50° 10' N. 95° 60' W. 03-vi-2011, 10-vi-2011, HH Namin
	0355506	
	0355507	
<i>Oc. diantaeus</i>	0355508	Canada: MB, Whiteshell Prov. Forest, Pine Point Rapids Trail 50° 10' N. 95° 60' W. 03-vi-2011, 10-vi-2011, 17-vi-2011, HH Namin
	0355509	
	0355510	
	0355511	Canada: MB, Churchill, Herchmer, 57°13' N. 94° 19' W. (11-22)-vii-2010, TS Woodcock and PG Kevan
<i>Oc. hexodontus</i>	0355512	
	0355513	Canada: MB, Lee Lake Camp, 13-vii-2009, TS Woodcock/PG Kevan
	0355514	
	0355515	
	0355516	
	0355517	
	0355518	
	0355519	Canada: MB, Fletcher Lake, 13-vii-2009, TS Woodcock/PG Kevan
	0355520	
	0355521	
	0355522	
	0355523	Canada: MB, Dene Village, 58° 44' N. 94° 06' W. 14-vii-2008, A Thielman
	0355524	
	0355525	
	0355526	
<i>Oc. impiger</i>	0355527	Canada: MB, Twin Lake Rd. 58° 37' N. 93° 48' W. 17-vii-2008, A Thielman
	0355528	
	0355529	
	0355530	Canada: MB, Hoot Creek Forest, 13-vii-2009, TS Woodcock/PG Kevan
	0355531	
	0355532	
	0355533	Canada: MB, Wapusk NP Botany 4, 12-vii-2009, TS Woodcock/PG Kevan
<i>Oc. implicatus</i>	0355534	
	0355535	Canada: MB, Fletcher Lake, 13-vii-2009, TS Woodcock/PG Kevan
	0355536	
	0355537	
	0355538	Canada: MB, Hoot Creek Forest, 13-vii-2009, TS Woodcock/PG Kevan
	0355539	
	0355540	Canada: MB, Lee Lake Camp, 13-vii-2009, TS Woodcock/PG Kevan
<i>Oc. pionips</i>	0355541	Canada: MB, Whiteshell Prov. Forest, Pine Point Rapids Trail 50° 10' N. 95° 60' W. 17-vi-2011, 30-vi-2011, HH Namin
	0355542	
<i>Oc. provocans</i>	0355543	Canada: MB, Whiteshell Prov. Forest, Pine Point Rapids Trail 50° 10' N. 95° 60' W. 03-vi-2011, HH Namin
	0355544	
<i>Oc. provocans</i>	0355545	Canada: MB, Birds Hill Prov. Pk. West Pine Ridge, 50° 00' N. 96° 90' W. 03-vi-2011, HH Namin
	0355546	Canada: MB, Sandilands Prov. Forest 49° 30' N. 96° 0' W. 27-v-2011, HH Namin

Table 3.1 Continued. Specimen locality and voucher numbers for the collected mosquito specimens used in this study.

species	Voucher No.	Label information
<i>Oc. punctor</i>	0355547	Canada: MB, Wapusk NP Botany 4, 12-vii-2009, TS Woodcock/PG Kevan
	0355548	Canada: MB, Lee Lake Camp, 13-vii-2009, TS Woodcock/PG Kevan
	0355549	
	0355550	Canada: MB, Fletcher Lake, 13-vii-2009, TS Woodcock/PG Kevan
	0355551	
	0355552	Canada: MB, Farnsworth Lake, 58° 07' N. 94° 05' W. 08-vii-2009, TS Woodcock
	0355553	
	0355554	Canada: MB, Hoot Creek Tundra, 13-vii-2009, TS Woodcock/PG Kevan
	0355555	Canada: MB, Twin Lake Rd. 58° 37' N. 93° 48' W. 17-vii-2008, A Thielman
<i>Oc. spencerii</i>	0355556	
	0355557	Canada: MB, Ian N. Morrison Research Farm, Carman, 11-vi-2011, HH Namin
<i>Oc. sticticus</i>	0355558	Canada: MB, Spruce Woods Prov. Forest, Marshs Lake, 49° 40' N. 99° 16' W. 24-vi-2011, HH Namin
	0355559	Canada: MB, Birds Hill Prov. Pk. West Pine Ridge, 50° 00' N. 96° 90' W. 03-vi-2011, HH Namin
	0355560	Canada: MB, Grand Beach Forest, 22-vii-2011, HH Namin
	0355561	Canada: MB, Ian N. Morrison Research Farm, Carman, 11-vi-2011, HH Namin
	0355562	Canada: MB, Sandilands Prov. Forest 49° 30' N. 96° 0' W. 02-vii-2011, HH Namin

3.2.2 DNA extraction, COI, ITS2 and RPS12 amplification, and RFLP analysis

Genomic DNA extraction was performed following the protocol published by Namin *et al.* (2013). Cytochrome c oxidase I (COI) and internal transcribed spacer 2 (ITS2) were amplified using primers LCOI490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994), and primers 5.8S (5'-TGTGAAGTGCAGGACACATG-3') and 28S (5'-ATGCTTAAATTAGGGGT-3') (Collins and Paskewitz 1996), respectively. The first intron of ribosomal protein S12 (RPS12) was amplified using the newly designed forward primer RPS12F (5'-ATGTCAGTYGAAGTGTAAAG) and reverse primer RPS12R (5'-

CRATCAGSGACTTCTTYAGSAC-3'), which are positioned in the upstream and downstream exons, respectively. The specific primer pairs were designed according to the first and second exon of *Aedes aegypti* (XM_001654385 and XM_001654386) using Primer3 software (Rozen and Skaletsky, 1998). Amplifications were carried out by a Bio-Rad PTC-0200 DNA Engine thermal cycler, and each PCR master mixture consisted of 1x Standard *Taq* Reaction Buffer (10 mM Tris-HCl; 50 mM KCl; 1.5 mM MgCl₂ pH 8.3, New England Biolabs, Ipswich, MA, USA), 200 µM dNTP (Invitrogen, Carlsbad, CA, USA), 0.4 µM of each primer, 1 unit *Taq* DNA polymerase (New England Biolabs), approximately 2 µg of genomic DNA, and purified water to a final volume of 50 µL. COI amplification was performed following the thermal cycling program published by Namin *et al.* (2013). PCR reactions for ITS2 and RPS12 were performed under the following thermal conditions: an initial denaturation at 94°C for 60 s, followed by 30 cycles of 40s at 94°C, 60s at 50°C, 80s at 72°C, and a final extension of 72°C for 5 min. PCR products were resolved and visualized on a 1% agarose gel, stained with Gel Red (Biotium Inc. California, USA), using a Gel Doc EZ imager (BioRAD Inc, USA). Sequencing was performed for both forward and reverse strands via the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), and using an Applied Biosystems 3730xl DNA Analyzer at the University of Kentucky, Advanced Genetic Technologies Center (UK-AGTC), USA to analyze the sequencing reaction products. The digestion reaction for PCR-RFLP analyses for COI sequences were performed following the RFLP protocol described by Namin *et al.* (2013), using the restriction enzyme *Alu*I.

3.2.3 Data analysis

COI sequence contigs were assembled and edited using Geneious version 5.4.5 (Drummond *et al.* 2011). COI sequences of some other *Ochlerotatus* species related to the *punctor* complex were obtained from GenBank [<http://www.ncbi.nlm.nih.gov/genbank>] and from Namin *et al.* (2013) (see Table 3.2). Multiple sequence alignments for COI and RPS12 exonic sequences were performed by hand using BioEdit version 7.1.3 (Hall 1999) as the alignments were trivial. The intron region of RPS12 was aligned using ClustalX version 2.0.11 software (Larkin *et al.* 2007) with the default parameter settings. Multiple sequence alignments for ITS2 sequences were based on a modified secondary structure model for *Oc. triseriatus* (Say 1823) published by Wesson *et al.* (1992). Modifications to the model accommodated variation in hairpin stem-loops across taxa determined using the mfold web server (Zuker 2003). Regions of expansion and contraction (REC), regions of slipped-strand compensation (RSC), and regions of ambiguous alignment (RAA) were delimited within the model following the methods of Gillespie (2004). Although homology is difficult to ascertain in these regions, they often contain phylogenetically informative characters and can improve phylogenetic reconstruction (Sharanowski *et al.* 2011). Thus, these regions were aligned independently using MAFFT (Katoh and Toh 2008) using default parameters and reinserted into the secondary structure alignment.

The best fitting model of DNA sequence evolution was determined independently for COI, ITS2, and the RPS12 exons and the intron using PAUP v.4.0 beta (Swofford 2000), ModelTest v.3.7 (Posada and Crandall 1998), and the ModelTest Server (Posada 2006) using the Akaike Information Criterion (AIC). For the RPS12 exons, the Tamura-Nei model with a

parameter for invariant sites and rate heterogeneity modeled under a gamma distribution (TrNef+I+G) was determined as the best fitting model of nucleotide substitution.

Table 3.2. List of the additional COI sequences analyzed in this study with their GenBank accession numbers and respective references.

Species	Accession No.	Reference	Species	Accession No.	Reference	
<i>Oc. abserratus</i>	GU907830	Cywinska <i>et al.</i> 2006	<i>Oc. churchillensis</i>	KC713599	Namin <i>et al.</i> 2013	
	GU907831			KC713607		
	GU907832			KC713604		
	GU907833			KC713602		
	GU907834			KC713601		
	GU907835			KC713603		
	GU907836		<i>Oc. communis</i>	KC713606		
	GU907837			KC713598		
	GU907838			KC713596		
	JX259620			KC713600		
<i>Oc. hexodontus</i>	JX259621	Gibson <i>et al.</i> 2012		KC713605		
	JX259622			KC713597		
	JX259623					
	JX259624					
	JX259625					
	JX259626					
	JX259627					
	JX259628					
	JX259629					
	JX259630					
	JX259631					
	JX259632					
	JX259633					
	JX259634					
	JX259638					
<i>Oc. implicatus</i>	GU907904	Cywinska <i>et al.</i> 2006				
	GU907905					
	GU907906					
	GU907907					
	GU907908					
	GU907909					
	GU907910					
	GU907911					
	GU907912					
	GU907913					
<i>Oc. punctor</i>	GU907914					
	APU48378	Miller <i>et al.</i> 1997				
	JX040508	Engdahl <i>et al.</i> 2012				

The general time-reversible model with a parameter for invariant sites and rate heterogeneity modeled under a gamma distribution (GTR+I+G) was selected for all other gene regions (COI, ITS2, and the RPS12 intron). *Oc. provocans* (Walker, 1848) was chosen as an outgroup based on the results of Cywinska *et al.* (2006). Five additional black-legged *Ochlerotatus* species were also analyzed along with the members of the *punctor* and *communis* complexes to illustrate the phylogenetic relationships of these species, including: *Oc. diantaeus* (Howard *et al.* 1913), *Oc. imipger* (Walker, 1848), *Oc. pionips* (Dyar, 1919b), *Oc. spencerii* (Theobald, 1901) and *Oc. sticticus* (Meigen, 1838). Bayesian inference was completed using two independent runs of four chains, with default priors using MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). These analyses were performed for 5,000,000 generations sampling every 1000th generation and the results from the two independent searches were summarized in a majority rule consensus tree after discarding the initial 25% of the trees for burn-in. Runs were considered at stationary when the standard deviation of split frequencies was below 0.02. Inter and intraspecific uncorrected p-distances for the analyzed sequences were calculated using MEGA 5 (Tamura *et al.* 2011) for all pairwise comparisons within and between species.

3.3 Results

3.3.1 COI sequences and Bayesian analyses

The alignment of COI sequences was straightforward as no indels or stop codons were observed. The data matrix included 108 taxa representing 12 *Ochlerotatus* species, and was 657bp in length. Of these, 164 sites were variable (24.9%) and 154 sites (23.4%) were parsimony informative. A high A-T content (67.3%) was detected for COI (A: 29%; C:

16.3%; G: 16.4%; T: 38.3%). The overall mean interspecific genetic variation was 5.03% (range 0.9 - 7.0%) while the mean intraspecific genetic variation was 0.78% (range 0.09 - 2.0%). The lowest interspecific sequence divergence was between *Oc. abserratus* and *Oc. punctor* (0.9%), and the highest interspecific sequence divergence was between *Oc. abserratus* and *Oc. provocans* (7.0%). Average inter- and intraspecific genetic distances for the analyzed species are shown in Table 3.3.

Table 3.3. Average inter- and intraspecific uncorrected p-distances for *Ochlerotatus* species based on a 657bp sequence of cytochrome oxidase 1. The numbers given in the brackets represent the number of analyzed sequences for each species.

	Taxa	Average interspecific p-distance											Average intraspecific
		1	2	3	4	5	6	7	8	9	10	11	
1	<i>Oc. abserratus</i> [13]												0.0049
2	<i>Oc. churchillensis</i>	0.040											0.0051
3	<i>Oc. communis</i> [6]	0.034	0.029										0.0051
4	<i>Oc. dianaeus</i> [4]	0.058	0.057	0.055									0.0178
5	<i>Oc. hexodontus</i> [37]	0.014	0.039	0.034	0.056								0.0119
6	<i>Oc. impiger</i> [7]	0.057	0.054	0.055	0.054	0.054							0.0066
7	<i>Oc. implicatus</i> [13]	0.028	0.044	0.032	0.064	0.028	0.060						0.0039
8	<i>Oc. pionips</i> [2]	0.046	0.040	0.031	0.057	0.043	0.057	0.044					0.0036
9	<i>Oc. provocans</i> [2]	0.070	0.056	0.062	0.047	0.066	0.059	0.069	0.062				0.0009
10	<i>Oc. punctor</i> [10]	0.009	0.038	0.034	0.056	0.010	0.055	0.028	0.044	0.067			0.0074
11	<i>Oc. spencerii</i> [2]	0.059	0.047	0.051	0.043	0.056	0.054	0.061	0.056	0.044	0.057		0.0204
12	<i>Oc. sticticus</i> [5]	0.067	0.061	0.065	0.063	0.068	0.070	0.067	0.069	0.051	0.067	0.051	0.0064

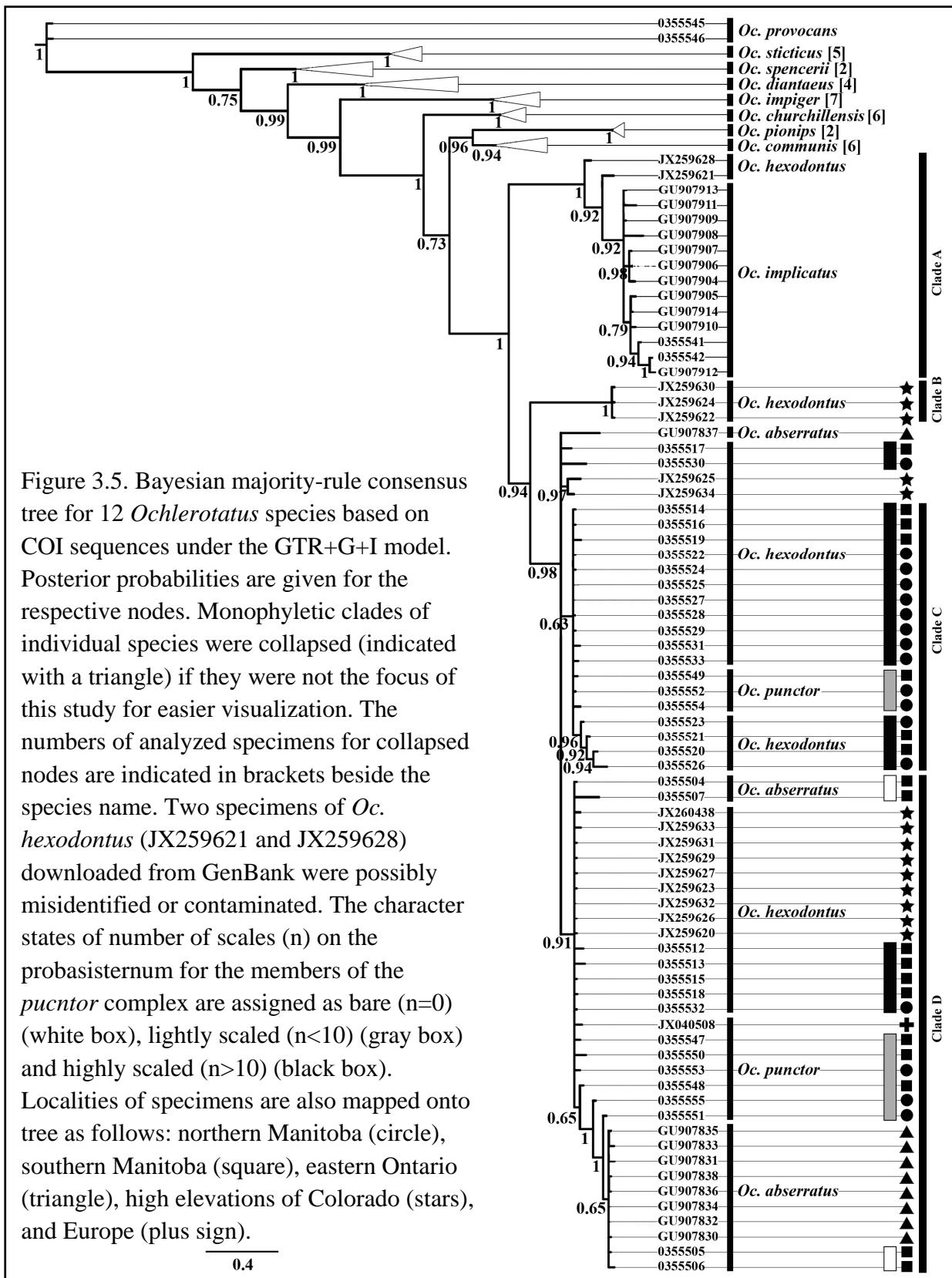
Based on the Bayesian analysis of the COI gene, the *punctor* complex was recovered as a well supported monophyletic clade (posterior probabilities (pp) = 0.94), and sister to *Oc. implicatus* (Fig. 3.5). However, two sequences of *Oc. hexodontus* downloaded from GenBank (JX259628 and JX259621) were recovered with the other *Oc. implicatus* species (Clade A, Fig. 3.5), indicating these sequences may have been contaminated or the specimens were

misidentified. It's also possible that these sequences belong to an undescribed species and the *punctor* complex may include additional species. However, the morphological examination of these specimens (JX259628 and JX259621) was not possible for this study and the relationships of these specimens to *Oc. implicatus* are still not clear. The members of the *punctor* complex included in this study were recovered in three main polyphyletic clades (Clades B, C, and D, Fig. 3.5). Clade B included only three individuals of *Oc. hexodontus*, and thus was recovered as paraphyletic with respect to the other specimens of *Oc. hexodontus* (Fig. 3.5).

The lowest interclade sequence divergence was observed between Clades C and D (2.2%), while the highest value was between Clades B and D (4.6%, Table 3.4). Genetic distances between these clades are shown in Table 3.4. The character states for the numbers of scales on the probasisternum as defined by Gimnig (2000) for *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor* were assigned as absent (no scales), lightly scaled (fewer than 10 scales) and highly scaled (more than 10 scales), respectively and then mapped on the phylogenetic tree for taxa sequenced in this study (Fig. 3.5).

Table 3.4. Average interclade uncorrected p-distances based on analyzing COI sequences for members of the *punctor* complex.

	Taxa	1 - Clade B	2 - Clade C	3 - Clade D
1	Clade B			
2	Clade C	0.043		
3	Clade D	0.046	0.022	
	Clade A (<i>Oc. implicatus</i>)	0.055	0.053	0.049



The geographic localities of the collected specimens for these three species were also mapped onto the tree (Fig. 3.5). The *communis* complex (excluding *Oc. nevadensis* and *Oc. tahoensis*) was recovered as a strongly supported polyphyletic clade with respect to *Oc. pionips* ($pp = 0.94$, Fig. 3.5). *Oc. communis* was recovered as sister to *Oc. pionips*, and this clade was recovered sister to *Oc. implicatus* + the *punctor* complex (Fig. 3.5).

3.3.2 ITS2 sequences and Bayesian analyses

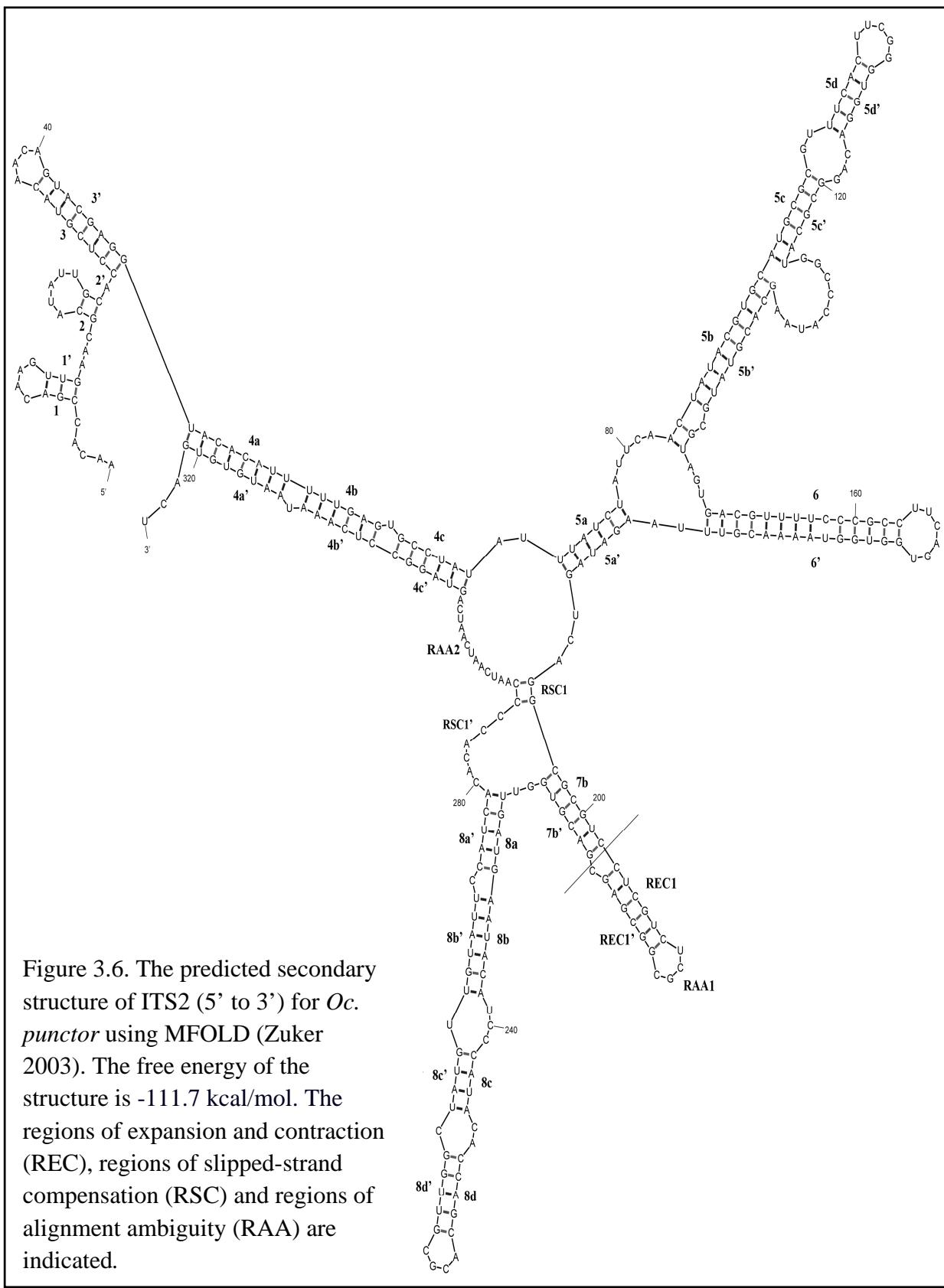
Internal transcribed spacer 2 (ITS2) has been used extensively for phylogenetic studies of taxa at family and species levels, and also for distinguishing cryptic species complexes in Culicidae (Miller *et al.* 1997; Wesson *et al.* 1992; Behbahani *et al.* 2005; Wilkerson *et al.* 2005; Shepard *et al.* 2006). Due to the maternal inheritance of mtDNA, ITS2 was selected as another independent source of molecular data to investigate the phylogenetic relationships among the members of the *punctor* complex. The ITS2 data matrix included 72 taxa and was 370bp in length, of which 126 sites were variable (34.0%) and 115 sites (31.0%) were parsimony informative. There was a 51.2% average A-T content for the analyzed sequences (A: 25.3%; C: 25.8%; G: 23.0%; T: 25.9%). The overall mean interspecific genetic distance was 8.2% (range 1.1 - 19.4%) while the mean intraspecific genetic variation was 1.12% (range 0 - 2.6%). The lowest interspecific sequence divergence was between *Oc. abserratus* and *Oc. punctor* (1.1%), and the highest interspecific sequence divergence was between *Oc. diantaeus* and *Oc. sticticus* (19.4%) (see Table 3.5).

The regions of ambiguous alignment (RAA), regions of slipped-strand compensation (RSC) and regions of expansion and contraction (REC) are indicated in the predicted secondary structure of ITS2 for *Oc. punctor* and in the ITS2 sequence alignment for all

Ochlerotatus species (Figs. 3.6 and 3.7, respectively). The length of REC1 varied from 3bp in *Oc. diantaeus*, *Oc. provocans*, and *Oc. spencerii* to 13bp in *Oc. sticticus* (Fig. 3.7). The length of RAA1 ranged from 2bp in *Oc. diantaeus* to 7bp in *Oc. spencerii* (Fig. 3.7). The predicted secondary structure of ITS2 was relatively consistent with the ITS2 model of *Oc. triseriatu*s suggested by Wesson *et al.* (1992). However a few structural differences, especially in stems 1, 2, 6, RSC1 and RAA2, were observed between Wesson *et al.*'s (1992) model and the one proposed here. Although the length and sequences of REC1, REC1', RSC1' and RAA1 were fairly distinct for most of the *Ochlerotatus* species, no significant sequence divergence or length differences were detected between the members of the *punctor* complex in these regions. The length and nucleotide sequence of RAA2 was distinct among the most black-legged *Ochlerotatus* species included here and the length of this region ranged from 10bp in *Oc. sticticus* to 24bp in *Oc. impiger* (Fig. 3.7).

Table 3.5. Average inter- and intraspecific uncorrected p-distances for *Ochlerotatus* species based on a 370bp sequence of internal transcribed spacer 2. The numbers given in the brackets represent the number of analyzed sequences for each species.

	Taxa	Average interspecific p-distance											Average intraspecific
		1	2	3	4	5	6	7	8	9	10	11	
1	<i>Oc. abserratus</i> [4]												0.0017
2	<i>Oc. churchillensis</i> [6]	0.035											0
3	<i>Oc. communis</i> [6]	0.034	0.016										0.0031
4	<i>Oc. diantaeus</i> [4]	0.079	0.078	0.080									0.0173
5	<i>Oc. hexodontus</i> [22]	0.016	0.046	0.045	0.087								0.026
6	<i>Oc. impiger</i> [7]	0.063	0.044	0.058	0.089	0.072							0
7	<i>Oc. implicatus</i> [2]	0.013	0.032	0.038	0.074	0.023	0.061						0.0033
8	<i>Oc. pionips</i> [2]	0.058	0.053	0.052	0.082	0.065	0.094	0.053					0.0066
9	<i>Oc. provocans</i> [2]	0.079	0.062	0.072	0.098	0.089	0.092	0.076	0.070				0.0235
10	<i>Oc. punctor</i> [10]	0.011	0.040	0.040	0.085	0.023	0.068	0.019	0.061	0.085			0.018
11	<i>Oc. spencerii</i> [2]	0.090	0.106	0.105	0.110	0.103	0.106	0.092	0.112	0.096	0.099		0.0201
12	<i>Oc. sticticus</i> [5]	0.157	0.156	0.163	0.194	0.169	0.152	0.167	0.173	0.139	0.164	0.150	0.0149



	1	1'	2	2'	3	3'	4a	4b	4c	5a	5b	
Abserratus	((()	1')	(()	(((((((((((((((((
	AACAC CGA CAAG TTG AAC GC ATATT GS A CCTCGTAC AAC A GTACGAGG TACACATT TTGAG TGCCTAT AT TTATCT ATTCA ACTATACGTGC	93										
Hexodontus	V..M. VS.	.	R.	.	S.	.	RVV.
Punctor	R..M.	.	S.	.	SC	.	R.
Implicatus	.	.	C
Churchillensis	.	.	C
Communis	S.	C
Pionips	C
Diantaeus	M.	C	M.S.W.
Impiger	C	T
Spencerii	C	A.	T	.	.	.	Y
Sticticus	SC	C	M.Y.T.	C	T
Provocans	C	T
	5c	5d	5d'	5c'	5b'	6	6	6'				186
Abserratus	(((((....	(((((....	((((((((((((((((((((((((((((((((((((((((
	ATGCCG GT TTCAC TTCGG GTGGA CAG GGGCAT GGGCCATAA GCACGTATGCGT AGT GACGTTTCCCG-CC TTCACT GGTTGAAACGTT TA											
Hexodontus	H.	-
Punctor	Y.	-
Implicatus	C.	-	G.
Churchillensis	C.	-	G.
Communis	C.	-	G.
Pionips	C.	C.	C.G	-	G.	.	.	.
Diantaeus	G.	Y.	C.	-	G.	.	.	.
Impiger	G.	C	C.	-	G.	C.	.
Spencerii	G.	C	G.	C.	S.	A.	G.
Sticticus	G.	AC	C	KM.	Y.	G.
Provocans	T.	C.	C.G	T.	G.	A.
	5a'	RSC1	7b	REC1	RAA1	REC1'	7b'	8a	8b	8c	8d	282
Abserratus	((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((
	AGATAG TCA GG CGCGTC	CTCGTC	TCGC	GCGAG	C GACGTG GT TGATG AATACA TCC CATA CA CCAGC ACCG-	.	.	.
Hexodontus	S.	M.	V.	D.	MN. H...
Punctor	S.	M.	M.	M.	M...
Implicatus
Churchillensis	CGAGGATCA	CAGC	T.GTCC	CAGC.-
Communis	CGAGGTG	GTAACA	CATCC	GAC.-
Pionips	CGAGGRY	CMYGC	RC.Y	WC.-
Diantaeus	CYC	TC	-	GIG	Y.
Impiger	CGG	C.VKCC	TCG	AG.GC	C..G
Spencerii	S.	G.	-	CTCGCGC	A.
Sticticus	T.	GTAGGTAC.CCGC	CTCG	C..G	CCAT.G.C.
Provocans	YKC	-RM.WG	A	C.-
	8d'	8c'	8b'	8a'	RSC1'	RAA2	4c'	4b'	4a'			370
Abserratus	((((((((((((((((((((((((((((((((((((((((((((((((((((((((((((
	GTTGG C TATGTTGTATT C CATCA CACACC C	A	AT	CA	GTAGGCCTCAA	T AATGTGTG ACT	.
Hexodontus	KK.	Y	YMY.M	M.M.	Y	RD..... M.Y
Punctor	S....	Y	M	M.M.M.	M	M.S.	M..
Implicatus	M.	C..	S.
Churchillensis	T	A.	CAG	CT
Communis	T	AA	CC	CAR	CT
Pionips	T	TC	GA	C..C.C	G
Diantaeus	T	G	A.TCAA	M	ATCTA	M.
Impiger	C....	T	G	A.	GM	ATGA
Spencerii	C....	T	G	A.	CATACGCA	T.GA	TGCATAT-ACC
Sticticus	C....	T	M..A	TC	C	A.T	ACC..A.
Provocans	T	G	CA.A.	ACCCAACCAA	CCA	C.A..A.S.C.C..K.K...

Figure 3.7. Multiple sequence alignment of ITS2 (5' to 3') for 12 *Ochlerotatus* species. The consensus sequence for each species were obtained using Geneious version 5.4.5 (Drummond *et al.* 2011). Sequence length and the general motif of RSC1', REC1, REC1', RAA1 and RAA2 were distinct for all the analyzed species except for members of the *punctor* complex and *Oc. implicatus*.

However, no sequence divergence or length differences were detected between the members of the *punctor* complex and *Oc. implicatus* in this region (Fig. 3.7). The length of RSC1' ranged from 1bp *Oc. impiger* to 10bp in *Oc. sticticus*, and this region was markedly distinct for *Oc. sticticus* (Fig. 3.7).

Based on the Bayesian analysis of the ITS2 sequence alignment, the *communis* complex was recovered as monophyletic and sister to *Oc. pionips*, although the entire clade had relatively low support (Clade A, pp = 0.67, Fig. 3.8). Members of the *punctor* complex and *Oc. implicatus* were recovered together in a well supported clade (pp = 0.99). However, none of the four species were recovered as monophyletic (Clade B, Fig. 3.8). Most other *Ochlerotatus* species were recovered as monophyletic. In general, there was little resolution among the members of the *punctor* complex. Although there was a higher rate of phylogenetically informative sites (31.1%) for ITS2 compared to COI (23.4%), there were few informative sites across members of the *punctor* complex as well as *Oc. implicatus*.

3.3.3 RPS12 sequences and Bayesian analyses

This is the first attempt to use the first intron of 40S ribosomal protein S12 (RPS12) as a molecular identification marker for *Ochlerotatus* species. RPS12 is involved in translation and contributes to the structural integrity of the ribosome (Marygold *et al.* 2013). RPS12 consists of two introns in *Aedes aegypti* (Linnaeus, 1762), and the first intron was targeted for amplification in this study. The final alignment of RPS12 was 158bp in length, of which 82bp were part of the flanking exons. Twenty-nine sites (18.3%) were variable, and 21 (13.2%) were parsimony informative. The data matrix consisted of an average A-T content of 54.6% (A: 30.7%; C: 18.3%; G: 26.5%; T: 24.6%). The overall mean interspecific and intraspecific

genetic distances were 2.55% (range 0.3-4.7%) and 1.0% (range 0.3-2.2%), respectively. The lowest interspecific divergence was between *Oc. abserratus* and *Oc. hexodontus* (0.3%), and the highest divergence was between *Oc. communis* and *Oc. sticticus* (4.7%). Average inter- and intraspecific genetic distances for the analyzed species are given in Table 3.6.

Table 3.6. Average inter- and intraspecific uncorrected p-distances for *Ochlerotatus* species based on a 160bp sequence of ribosomal protein S12 (RPS12). The numbers given in the brackets represent the number of analyzed sequences for each species.

	Taxa	Average interspecific p-distance											Average intraspecific
		1	2	3	4	5	6	7	8	9	10	11	
1	<i>Oc. abserratus</i> [4]												0.0029
2	<i>Oc. churchillensis</i> [6]	0.020											0.009
3	<i>Oc. communis</i> [6]	0.025	0.022										0.0188
4	<i>Oc. diantaeus</i> [4]	0.018	0.026	0.032									0.0058
5	<i>Oc. hexodontus</i> [22]	0.003	0.020	0.024	0.018								0.0025
6	<i>Oc. impiger</i> [7]	0.022	0.028	0.036	0.015	0.022							0.005
7	<i>Oc. implicatus</i> [2]	0.013	0.032	0.035	0.030	0.012	0.034						0.0043
8	<i>Oc. pionips</i> [2]	0.018	0.007	0.023	0.022	0.018	0.024	0.030					0.0087
9	<i>Oc. provocans</i> [2]	0.018	0.037	0.039	0.035	0.017	0.039	0.018	0.035				0.0224
10	<i>Oc. punctor</i> [10]	0.006	0.021	0.024	0.019	0.004	0.023	0.015	0.020	0.019			0.0061
11	<i>Oc. spencerii</i> [2]	0.018	0.032	0.034	0.034	0.016	0.038	0.027	0.034	0.029	0.016		0.022
12	<i>Oc. sticticus</i> [5]	0.026	0.042	0.047	0.041	0.026	0.044	0.030	0.041	0.043	0.028	0.021	0.0113

The multiple sequence alignment of the RPS12 gene region is indicated in Fig. 3.9. Among the analyzed *Ochlerotatus* species, *Oc. churchillensis*, *Oc. communis*, *Oc. impiger* and *Oc. pionips* are distinct from the other species by having an indel of Valine (GTC) in the 11th position of the coding region (in exon 2), based on the coding sequence (CDS) for RPS12 in *Aedes aegypti* (XM_001654385) (Fig. 3.9).

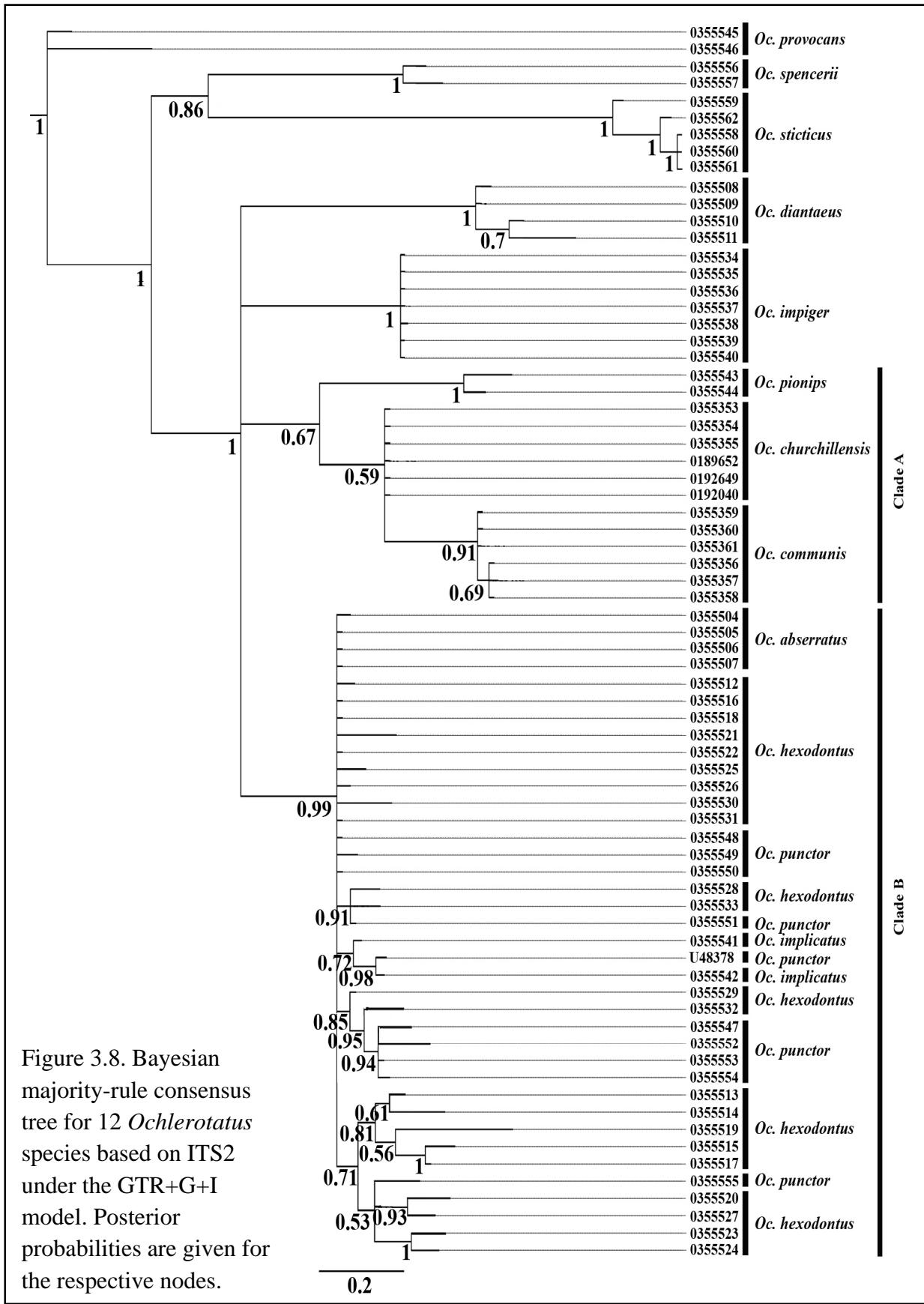


Figure 3.8. Bayesian majority-rule consensus tree for 12 *Ochlerotatus* species based on ITS2 under the GTR+G+I model. Posterior probabilities are given for the respective nodes.

	Exon 1	Intron 1	Exon
Abserratus	ATGTCGGACGTGAAAGT	GTAAGTAAACTGATTATTCGGAAGAAGCGTTGCAATAATAAGAAAAATGTTACAG	TGAAGTCCCGGTC GCTCC 100
Hexodontus
Punctor
Implicatus	C.....
ChurchillensisT.....A.....GGTC.....
CommunisG.....A.C.....GGTC.....
PionipsA.....GGTC.....
DiantaeusT.....G.....A.....
ImpigerG.....A.....G.....GGTC.....
SpenceriiT.....
SticticusT.A.....C.....
ProvocansA.....G.....
Abserratus	AGTCGACGGAACGATGGATGTCAACACAGCTCTGCAGGAAGTCCTAAAGAAGTCCCTGATC	160	
Hexodontus	
Punctor	
Implicatus	C.....T.....	
ChurchillensisG.C.....	
CommunisG.....	
PionipsG.C.....T.....	
Diantaeus	
ImpigerT.....G.....	
Spencerii	
Sticticus	C.....	
ProvocansT.....	

Figure 3.9. Multiple sequence alignment of exon 1, intron 1, and partial exon 2 of 40S ribosomal protein S12 (RPS12) for 12 *Ochlerotatus* species. *Oc. churchillensis*, *Oc. communis*, *Oc. impiger* and *Oc. pionips* are distinct from the other *Ochlerotatus* species included here by having an insertion/deletion of Valine (GTC) in the 11th amino acid position of RPS12 (outlined in a box), based on the coding sequence in *Aedes aegypti* (XM_001654385).

Interestingly no mutations were detected between *Oc. abserratus*, *Oc. hexodontus*, *Oc. punctor* and *Oc. implicatus* in the intronic region of RPS12 (Fig. 3.9), and no mutations in the exons for the former three species. Not surprisingly, the Bayesian analysis of the RPS12 gene region was poorly resolved, due to the low number of informative sites between the

Ochlerotatus species studied here (Fig. 3.10). However, *Oc. sticticus*, *Oc. implicatus*, *Oc. dianeus*, and *Oc. impiger* were recovered as monophyletic.

3.3.4 Sequences and Bayesian analyses for concatenation of all genes

For the concatenated dataset, only the taxa that were sequenced in this study were included, as GenBank sequences were not available for additional taxa for RPS12 and ITS2. The final aligned concatenated data set across the three included genes consisted of 1184 characters (657 sites from COI, 369 sites from ITS2 and 158 sites from the RPS12). Of these, 312 (26.3%) were variable and 284 (23.9%) phylogenetically informative. The data matrix consisted of a relatively high AT content of 61.3% (A: 28.3%; C: 19.2%; G: 19.5%; T: 33.0%) due to the larger number of sites from COI.

Based on the Bayesian analysis, the *punctor* complex was recovered as monophyletic ($pp = 0.98$), and sister to *Oc. implicatus* (Clade B, Fig. 3.11). However, the members of this complex collected in Manitoba (not including *Oc. aboriginis* and *Oc. punctodes*) were recovered as polyphyletic (Clade B, Fig. 3.11). The two members of the *communis* complex included here (*Oc. churchillensis* and *Oc. communis*) were both recovered as monophyletic. However, *Oc. pionips* was recovered within the *communis* complex in a well supported clade ($pp = 1$), and sister to *Oc. communis*, thereby rendering the *communis* complex paraphyletic (Clade A, Fig. 3.11). However, this study did not include the two other members of the *communis* complex: *Oc. nevadensis* and *Oc. tahoensis*.

The *communis* complex + *Oc. pionips* was recovered as sister to *Oc. implicatus* + the *punctor* complex ($pp = 1$, Fig. 3.11). Thus, the separation of the *communis* complex from the *punctor* complex was strongly supported in this analysis.

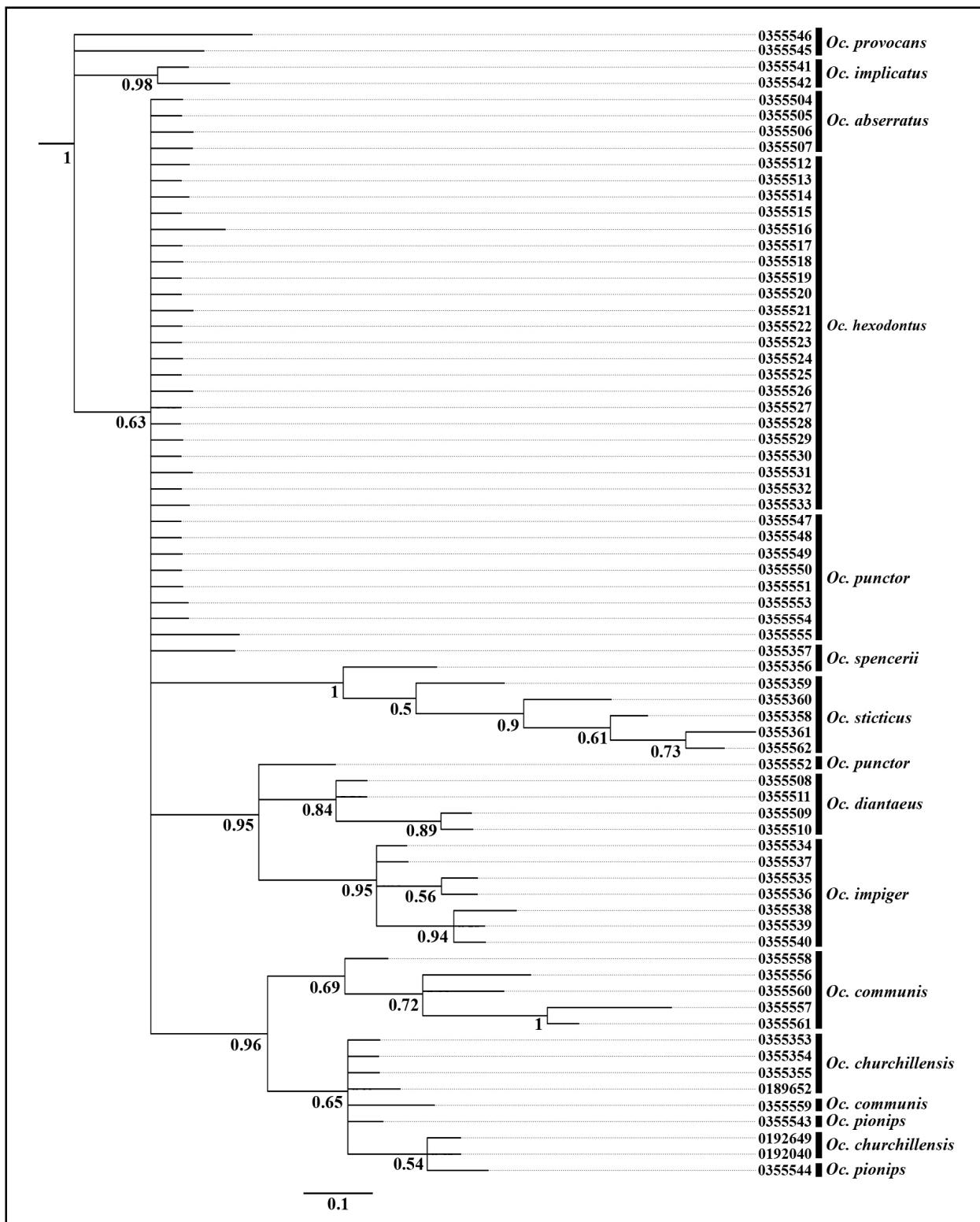
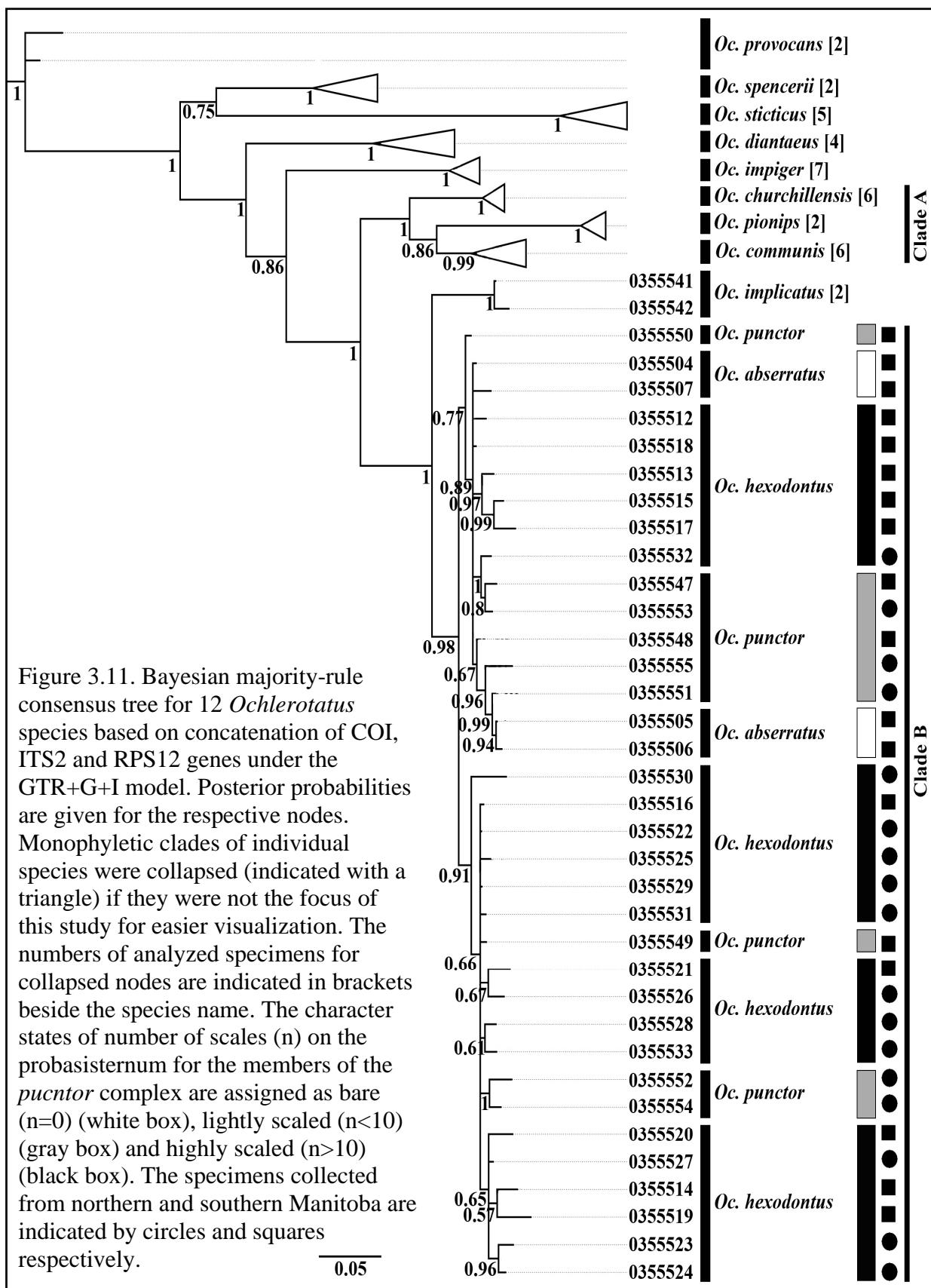


Figure 3.10. Bayesian majority-rule consensus tree for the analyzed *Ochlerotatus* species based on RPS12 intron one and its flanking exons under the TrNef+G+I model. Posterior probabilities are given for the respective nodes.



3.3.5 PCR-RFLP analysis for members of the *punctor* complex

The PCR-RFLP diagnostic patterns for members of the *punctor* complex were developed using *AluI* (5'-AGCT-3') on PCR products of COI. The RFLP patterns for individuals of *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor* were consistent with the predicted digestion patterns obtained through NEB Cutter 2.0 (Vincze *et al.* 2003). A similar RFLP pattern was detected in all three members of the *punctor* complex included here, characterized by fragments of approximately 400, 150, 120, and 50bp (lanes 1-15, Fig. 3.12). This digestion RFLP profile was also consistent with those predicted using NEB Cutter 2.0 (Vincze *et al.* 2003) for all the additional sequences belong to *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor* downloaded from GenBank and BOLD (Table 3.2). Among all examined commercially available restriction enzymes on NEB Cutter 2.0 (Vincze *et al.* 2003), no restriction enzyme was found to produce a species specific cut pattern for *Oc. abserratus*, *Oc. hexodontus*, or *Oc. punctor* for the barcoding region of COI.

Relative to the *punctor* complex, a diagnostic RFLP pattern for *Oc. implicatus* was observed using *AluI*, which was characterized by fragments of approximately 600, 120, and 50bp (lanes 16 and 17, Fig. 3.12). A transition mutation (A to G) in the third codon position of amino acid 53, and a transversion mutation (T to G) in the third codon position of amino acid 55 cause this different RFLP pattern in *Oc. implicatus* compared to members of the *punctor* complex. The observed RFLP profile was also consistent with the predicted RFLP pattern for all the sequences of *Oc. implicatus* from GenBank (GU907904- GU907914) using NEB Cutter 2.0 (Vincze *et al.* 2003).

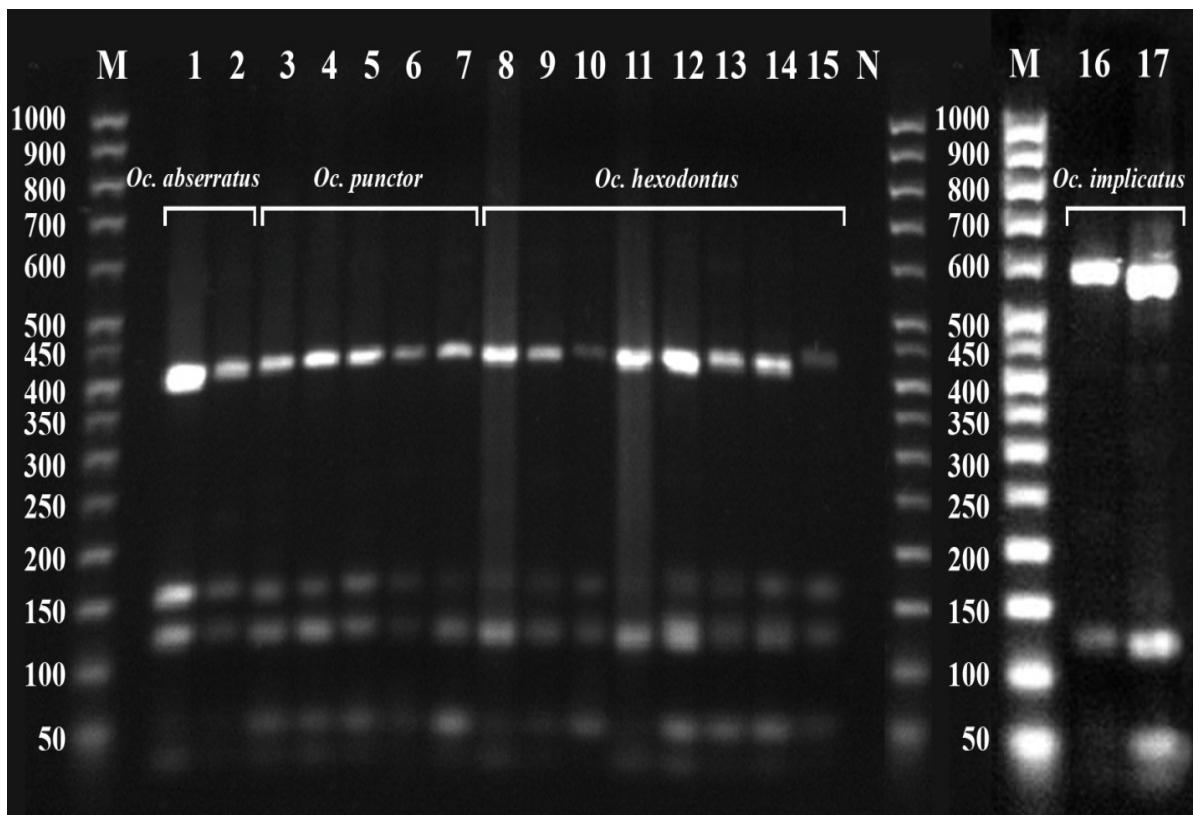


Figure 3.12. COI PCR-RFLP profiles for members of the *punctor* complex and *Oc. implicatus* using *Alu*I. Gel lanes are labeled across the top as follows: M: 50bp DNA ladder, 1 and 2: *Oc. abserratus* (0355305-6), 3-7: *Oc. punctor* (0355348, 0355350-5), *Oc. hexodontus* (0355313, 0355318-21, 0355323, 0355326 and 0355331), N: Negative control, and 16, and 17: *Oc. implicatus* (0355341-2). The RFLP pattern for members of the *punctor* complex (with no bands larger than 400bp) was distinct from that for *Oc. implicatus* (with the largest fragment at 600bp) after digestion using *Alu*I.

3.4 Discussion

Based on phylogenetic analysis of COI by Namin *et al.* (2013), the *communis* complex was recovered as polyphyletic with respect to *Oc. abserratus* + *Oc. implicatus*. However, based on the concatenated data including COI, ITS2 and RPS12 sequences the *communis* complex along with *Oc. pionips* was recovered as a distinct clade sister to *Oc. implicatus* + the *punctor* complex. The *punctor* complex was also recovered as a well supported monophyletic clade ($pp = 0.98$) sister to *Oc. implicatus* in this study (Fig. 3.11). This confirms the previous conclusions by other researchers (Rokas *et al.* 2003, Rubinoff and Holland 2005,

and Sharanowski *et al.* 2011) that one gene phylogenies are typically inadequate for resolving phylogenetic relationships and additional genetic sampling improves phylo-genetic inference. A sequence divergence of about 2% has been shown by Cywinska *et al.* (2006) for species delineation of *Ochlerotatus* and *Aedes* species using DNA barcoding with COI. The average interspecific sequence divergence of COI between *Oc. implicatus* and members of the *punctor* complex was 2.8% (Table 3.3). Based on the number and size of larval prothoracic setae, and the number of scales on the katepisternum and mesepimeron and the shape of gonocoxite in the adult stage, *Oc. implicatus* can be morphologically identified from members of the *punctor* complex. In addition to the morphological differences between *Oc. implicatus* and members of the *punctor* complex, *Oc. implicatus* has a distinct RFLP profile and DNA barcoding sequence of COI. This supports separation of *Oc. implicatus* from the *punctor* complex, even though they were recovered in the same polyphyletic clade in the ITS2 single gene analysis.

Among all the analyzed black-legged *Ochlerotatus* species in this study, relatively low interspecific COI genetic distances were observed between members of the *punctor* complex: 0.9% between *Oc. abserratus* and *Oc. punctor*, 1.0% between *Oc. hexodontus* and *Oc. punctor*, and 1.4% between *Oc. abserratus* and *Oc. hexodontus* (Table 3.3). These results indicate that no discernable boundaries between these three species based on DNA barcoding using COI. However, there was a considerable genetic distance (more than 4%) observed for the specimens of *Oc. hexodontus* of clade B and the members of the *punctor* complex from clade C and D (Table 3.4). This may indicate a lack of gene flow between the *Oc. hexodontus* specimens of Clade B and all other taxa sampled. However, further inquiries regarding the

morphology and ecology of the specimens of this clade are needed to determine the validity of their species identification.

The lowest ITS2 divergence for the studied black-legged *Ochlerotatus* species was observed between *Oc. abserratus* and *Oc. punctor* (1.1% - Table 3.5), which was considerably lower than the interspecific distances between other species of *Ochlerotatus* (Table 3.5) and the ITS2 divergence (3.6%) observed by Porter and Collins (1991) between two cryptic species of the *Anopheles maculipennis* complex: *Anopheles freeborni* (Aitken 1939) and *A. hermsi* (Barr and Guptavanij, 1988). In general, little variability was detected in ITS2 and RPS12 sequences between members of the *punctor* complex relative to the other black-legged *Ochlerotatus* species, which further suggests little or no divergence among these species. However, the members of the *communis* complex included here (*Oc. churchillensis* and *Oc. communis*), *Oc. impiger* and *Oc. pionips* were distinct from the other black-legged *Ochlerotatus* species by having an extra Valine (GTC) in the 11th amino acid position of RPS12 (second exon, Fig. 3.9). This indel event provides additional support to distinguish the members of the *communis* and *punctor* complexes from each other.

Although isoenzyme analysis of *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor* has been shown as a successful method to identify members of the *punctor* complex by Gimnig (2000), our PCR-RFLP analysis revealed a high degree of COI similarity between these species, and presents the limitations of this molecular technique to distinguish members of the *punctor* complex included here. However, the developed RFLP patterns for *Oc. implicatus* will be useful as a rapid method to distinguish the eggs as well as damaged specimens of this species from members of the *punctor* complex distributed in sympatric regions such as Manitoba.

As previously stated, the lack of sufficient morphological characters in all life stages greatly increases the risk of misidentification for members of the *punctor* complex (Beckel 1954; Vockeroth 1954; Carpenter and LaCasse 1955). The number of scales on probasisternum has been suggested by Gimnig (2000) as the most reliable female character for species identification in *Oc. abserratus*, *Oc. hexodontus* and *Oc. punctor*. However, based on the Bayesian analysis of COI, this adult female character is not phylogenetically conserved across species or individual clades within the *punctor* complex (Fig. 3.5). There were also no recovered clades that were associated with distinct geographical localities in this analysis (Fig. 3.5) that might suggest discrete populations. As indicated in Fig. 3.5, specimens of *Oc. hexodontus* collected from high elevation regions in Colorado (approximately 2800 m, indicated by stars), and those collected from southern regions of Manitoba (altitude 300-400 m, indicated by squares), were recovered in the same clade (Clade D). Therefore, no logical association with the distribution of the collected specimens and their COI sequence evolution was inferred in this analysis. These results can be interpreted in different ways. All the analyzed specimens in this study, including those obtained from our collected specimens in sympatric areas of Manitoba and those from GenBank, were acquired from identified adult female specimens. The results suggest that the presence and number of scales on the female probasisternum is not a reliable character for identifying members of the *punctor* complex contrary to Gimnig (2000). If this is true, then the identification of all specimens analyzed in this study (including those from GenBank) is called into question. If the identity of the specimens is uncertain, we would still expect the DNA sequences to be related based on biogeography, separating reproductively isolated groups into clades. However, of the three identified clades within the *punctor* complex based on COI (Clades B, C, and D - Fig. 3.5),

there was no logical association with biogeography. These results suggest that these three species of the *punctor* complex (*Oc. abserratus*, *Oc. hexodontus*, and *Oc. punctor*) may be one widely distributed species. It is possible that COI, as a mitochondrial gene, is not appropriate for teasing apart the biogeographical patterns for these species. Unfortunately, as the sampling for this study was focused in Manitoba, analysis of a wider range of localities was not possible for ITS2 and RPS12 sequences.

Given the lack of discrete larval characters and unreliability of the adult female probasisternum scales across species, morphology-based identification at both larval and adult stages seems essential to distinguish the *punctor* complex accurately, if they are indeed separate species. Therefore, a comprehensive taxonomic revision of the *punctor* complex based on a larger sample size in North America, including the adult and larval associated specimens is necessary to determine the validity of these species.

Chapter 4: Overall summary and future directions

Only through accurate identification of cryptic species can the vector potential of each species be determined, and this is critical for disease epidemiology. This work has provided the first COI barcodes for several black-legged *Ochlerotatus* species including *Oc. churchillensis*, *Oc. impiger*, and *Oc. pionips*. Additionally, this was the first attempt to test the efficacy of the first intron of ribosomal protein S12 (RPS12) as a DNA barcoding tool and as a marker for inferring phylogenetic relationships. The designed RPS12 primer pairs were used to amplify the first intronic region of the RPS12 gene for black-legged *Ochlerotatus* species. Based on a Blast (Altschul *et al.* 1990) search of the primers, however, this primer set will likely amplify this gene from several other insect lineages, including Diptera, Lepidoptera, and Hymenoptera. Thus, the newly designed primer pairs can be used for molecular identification and phylogenetics studies in a relatively broad range of insect species. In spite of frequent indels in the regions of expansion and contraction (REC) and the regions of ambiguous alignment (RAA) of the ITS2 sequences for the black-legged *Ochlerotatus* species included here, the low numbers of informative sites for these genes clearly limited the power of these DNA markers to resolve the phylogenetic relationships among these species. However, the predicted secondary structure of ITS2 in this study can be useful to establish more accurately future phylogenetic studies of *Ochlerotatus* species based on homology assessment.

At least three members of the *communis* complex have diagnostic DNA barcodes using COI, including *Oc. churchillensis*, *Oc. communis*, and *Oc. tahoensis*. However, no specimens of *Oc. nevadensis* were included in this study and analyzing DNA sequences from this species will provide more clarification about the species validity of *Oc. nevadensis* and

the efficacy of DNA barcoding for identification of the entire *communis* complex in North America. This is the first study to develop PCR-RFLP as a molecular based identification tool for two sympatric, cryptic members of the *communis* complex: *Oc. communis* and *Oc. churchillensis*. The developed RFLP profiles can be used as a quick diagnostic tool along with a brief morphology-based identification for the black-legged *Ochlerotatus* species, to identify *Oc. communis* from *Oc. churchillensis*. This molecular method will help facilitate monitoring of *Oc. communis* to predict future outbreaks of Jamestown Canyon and Snowshoe Hare Viruses, and will also facilitate future research to assess the potential of *Oc. churchillensis* as a vector of other emerging diseases.

Among all the analyzed black-legged *Ochlerotatus* species in this study, relatively low interspecific COI genetic variation relative to intraspecific variation within species has been observed between members of the *punctor* complex, which indicates no distinctive boundaries between these species based on DNA barcoding. Moreover, by evaluating about 350 commercially available restriction enzymes in NEB Cutter 2.0 (Vincze *et al.* 2003), no restriction enzyme was found to produce unique RFLP digestion pattern for the individuals of *Oc. abserrarus*, *Oc. hexodontus* and *Oc. punctor*. This indicates these species are indistinguishable using PCR-RFLP analysis of COI sequences due to a high degree of sequence similarity between these species. However, no specimens of *Oc. aboriginis* and *Oc. punctodes* were analyzed in this study, and thus further work is needed to determine whether these two species represent distinct and separate species from the other members of complex or whether all the five members of the *punctor* complex belong to one widely distributed species.

Based on the Bayesian analysis of the concatenated COI, ITS2 and RPS12, all the members of the *communis* complex (excluding *Oc. nevadensis* and *Oc. tahoensis*) were recovered in monophyletic clades. However, *Oc. pionips* was recovered within the *communis* complex, and thus rendered this complex as a polyphyletic assemblage. Analyzing COI, ITS2, and RPS12 sequences of *Oc. nevadensis* and *Oc. tahoensis* along with the other members of this complex will provide more clarification for the phylogenetic relationships of these species, and the taxonomic status of the species within this complex. Based on the Bayesian analysis of the concatenated genes (1184bp), the *punctor* complex (excluding *Oc. aboriginis* and *Oc. punctodes*) was recovered as a strongly supported monophyletic clade, and sister to *Oc. implicatus*. These results clearly support the separation of the *punctor* complex from the *communis* complex contrary to Namin *et al.* (2013). However, members of the *punctor* complex were recovered as polyphyletic with no clear association between the female morphological characters or their distribution with the recovered phylogenetic clades. At minimum, these results indicate that the scales on the probasisternum are not reliable to distinguish adult female members of the *punctor* complex, and suggest morphology-based identification of both larval and adult specimens are necessary for accurate identification of the members of this complex.

The members of the *punctor* complex including *Oc. abserrarus*, *Oc. hexodontus* and *Oc. punctor* have a sympatric distribution in woodland and tundra areas of Manitoba. These three species have similar biologies as their larvae hatch in the same ecological niches during early summer. All three species are also reported as having similar vector competencies. Additionally, the molecular analyses in this study revealed a high degree of DNA similarity between these species. All this evidence supports the hypothesis that these three species may

be one widely distributed species in North America. Since female and larval associated specimens are necessary for accurate identification of these species, examination of additional larval and female specimens for all five members of the *punctor* complex are still required to elucidate the species boundaries for the members of this complex. Immature rearing of the mosquitoes can be difficult due to the need to provide suitable temperature and humidity, and to eliminate the risk of infection by the fungal pathogens. In order to analyze the adult and larval associated mosquito specimens, the larval specimens should be reared in separate plates, and be identified morphologically. It is worth mentioning that the genomic DNA can be extracted and amplified from the larval and pupal skins of larvae, so it would be possible to keep the larvae alive and in separate cages for their emergence to the adult stage. Subsequently, the adult mosquito specimens should be identified morphologically and subjected to DNA extraction and amplification as well. Finally, analyzing the molecular data obtained from DNA materials of larval and adult specimens will be necessary to validate any species-specific morphological characters and also to clarify the species validity of the members of the *punctor* complex.

PCR-RFLP has several advantages over morphology-based identification in mosquito-borne surveillance programs. PCR-RFLP can be applied for a large number of specimens, and can be used to identify specimens in all developmental stages. More importantly, it can be extremely useful for non-taxonomists to identify mosquito specimens quickly and accurately. However, using RCP-RFLP for distinguishing all the black-legged *Ochlerotatus* species found in Manitoba, may not be realistic for several reasons. Firstly, RFLP patterns can vary due to intraspecific genetic variation between populations of one species. Thus, analyzing DNA samples from individuals of each black-legged *Ochlerotatus* species from multiple

populations is necessary to predict and characterize the possible RFLP patterns for each species. Secondly, although PCR-RFLP may be a more cost-effective technique compared to other molecular-based identification methods such as DNA barcoding, it still does not seem to be economical to use for identification of morphologically distinct mosquito species. Therefore, using molecular techniques such as RFLP and DNA barcoding as routine tools of identification is likely only practical for distinguishing the most important cryptic vector species in terms of health impact and disease prevalence.

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