Phylogenetics and molecular identification of the *Ochlerotatus communis* complex (Diptera: Culicidae) using DNA barcoding and polymerase chain reaction-restriction fragment length polymorphism

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Abstract—The *Ochlerotatus communis* (De Geer, 1776) complex consists of four cryptic mosquito species in North America, including: *O. communis*, *Ochlerotatus churchillensis* (Ellis and Brust, 1973), *Ochlerotatus nevadensis* (Chapman and Barr, 1964), and *Ochlerotatus tahoensis* (Dyar, 1916). Most of the morphological characters used for the identification of these species are quantitative and overlap across species. Here we evaluated the efficacy of DNA barcoding for identification of three members of the *communis* complex (*O. nevadensis* is not included in this study) and developed diagnostic restriction fragment length polymorphism (RFLP) patterns for *O. communis* and *O. churchillensis*. A phylogeny of 23 *Ochlerotatus* Lynch Arribálzaga, 1891 species was inferred using mitochondrial cytochrome *c* oxidase subunit I gene sequences. All species included in our analysis within the *O. communis* complex were delineated using cytochrome *c* oxidase subunit I barcodes. However, this complex was recovered as paraphyletic with respect to *Ochlerotatus abserratus* (Felt and Young, 1904) and *Ochlerotatus implicatus* (Vockeroth, 1954), indicating the need for increased genetic and taxonomic sampling to infer the phylogenetic relationships of these taxa. The RFLP profile for multiple field specimens of *O. communis* was distinct from all RFLP patterns for *O. churchillensis*, and this method can be used as an efficient molecular method for the identification these species.

Résumé—Le complexe d'*Ochlerotatus communis* (De Geer, 1776) comprend quatre espèces cryptiques de moustiques, soit *O. communis*, *O. churchillensis* (Ellis et Brust, 1973), *O. nevadensis* (Chapman et Barr, 1964) et *O. tahoensis* (Dyar, 1916). La plupart des caractères morphologiques utilisés pour l'identification de ces espèces sont quantitatifs et il y a du recoupement entre les espèces. Nous évaluons l'efficacité de la codification à barres d'ADN pour l'identification de trois membres du complexe de *communis* (*O. nevadensis* n'est pas inclus dans notre étude) et mettons au point des patrons RFLP pour *O. communis* et *O. churchillensis*. Nous avons déduit une phylogénie de 23 espèces d'*Ochlerotatus* Lynch Arribálzaga, 1891 à partir des séquences du gène mitochondrial de la sous-unité I de la cytochrome c oxydase. Il a été possible de délimiter toutes les espèces du complexe de *communis* incluses dans notre analyse à l'aide des codes de barres de COI. Cependant le complexe s'est avéré paraphylétique en ce qui a trait à *O. abserratus* (Felt et Young, 1904) et *O. implicatus* (Vockeroth, 1954), ce qui indique qu'il faudra un plus important échantillonnage génétique et taxonomique afin de déduire les relations phylogénétiques de ces taxons. Le profil RFLP de nombreux échantillons de terrain d'*O. communis* se distingue des patrons RFLP d'*O. churchillensis* et la méthode peut servir d'outil moléculaire efficace pour l'identification de ces espèces.

Introduction

According to Ellis and Brust (1973), the *Ochlerotatus communis* complex consists of four species in North America, including: *O. communis* (De Geer, 1776), *O. churchillensis* (Ellis and Brust, 1973), *Ochlerotatus nevadensis* (Chapman and Barr, 1964), and *Otahoensis tahoensis*

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(Dyar, 1916). These species are morphologically cryptic and thus identification based on morphology is exceedingly difficult. Most characteristics are diagnostic for only one species within the complex or for a specific developmental stage or sex. For example, the shape of the larval comb scales is only diagnostic for the identification of *O. nevadensis* in the fourth larval instar (Ellis and Brust 1973). In the adult stage, male members of this complex may be distinguished by the size of genital gonocoxites (Ellis and Brust 1973). Females, however, are much more difficult to identify. The shape of the metatarsal claw is diagnostic for *O. communis* from other members of the complex, but this character is not always reliable for distinguishing the other species (Brust and Munstermann 1992).

Species within the *O. communis* complex have interesting distributions suggesting that both allopatric and sympatric speciation have played a role in their evolution. *Ochlerotatus communis* has a Holarctic distribution and has been collected throughout much of North America (Wood *et al.* 1979). *Ochlerotatus nevadensis* has been reported from southeastern British Columbia, Canada and northwestern parts of the United States of America (Belton 1983) but has only been collected in mountainous regions suggesting that this species might be reproductively isolated from other species within the complex (Belton 1982; Schutz and Eldridge 1993). *Ochlerotatus tahoensis* has a restricted range of distribution from the Sierra Nevadas to the southern Cascades and Klamath mountains in California, United States of America but has only been collected from high elevations (1500–3000 m; Schutz and Eldridge 1993). *Ochlerotatus tahoensis* can be distinguished from *O. nevadensis* based on larval morphological characters and from all species in the complex by collection locality (Darsie 1995).

Ochlerotatus communis and *Ochlerotatus churchillensis* have a sympatric distribution in northeastern and southeastern Manitoba and western Alberta, Canada (Ellis and Brust 1973; Wood *et al.* 1979) and thus are particularly difficult to identify. These two species have similar 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, *O. churchillensis* females have smaller salivary glands than *O. communis* due to their autogenous ovarian development. This character may be useful to separate populations of *O. churchillensis* from all other members of the complex (Ellis and Brust 1973). Morphometric characteristics of the metatarsal claws are the primary characters used for the identification of adult females, although the measurements are not discrete with considerable overlap across species (Brust and Munstermann 1992).

The combination of reproductive differences in addition to small morphological differences, particularly for males, originally led Ellis and Brust (1973) to describe *O. churchillensis* as a distinct species from *O. communis*. Brust and Munstermann (1992) suggested isozyme analysis as a method for distinguishing members of the *O. communis* complex. This method, however, lacks consistency and requires very fresh specimens, thereby limiting the use of this technique as a routine tool for species identification (Schutz and Eldridge 1993). Currently, there is no reliable method for quickly and accurately separating *O. communis* from *O. churchillensis*.

Accurate and rapid identification of pathogens and vectors is essential in epidemiological studies of mosquito-borne diseases. *Ochlerotatus communis* is a vector of Jamestown Canyon virus (JCV) and snow shoe hare virus (SSHV) in North America (McLean *et al.* 1981; Heard *et al.* 1990), but the clinical significance of *O. churchillensis* has not yet been studied. Both viruses are widely distributed across temperate areas of North America, and several boreal *Aedes* Meigen, 1818 and *Ochlerotatus* Lynch Arribálzaga, 1891 species are the primary vectors (Grimstad 1988; Andreadis *et al.* 2008; Bennett *et al.* 2012). Although JCV antibodies have been detected in moose, elk, bison, mule deer, domestic bovine and equine, white-tailed deer is the only known 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, acute central nervous system infection, and respiratory system involvement (Grimstad 1988, 2001). SSHV was isolated for the first time from snowshoe hare (*Lepus americanus* Erxleben, 1777; Mammalia: Leporidae) in Montana, United States of America in 1959 (Burgdorfer *et al.* 1961). However, its antibodies also have recently been detected in several small vertebrates and domestic animals such as equine, cattle, dog, and chicken

(Bennett *et al.* 2012). The symptoms of SSHV 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 *O. communis* complex and greatly facilitate mosquito vector surveillance.

Unlike morphological characters, molecular tools can be applied for the identification of species in all developmental stages and for damaged specimens. Cytochrome *c* oxidase subunit I (COI) has been used for species identification in many insect species (Hebert *et al.* 2003; Smith *et al.* 2006; Rivera and Currie 2009; Boring *et al.* 2011; Hernández-Triana *et al.* 2012). Cywinska *et al.* (2006) also used DNA barcodes for the identification of several species of *Aedes* and *Ochlerotatus* in Canada; however, *O. churchillensis* was not included in their analysis. Polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) has been successfully used as an effective molecular tool for the identification of two other cryptic *Ochlerotatus* species: *Ochlerotatus triseriatus* (Say, 1823) and *Ochlerotatus hendersoni* (Cockerell, 1918) (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. This study was part of a larger ongoing project to develop RFLP profiles for all black-legged *Ochlerotatus* species in Manitoba, Canada. Due to the cryptic morphology and sympatric distribution of *O. communis* and *O. churchillensis* in Canada, the major aim of this study was to develop diagnostic RFLP patterns for these species and assess the applicability of this technique for rapid identification for use in vector surveillance programs in Manitoba. Additionally, we evaluated the efficacy of DNA barcoding for species identification of the members of the *O. communis* complex in North America (excluding *O. nevadensis*). Additional species of *Ochlerotatus* were added to the data matrix for comparative examination of the genetic variability in the DNA barcoding gene COI within and between species.

Materials and methods

Mosquito collection

Adult mosquito specimens were collected from multiple localities in Manitoba (including known localities for *Ochlerotatus churchillensis*) using aspirators between May and July 2011 (Table 1). The specimens of *O. churchillensis*, collected by Brust (Table 1), were obtained from the Wallis–Roughley Museum of Entomology (University of Manitoba, Winnipeg, Manitoba, Canada). Additional specimens of this species were obtained from the collected materials of a project entitled "Insect biodiversity in the Churchill region and Wapusk National Park", collected using a malaise trap in July 2010. Although malaise traps are not an appropriate method of collecting adult female specimens, the specimens were adequately preserved to make putative morphological identifications of *O. churchillensis* and allowed for increased sampling of this taxon. Collected female specimens were identified using the following morphological keys (Ellis and Brust 1973; Wood *et al.* 1979; Thielman and Hunter 2007) and deposited in the Wallis–Roughley Museum of Entomology at the University of Manitoba. Collection information for all specimens is provided in Table 1.

DNA extraction and amplification

Genomic DNA was extracted from legs of the collected specimens and from the whole body of previously mounted museum specimens. DNA extraction was carried out using the Qiagen DNEasy extraction kit (Qiagen Inc., Valencia, California, United States of America) following the manufacturer's instructions. Universal primers LCOI490 (5′-GGTCAACAAATCATAAA GATATTGG-3′) and HCO2198 (5′-TAAAC TTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.* 1994) were used to amplify an ~660 base pair (bp) fragment of COI. All PCR reaction mixtures contain Chapman d 1× Standard *Taq* Reaction Buffer (10 mM Tris-HCl; 50 mM KCl; 1.5 mM $MgCl_2$ pH 8.3, New England Biolabs, Ipswich, Massachusetts, United States of America), 200 μM dNTP (Invitrogen, Carlsbad, California, United States of America), 0.4 μM of each primer, 1 unit *Taq* DNA polymerase (New England Biolabs), ~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 seconds, followed by 30 cycles of 60 seconds at 94 °C, 60 seconds at 50 °C, 60 seconds at 72 °C, and a final extension of 72 °C for seven minutes. The PCR products

Table 1. Specimen locality, voucher, and GenBank numbers for *Ochlerotatus communis* and *Ochlerotatus churchillensis* specimens collected in this study.

Species	Voucher no.	Accession no.	Label information
O. churchillensis	0189652	KC713599	Canada, MB, Churchill, near Stephens' Lake 56°N.,
	0192649	KC713607	94°W., 22-VI-1983, R. A. Brust
	0192040	KC713604	Canada, MB, Churchill, SE of Churchill harbor, 58°N., 94°W., 18-VI-1985, R. A. Brust
	0355353	KC713602	Canada, MB, Churchill, Herchmer, 57°13′N., 94°19′W.,
	0355354	KC713601	(11-22)-VII-2010, T. S. Woodcock and P. G. Kevan
	0355355	KC713603	
O. communis	0355356	KC713606	Canada, MB, Winnipeg, Sandilands Prov. Forest 49°30′N., 96°0′W., 27-V-2011, H. H. Namin
	0355357	KC713598	Canada, MB, Whiteshell Prov. Forest, Pine Point Rapids
	0355358	KC713596	Trail 50°10′N., 95°60′W., 03-VI-2011, 10-VI-2011,
	0355359	KC713600	17-VI-2011, H. H. Namin
	0355361	KC713605	Canada, MB, Spruce Woods Prov. Forest, Marshs lake, 49°40′N., 99°16′W., 24-VI-2011, H. H. Namin
	0355360	KC713597	Canada, MB, Winnipeg, Birds Hill Prov. Pk., West Pine Ridge, 50°00′N., 96°90′W., 30-VI-2011, H. H. Namin

were purified with ExoSAP-IT (Affymetrix, Santa Clara, California, United States of America) using 25% of the recommended reagent amount, but otherwise following the manufacturer's instructions. Sequencing was carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, United States of America), with reaction products analysed on an Applied Biosystems 3730×l DNA Analyzer at Eurofins MWG Operon (Huntsville, Alabama, United States of America).

Data analysis

Sequence contigs were assembled, trimmed, and edited using Geneious version 5.4.5 (Drummond *et al.* 2011), and deposited in GenBank (see Table 1 for accession numbers). Additional COI sequences of *Ochlerotatus* species were obtained from the sequence data from Mitchell *et al.* (2002), Morlais and Severson (2002), Cywinska *et al.* (2006), and Gibson *et al.* (2012), and are listed in Supplementary Table 1.

Multiple sequence alignments were performed using ClustalX v. 2.0.11 (Larkin *et al.* 2007) with default settings and checked for reading frame accuracy. Interspecies and intraspecies genetic 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 chosen as the best fitting model of nucleotide substitution using the Akaike Information Criterion. 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). 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. *Culex territans* Walker was chosen as the outgroup for the phylogenetic 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 <0.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Trees were summarised from the two independent searches using majority rule consensus after discarding 25% of the samples for burn-in.

Restriction digestion

*Alu*I was determined as the best restriction enzyme for producing a unique RFLP pattern for each species using NEB Cutter 2.0 (Vincze *et al.* 2003). The digestion mixtures consisted of 5–7 μL PCR-products (~50–150 ng), 5 units of AluI (Invitrogen), 1× incubation buffer (1.5 mM

Table 2. The interspecific and intraspecific p-distances among the members of the *Ochlerotatus communis* complex calculated using MEGA 5 (Tamura *et al.* 2011) based on a 658 bp sequence of cytochrome oxidase 1 and the number of analysed specimens for each species.

Taxon	Average interspecific p-distance		Number of specimens	Average intraspecific p-distance
	1	2		
1. *Ochlerotatus churchillensis*			6	0.0094
2. *O. communis*	0.051		39	0.0084
3. *Ochlerotatus tahoensis*	0.030	0.045	2	0

Tris-HCl, pH 7.5; 1.5 mM $MgCl_2$; 0.15 mM Dithiothreitol) and purified water to a final volume of 15 μL. The digested mixture was incubated at 37 °C for two hours and the digested fragments were resolved on a 1.5% agarose gel, stained with Gel Red (Biotium Inc., Hayward, California, United States of America). The restricted DNA patterns were photographed on a Gel Doc EZ imager (BioRAD Inc, Mississauga, Ontario, Canada) and the length of each restriction fragment was estimated using a 50-base DNA ladder (Novagen, Darmstadt, Germany).

Results and discussion

Sequence and phylogenic analyses

The final alignment was 658 bp in length of which 236 (35.9%) sites were variable and 224 (34%) were parsimony informative. The COI sequences showed a strong AT base composition bias ranging from 65.4% (*Ochlerotatus implicatus* (Vockeroth, 1954)) to 70.6% (*Aedes vexans* (Meigen, 1830)), with an average of 68% for all taxa. No stop codons or indel events were observed among the studied sequences. Intraspecific genetic variation within the *O. communis* complex ranged from 0% for *O. tahoensis* to 0.94% for *O. churchillensis* (Table 2). However, only two sequences of *O. tahoensis* were included in this analysis and no reference sequence for *O. nevadensis* was available for study. The highest interspecific genetic variation was observed between *O. churchillensis* and *O. communis* (5.1%) while the lowest value was detected between *O. churchillensis* and *O. tahoensis* (3.0%; Table 2). Although there is no widely accepted threshold of interspecific genetic variation for species identification using DNA barcoding, a sequence divergence of <2% has been observed by Cywinska *et al.* (2006) for species delineation of *Ochlerotatus* and *Aedes* species. Given the low intraspecific variability and high interspecific distances, *O. churchillensis*, *O. communis*, and *O. tahoensis* can be delineated using COI barcodes (Table 2). These distinct barcodes support the conclusions of Ellis and Brust (1973) that *O. churchillensis* is a distinct species from *O. communis*. Ellis and Brust (1973) and Brust and Munstermann (1992) suggested *O. tahoensis* is a valid species distinct from *O. communis* based on morphological and allozyme evidence. The DNA barcode data from this study also supports *O. tahoensis* as a genetically distinct species.

On the basis of Bayesian inference of phylogenetic relationships, *Aedes* and *Ochlerotatus* were recovered as well-supported monophyletic sister taxa (Fig. 1). These results are congruent with the results of Shepard *et al.* (2006) based on 18s rDNA, and also support the proposed classification of Culicidae by Reinert (2000) with *Ochlerotatus* as a distinct, valid genus from *Aedes*. Although the recognition of *Ochlerotatus* as a distinct genus has been controversial (Black 2004; Savage and Strickman 2004; Edman 2005), the current study provides additional evidence supporting recognition of *Ochlerotatus* by Reinert (2000). Furthermore, we agree with Black (2004) that taxonomic stability is best achieved when taxonomy follows phylogeny.

Ochlerotatus churchillensis was recovered as sister to *O. tahoensis* (Fig. 1). However, one sequence of *Ochlerotatus aurifer* (GU907850) was recovered within the *O. churchillensis* clade. Since all the other sequences of *O. aurifer* were recovered as a monophyletic group sister to *O. cantator* + *O. canadensis*, this sequence (GU907850) was likely contaminated or the specimen was misidentified prior to sequencing

Fig. 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 visualisation. The number of analysed specimens for collapsed nodes is indicated in brackets beside the species name. GenBank and BOLD Accession numbers for specimens within collapsed clades can be found in Supplementary Table 1. For other sequences used from previously published data sets, the accession numbers are listed on the figure and in Supplementary Table 1. For specimens collected and sequenced in this study, voucher numbers are listed on the figure and accession numbers are listed in Table 1. One sequence of *Ochlerotatus aurifer* (GU907850) downloaded from GenBank was possibly misidentified or contaminated. COI, cytochrome *c* oxidase subunit I.

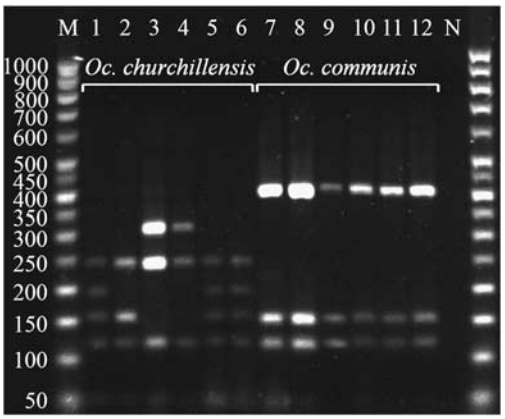
(Fig. 1). This sequence of *O. aurifer* (GU907850) was downloaded from GenBank and has not been published in a peer-reviewed journal, highlighting the risks with GenBank sequences, particularly from unpublished data. *Ochlerotatus communis* was recovered as paraphyletic with respect to *Ochlerotatus abserratus* + *O. implicatus* (Fig. 1). Although there are distinct morphological differences that separate *O. abserratus* and *O. implicatus* from members of the *O. communis* complex (Carpenter and LaCasse 1955; Wood *et al.* 1979), the phylogenetic relationships recovered here suggest that the *O. communis* complex may include additional species. However, additional genes and taxon sampling will be necessary to establish the limits of the *O. communis* complex, as not all taxa within *Ochlerotatus* 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).

All specimens of *O. churchillensis* were collected in northern Manitoba, near Churchill. Even though efforts were made to collect this species from known localities in southern Manitoba, *O. churchillensis* was not collected from the known sympatric regions in Manitoba. It may be possible that this species now has a more restricted distribution, similar to *O. tahoensis* and *O. nevadensis*, which would facilitate identification of all species within the *O. communis* complex based on locality.

PCR-RFLP analysis

The PCR-RFLP banding patterns were developed for the identification of *O. churchillensis* and *O. communis* based on species specific restriction sites of *Alu*I in the COI sequences. The RFLP patterns were consistent with the predicted digestion patterns of *O. communis* and *O. churchillensis* sequences from GenBank, BOLD (www.boldsystems.org), and multiple field collected specimens. Three different RFLP patterns, corresponding to those clades found in the Bayesian analysis, were observed for *O. churchillensis* specimens after digestion with *Alu*I. The first RFLP pattern was characterised by fragments of ~250, 200, 160, and 120 bp and was observed in three specimens of *O. churchillensis* (0355353, 0192649, and 0192040 in lanes 1, 5, and 6, respectively, Fig. 2). The second RFLP profile was typified by fragments of ~320, 250, 120, and 50 bp, and was observed in two specimens of *O. churchillensis* (0355355 and 0189652 in lanes 3 and 4, respectively, Fig. 2). The third RFLP pattern included fragments of ~250, two fragments of 150, and one fragment at 120 and 50 bp. This pattern was only observed in one specimen (0355354 in lane 2, Fig. 2). Variation at the third codon position of amino acids 139 and 175 cause the three different RFLP patterns observed in *O. churchillensis*. Alternatively, digestion with *Alu*I produced one consistent diagnostic RFLP pattern for all specimens of *O. communis* tested in this study. The *O. communis* RFLP profile was characterised by fragments of ~400, 150, 120, and 50 bp (0355356-0355361 shown in lanes 7–12, Fig. 2). This digestion RFLP pattern was also consistent with the predicted RFLP pattern for the sequences of *O. communis* from GenBank using NEB Cutter 2.0 (Vincze *et al.* 2003). Although the analysed specimens of *O. communis* and *O. churchillensis* were not collected from the

Fig. 2. Digestion patterns of COI PCR products with *Alu*I restriction enzyme for *Ochlerotatus communis* and *Ochlerotatus churchillensis*. Gel lanes are labeled across the top as follows: M: 50 bp DNA ladder, 1–5: *O. churchillensis* (0355353, 0355354, 0355355, 0189652, 0192649, 0192040), 7–12: *O. communis* (0355356–0355361) and N: Negative control. According to the RFLP patterns, a 250 bp band was detected in all specimens of *O. churchillensis* (never observed in *O. communis*), and a 400 bp band was observed for all specimens of *O. communis* (never observed in *O. churchillensis*). COI, cytochrome *c* oxidase subunit I; PCR, polymerase chain reaction.



sympatric areas, the RFLP pattern for *O. communis* (with the largest fragment at 400 bp) was distinct from all RFLP profiles for *O. churchillensis* (with no bands larger than 320 bp) after digestion using *Alu*I.

These distinctive profiles were created because the 73rd and 74th amino acids (based on the complete CO1 gene from *Aedes aegypti* Linnaeus, 1762, NC_010241.1) are conserved glycine and alanine residues in the *O. communis* complex, respectively. In all members of the complex analysed here, the codon for the glycine residue is always GGA. However, the downstream alanine residue codon is different in *O. churchillensis* (GCT) and *O. communis* (GCC), causing an additional cut at this site for *O. churchillensis*. Thus, it is this mutation that confers the diagnostic RFLP patterns for these species. It is predicted that *O. tahoensis* would not be cut by *Alu*I at this site, as the alanine residue is encoded by GCA, based on the two downloaded sequences analysed in this (Supplementary Table 1). Thus, this species would exhibit the same 400 bp fragment as found in *O. communis* (Fig. 2).

The PCR-RFLP method allows for accurate and relatively inexpensive molecular identification compared with DNA-barcoding for distinguishing *O. churchillensis* from *O. communis*. RFLP-based identification is quick and requires approximately six hours to finish, 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 visualise the RFLP banding patterns. This method can be used to reduce taxonomic uncertainty and can facilitate the efficient identification of these cryptic species in all life stages.

Conclusion

This work has provided the first COI barcodes for *O. churchillensis* and developed the first RFLP-based identification tool for two sympatric members of the *O. communis* complex in Canada: *O. communis* and *O. churchillensis*. At least three members of the *O. communis* complex have diagnostic DNA barcodes using COI, including *O. churchillensis*, *O. communis*, and *O. tahoensis*. Unfortunately, *O. nevadensis* was not included in this study. The developed RFLP profiles can be used as a quick diagnostic tool to identify *O. communis* from *O. churchillensis*. Only through accurate identification of cryptic species can the vector potential of each species be determined, and this is critical for disease epidemiology. The molecular identification tools developed in this study will help facilitate monitoring of *O. communis* to predict future outbreaks of JCV and SSHV and will also facilitate future research to assess the potential of *O. churchillensis* as a vector of these diseases.

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Supplementary materials

To view supplementary material for this article, please visit http://dx.doi.org/10.4039/tce.2013.60

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