DNA Barcodes Identify Medically Important Tick Species in Canada

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Running Title: DNA barcodes identify ticks in Canada

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Abstract

Medically important ticks (Acari: Ixodidae) are often difficult to identify morphologically. A standardized, molecular approach using a 658 base pair DNA barcode sequence (from the 5' region of the mitochondrial cytochrome *c* oxidase subunit I gene) was evaluated for its effectiveness in discriminating ticks in North America, with an emphasis on Canadian ticks. DNA barcodes were generated for 96 of 154 specimens representing 26 ixodid species. A genetic cluster analysis was performed on the barcode sequences, which separated specimens into haplogroups closely corresponding with morphologically identified species. The tree topology was further supported by a BIN analysis. COI sequences generated were found to have a mean maximum intraspecific divergence of 1.59% and a mean nearest neighbour divergence of 12.8%, indicating a significant “barcode gap”. This study also revealed possible cryptic diversity among specimens morphologically identified as *Ixodes soricis* and *Ixodes texanus*. A PCR-based test for *Borrelia burgdorferi* determined that 18.1% of Lyme-competent ticks in this study were positive. This study is also the first to record a *B. burgdorferi*-positive exoskeleton. In conclusion, DNA barcoding is a powerful tool that clinicians can use to determine the identification of tick specimens which can help them to suggest whether an attached tick is a potential health risk.

Keywords: *Borrelia burgdorferi*, COI, cryptic diversity, tick identification, DNA barcoding

Introduction

The family Ixodidae, the hard-bodied ticks, is comprised of 702 species within 14 genera (Guglielmone et al. 2010) and includes species capable of transmitting bacterial, viral, and protozoan pathogens to their animal and human hosts. One of the most common tick-borne zoonotic diseases in Canada is Lyme disease, caused by the spirochete bacteria *Borrelia burgdorferi*. In North America, the primary vectors of *B. burgdorferi* include *Ixodes scapularis* and *I. pacificus*; meanwhile, *I. affinis*, *I. dentatus*, *I. spinipalpis* (Eisen and Lane, 2006), and *I. muris* (Dolan et al. 2000) are also suitable vectors. A study published by Besansky et al. (2003) argues that taxonomic revisions should focus on vectors, such as ticks, that are responsible for the spread of medically relevant pathogens and that the use of DNA-based approaches for species discrimination should be incorporated into these revisions.

Traditionally, ticks have been identified morphologically. However, difficulties can arise due to significant variation within larval, nymphal, and adult tick life stages. The immature stages (larvae and nymphs) are the most difficult to identify (Anderson et al. 2004). These specimens have minuscule physical characteristics that are hard to distinguish even with a stereoscopic microscope. Further difficulty ensues if the ixodid tick is engorged or if the specimen has been disfigured, which often occurs when removing a tick from its host (Anderson et al. 2004). The aforementioned difficulties with identification are compounded by the fact that the most recent morphological key for ixodid ticks in Canada was published over 50 years ago (see Gregson, 1956). This key is now incomplete based on current knowledge of tick diversity and distribution in the country.

A DNA-based approach to specimen identification of ixodid ticks is advantageous in that it can be used to identify immature ticks and destroyed samples to species level. The DNA barcode methodology represents a prime candidate to undertake this task as it is the most comprehensive initiative of all the DNA-based approaches (IBOL.org). DNA barcoding uses short, standardized gene sequences for specimen identification and species discovery (Herbert et al. 2003a). For animals, the standard barcode

consists of 658 base pair (bp) of the 5' region of the mitochondrial cytochrome *c* oxidase subunit I gene (herein referred to as the COI gene).

Another advantage of the DNA barcoding approach is the increased transparency and reproducibility of sequence data enabled by the Barcode of Life Data System (BOLD; www.barcodinglife.org), an online workbench for the management, analysis, and archival of barcode records that includes specimen images and the Barcode Index Number (BIN) framework for organizing records into species-like groupings and for delineating cryptic taxa (Ratnasingham and Hebert 2013).

A bioidentification system for all animals has been proposed using this gene, and scientists have successfully demonstrated its success for diverse taxonomic groups (Hebert et al. 2003b; Witt et al. 2006; Clarke et al. 2006) including various parasitic taxa (Ogedengbe et al. 2011; Webster et al. 2012; Cywinska et al. 2006) and even limited numbers of Ixodidae species (Barrett et al. 2005; Lv et al. 2013). A recent study of Ixodidae collected in China found that as a single gene identification system, COI was more effective at delineating Ixodidae species than 16S or 18S (Lv et al. 2013). A large-scale DNA barcode study of Ixodidae has not been undertaken in Canada or North America in general. In addition, the vast majority of publicly available tick barcodes on GenBank and BOLD are focused on the major vectors of Lyme disease.

Further development of molecular approaches may also prove useful for detecting both the host species and the presence of infection, contributing to public health by improving diagnoses and in epidemiological studies. Once barcoded, Lyme-competent vector ticks in this study were screened for systemic infection of *B. burgdorferi* using a nested PCR-based assay. This test is specific for the *OspA* gene, which encodes an outer surface protein of *B. burgdorferi* (Matuschka et al. 1996).

The objectives of this study were to 1) construct a DNA barcode library for Ixodidae in Canada based on morphologically identified specimens; 2) assess the prospect of a DNA barcode-based specimen identification system for the identification of medically relevant ticks compared to the morphological

identification of tick specimens to species, through the quantification of intraspecific and interspecific genetic variability; and 3) assess the ability to make species-level diagnoses for ticks and detect the presence of *B. burgdorferi* in Lyme-competent vectors from within the same genomic DNA sample. In doing so, this study aims to provide support for the use of DNA barcoding as a tool clinicians can use to make tick specimen identifications to assist in assessing potential health risks. In addition to its use as a tool for medical diagnostics, this tool would also help contribute to wider public health goals, including monitoring, the preparation of public health warnings, and in the conduction of epidemiological studies

Methods

Specimens:

A total of 163 specimens were obtained from either the Lyme Disease Association of Ontario (LDAO) or the Biodiversity Institute of Ontario, University of Guelph. 149 specimens were obtained from the LDAO and represent an accumulation of specimens derived from multiple projects carried out over the past two decades (1993-2012). The authors attempted to generate DNA barcodes for all the LDAO specimens following the protocols listed below. 14 specimens were obtained from the Biodiversity Institute of Ontario (BIO), which were collected during the 2010 BIOBus field season. The BIOBus is a mobile collection vehicle that travels to North America's parks each summer to collect specimens to DNA barcode (www.biobus.ca). The 14 BIO sequences were generated using standardized and published methods at their high-throughput facility. The majority of the specimens were collected primarily from Canada, with a few collected from mammalian hosts from the United States, Mexico, and Costa Rica (Figures 1 and 2).

Ixodid ticks were identified morphologically employing keys by Durden and Keirans (1996) as well as Keirans and Clifford (1978). Specimens from the LDAO collected prior to 2009 were stored in isopropyl alcohol, and those collected after 2009 were stored in 95% ethanol. Specimens collected by the BIOBus were store in 95% ethanol. Voucher specimen information, digital images, and all other relevant metadata

available (e.g. parasite host species, method of collection, location of collection) were deposited in BOLD under the publicly accessible projects named “Ticks of North America (project code: TODO)” or “BIO Mammal Parasites (project code: CSBMP).” 96 of the collected specimens were of appropriate condition after use in this study for vouchering and have been deposited as voucher specimens in the Biodiversity Institute of Ontario arthropod collection.

DNA Extraction, PCR Amplification and Sequencing

Where possible, a single leg or single whole molted exoskeleton was used for molecular work. Whole genomic DNA was extracted from this sub-sample using a QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA) or a XYTOGEN Insect Extraction kit (XYTOGEN, Perth, Australia) according to manufacturer’s instructions to a final elution volume of 2 μ l. Specimens which had extractions fail were attempted again using the identical protocol. PCR reaction volumes of 12.7 μ l included: 0.0625 μ l dNTPs, 0.625 μ l $MgCl_2$, 1.25 μ l 10X buffer, 6.25 μ l 10% trehalose, 0.06 μ l platinum taq, 0.1 μ l of each primer cocktail, and 2 μ l of template DNA. PCR primers were used in succession until successful amplification was achieved (Table 1): 1) LCO1490 and HCO2198 (Folmer et al. 1994), 2), LepF1 and LepR1 (Herbert et al. 2004), and 3) Lep Folmer cocktail. For those samples that failed to amplify, PCR primers were used to amplify two halves of the barcode region separately to be edited together to recreate the full barcode sequence (Table 1): C_LepFolF (LCO1490 and LepF1 cocktail) and C_ANTMR1D (Ronldeg_R and AMR1deg_R cocktail) (Folmer et al. 1994, Hebert et al. 2004, Simon et al. 1994, and Smith et al. 2005) for the preceding half and MLepF1 and C_LepFolR (HCO2198 and LepR1 cocktail) (Hajibabaei et al. 2006, Folmer et al. 1994, and Hebert et al. 2004) for the following half. The PCR thermocycle profile for all reactions consisted of: 1 cycle of 94 °C for 1 min; 5 cycles of 94 °C for 5 min, 45 °C for 1.5 min, and 72 °C for 1 min; followed by 35 cycles of 94 °C for 1 min, 51 °C for 40 sec, and 72 °C for 1 min; and a final cycle of 72 °C for 5 min. PCR products were visualized on a 2% agarose gel using an E-Gel 96 Pre-cast Agarose Electrophoresis System (Invitrogen). Successful amplicons underwent forward and reverse sequencing reactions with the following reagents: 1 μ l of big dye, 1 μ l of

5X SeqBuffer, 10µl ddH₂O, 1µl PCR primer, 1µl PCR template. The sequencing profile consisted of: initial hot start of 96 °C for 2 min, 30 cycles [denaturation at 96 °C for 30 sec, annealing at 55 °C for 15 sec, and extension at 60 °C for 4 min]. The products of the sequencing reaction were bi-directionally sequenced using the Big Dye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Mississauga, ON) on an ABI 3730 capillary sequencer.

DNA Sequence Analysis

Bi-directional sequence contig assemblies were created and edited using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI). High quality sequences were subsequently aligned via the CLUSTAL W algorithm within MEGA version 6.06, and the alignment was translated to amino acids for verification of proper alignment against the Invertebrate Mitochondrial genetic code (Tamura et al. 2011). Contig assemblies were uploaded to BOLD along with the raw electropherogram files and primer sequences used to generate them. All sequences were pooled into one dataset on BOLD for analysis. Conspecific and congeneric genetic distances were calculated using the Kimura 2-parameter (K2P) model (Kimura, 1980) in BOLD 3.0. A neighbour-joining tree (Nei and Kumar, 2000) was constructed in MEGA 6.06 using the K2P model to visualize patterns of genetic divergence within and between barcode clusters.

To further examine the significance of barcode clusters, the Barcode Index Number (BIN) system was applied to all sequences longer than 500 base pairs (bp) within this study and more broadly against all specimens on BOLD. The BIN system assigns specimens from various studies to operational taxonomic units (OTUs) based on nucleotide variation in the COI gene using the RESL algorithm (Ratnasingham and Hebert, 2013). These OTU's are examined and species assignments are first deemed as concordant (if all specimens in one OTU have the same species assignment within the dataset) or discordant (if species assignments vary within one OTU). Next, BINS are further classified into 4 categories (MATCH, SPLIT, MERGE, or MIXURE). A MATCH occurs when all members of a species are placed in one discrete BIN

with no other members. A SPLIT occurs when a single species clusters into multiple discrete BINS with no other members, often indicating cryptic diversity. A MERGE occurs when 2 or more species are placed in a single BIN together, which may indicate misidentifications or reveal that the species are indistinguishable through barcodes. Lastly, a MIXTURE occurs when a species has complex portioning with more than one SPLIT and MERGE.

Morphological assignments that did not match barcode clusters were subject to re-examination by an expert. The number of misidentifications were noted and corrected as a result of this iterative process.

Testing for B. burgdorferi

Once barcoded, 22 Lyme-competent vector ticks (eight *Ixodes scapularis*, three *I. pacificus*, two *I. affinis*, two *I. dentatus*, three *I. spinipalpis*, three *I. muris*, and one *I. baergi*) were screened for systemic infection of *B. burgdorferi* using a nested PCR based test. Only ticks belonging to the genus *Ixodes* were screened for *B. burgdorferi* as there have been no confirmed cases of Lyme-competency in ticks of other genera (Burgdorfer et al. 1982). The whole genomic extraction used in DNA barcoding was screened; therefore, the source of DNA was from the tick leg or exoskeleton. In addition, five *I. scapularis* exoskeletons that were not barcoded (due to repetition of species) were also tested for *B. burgdorferi*. Whole exoskeletons from freshly molted ticks were placed in 95% ethanol within three days of molting. The nested PCR was specific for the gene encoding *OspA* of *B. burgdorferi* (Matuschka et al. 1996). Each PCR run included 2 positive controls collected from Lyme-endemic areas and 2 negative controls. Each PCR reaction totalled 15.7µl and included 0.0625µl dNTPs, 0.625µl MgCl₂, 1.25µl 10X buffer, 6.25µl 10% trehalose, 0.06µl platinum taq, 0.2µl of each primer cocktail, and 5µl of template DNA or 5µl of PCR product. For PCR primer sequences and position in the *OspA* gene, see Table 2. The PCR thermocycle profile included heating for 2 min at 95°C and 40 cycles as follows: 20 seconds denaturation at 95 °C, 20 seconds at 59°C for the first annealing reaction and 61°C for the nested reaction, and 20 seconds of extension at 72°C

(Matuschka et al. 1996). PCR products were visualized on a 2% agarose gel using an E-Gel 96 Pre-cast Agarose Electrophoresis System (Invitrogen).

Results

Species Barcoded and Barcode Quality

This study generated DNA barcodes for 25 morphologically identified species from 5 genera and one unknown *Amblyomma* specimen. Barcodes were successfully generated for 3.69 ± 0.660 (mean $\pm$ SEM) specimens per species, with a range of 1 to 15 barcodes produced per species. The most extensive coverage was of the genus *Ixodes*, with 16 species barcoded, consisting of 1 to 9 specimens per species (Figure 3). Full length DNA barcodes (> 500 bp) were successfully recovered for 84 specimens and partial barcodes for 13 specimens out of a total of 167 specimens examined. The partial barcodes were primarily produced due to successful sequencing in only one direction. Thus, to ensure accuracy, part of the barcode was trimmed due to a large number of ambiguous bases. The edited sequences had no stop codons and well defined chromatogram peaks, suggesting the absence of pseudogene co-amplification. Nucleotide composition showed an AT bias within Ixodidae, with a mean of 38.07% T, 29.07% A, 18.43% C, and 14.2% G.

Upon examination of the sequencing results and consultation with ixodid tick experts, five cases of misidentified specimens were detected. Morphologically misidentified specimens (i.e., a *Dermacentor occidentalis* nymph, an *Ixodes dentatus* nymph, an *Ixodes spinipalpis* larva, an *Amblyomma sabanerae* nymph, and an *Ixodes angustus* larva) were subject to morphological re-examination and found to be *Rhipicephalus sanguineus*, *Ixodes muris*, *Ixodes pacificus*, *Amblyomma imitator*, and *Ixodes soricis*, respectively.

Once misidentifications were resolved and corrected, K2P analysis showed that all but two tick species (*I. texanus* and *I. soricis*) separated into distinct haplogroups or “barcode clusters” (Figure 3). These two species each contained 2 distinct clusters (clusters A through D as indicated in Figure 3), suggesting the

presence of cryptic diversity. Notably, *I. texanus* cluster A consisted of 2 specimens with an average intracluster K2P divergence of 0.67% and an average pairwise divergence of 14.97% from cluster B. *Ixodes texanus* cluster B consisted of 4 specimens with an average intracluster K2P divergence of 0.64% (range 0-0.80%). Secondly, *I. soricis* cluster C consisted of 2 specimens with an average intracluster K2P divergence of .29%. The *I. soricis* cluster D consisted of 1 specimen with a mean K2P divergence of 4.29% from cluster C.

Due to the 2 *I. texanus* clusters exhibiting deep genetic divergence similar to that seen at the interspecific level (Figure 3), these specimens were morphologically reexamined by an expert. Upon morphological re-examination where information for which individuals sorted into which clusters was withheld, subtle but distinguishable patterns of morphological co-variation were noted between the 2 clusters. When morphological differences and significant genetic divergence are combined, the cryptic species hypothesis is strengthened.

A barcode gap analysis was conducted using the K2P distance model and MUSCLE alignment on the remaining 24 species with distinct haplogroups for sequences ≥ 500 bp in length, which revealed a mean maximum intraspecific divergence of 1.59%, with a range of 0 – 14.5%, and a mean nearest neighbour divergence of 12.8%, with a range of 3.6-17.94% (Figure 4), suggesting that ixodid ticks display a substantial “barcode gap”. The means and ranges of maximum intraspecific divergences within genera were additionally determined and are reported (Table 4). It is important to note that for 3 species (*Ixodes baergi*, *Ixodes marxi*, *Amblyomma tuberculatum*) only one DNA barcode was generated, and no other reference sequences were publicly available; therefore, the reliability of DNA barcoding for identifying these 3 species cannot be assessed.

To further investigate the barcode clusters generated by the neighbour-joining tree, the BIN system analysis was computed for 90 of the 96 sequences on June 1st, 2015. Six sequences did not have sufficient length to be included in this analysis (<500 bp). These 90 sequences were placed in 28 BINS, with 23

BINS containing more than one specimen along with 4 singleton BINS. When these 28 BINS were computed against all other BIN records on BOLD at the time of writing, one instance of discordance was observed. This discordant BIN contained 48 members, with 47 *Rhipicephalus sanguineus* sequences and one *R. leporis* sequence. When the BIN analysis is examined at a species level, it further supports the neighbour-joining tree as BIN assignments are congruent with haplotypes on the tree. This analysis revealed the *Ixodes texanus* and *Ixodes soricis* species were categorized as SPILTS, further supporting the hypothesis of cryptic diversity. In addition, the BIN system was utilized to identify the unknown *Amblyomma* specimen to the species *Amblyomma tapirellum*, whereas it had previously been identified to genus only based on morphology.

B. burgdorferi Testing Results

Lyme-competent ticks were screened for systemic infection of *B. burgdorferi*. 17 successfully barcoded ticks were tested in addition to 5 non-barcoded exoskeletons (whose species identities were known). Of 22 ticks tested, 4 (18%) were positive for systemic *B. burgdorferi* infection (Table 3). Of all specimens tested, only *I. scapularis* ticks were found to test positive, of which 50% of specimens assessed tested positive for the presence of *B. burgdorferi*.

Discussion

This study represents the first effort at creating a reference DNA barcode library based on morphologically identified tick specimens in Canada. In addition, this study significantly increases the diversity of Ixodidae species represented on BOLD, as it added 21 unrepresented species to BOLD. A limitation of this study was the incomplete representation of all Ixodidae species in Canada, being able to only include 24 of the approximately 40 species known to occur in Canada (Canadian Lyme Disease Foundation 2016).

This study further demonstrates the ability of DNA barcodes to effectively discriminate tick species due to the presence of a substantial “barcode gap,” with the minimum pairwise distance to the nearest

neighbouring species being 8x greater than the mean maximum intraspecific distance. In addition to the barcode gap, there was no overlap seen between intra and interspecies variation.

DNA barcoding also has the ability to reveal interesting findings that can be difficult or impossible to reveal through morphological examination. Firstly, DNA barcoding revealed 5 morphological misidentifications, which demonstrates the difficulty, even for experts, in identifying immature life stages, and the utility of DNA barcoding to aid in these challenging specimen identifications. Secondly, this study revealed two cases of deep genetic divergence in species morphologically identified as *Ixodes texanus* and *Ixodes soricis*. For *Ixodes texanus*, this genetic divergence was further supported by subtle morphological variation which further strengthens the cryptic species hypothesis. When evaluating species that have not been previously barcoded, cryptic species are often revealed (Clarke et al. 2006; Hebert et al. 2004; Smith 2008; Witt et al. 2006).

The revelation of cryptic diversity is of particular relevance for numerous ixodid tick species. In a study comparing 400 successfully barcoded ixodid ticks by Zhang and Zhang (2014), 12 species of ticks were found to exhibit exceptionally high intraspecific divergences out of a total 70 species across 14 genera. The authors suggest that this could represent the presence of cryptic diversity, but could also be a result of inaccurate identification (possibly due to mitochondrial introgression and hybridization). In a study of 130 immature tick sequences compared to an assembled 96 sequence adult tick barcode library, Miller et al. (2014) found the presence of possible cryptic diversity in *Haemaphysalis juxtakochi* and *Amblyomma ovale*. Miller et al. (2014) found both species split into two barcode clusters, and a BIN analysis showed each species was experiencing a SPLIT pattern (see Ratnasingham and Hebert, 2013) of BIN organization, similarly to what was observed within *I. texanus* and *I. soricis* in the current study. These studies, in addition to the current study, highlight the complexity of tick specimen identification and the need for taxonomic revision of potentially cryptic tick species.

A limitation present in this study and in all genetic methods for identifying species is that sequence generation rates can be less than ideal if the specimens are aged or not properly preserved (Watts et al. 2007; King and Porter, 2004). The authors suspect that DNA barcodes were only generated for 96 out of 166 specimens in this study due to DNA degradation caused by specimens age (up to 18 years), and the majority of the specimens being improperly stored in isopropyl alcohol as opposed to standard 95% ethanol.

The first tick-focused DNA barcode study was published by Lv et al. (2014a). Lv et al. (2014a) argue for the use of a three-gene DNA barcode system, including CO1, 16S, and 18S, for ticks as opposed to using COI alone. The reasoning behind using three-gene systems is not because it more accurately provides species delineations, but rather that there is not a sufficient COI sequence library on BOLD or GenBank to provide species-level matches for some tick species. While Lv et al. provide a novel solution to the lack of reference sequence library, this solution creates additional costs and efforts for limited added benefit and primarily relies on GenBank data that lacks the data integrity that BOLD offers. BOLD requires that all sequences must be associated with the following criteria in order to be considered a “barcode-compliant” sequence: 1) species name, 2) voucher information (including catalogue number and institution storing), 3) collection information (collector, collection date, and location with GPS coordinates), 4) identifier of the specimen, 5) for animals a COI sequence of at least 500 bp, 6) polymerase chain reaction (PCR) primers used to generate the amplicon, 7) raw trace files (Ratnasingham et al. 2007). These community standards increase the level of transparency, reproducibility, and taxonomic confidence of DNA barcoding (Ondrejicka et al. 2014) and even allow scientists to request voucher specimens when there are discrepancies between specimen identifications. In a follow up study, Lv et al. (2014b) found that there was no significant difference in the efficacy of 12S, 16S, ITS2, or COI for use in the identification of tick specimens; however, they found the use of COI ideal due to the utility and large COI sequence collection of BOLD.

In addition to increasing data integrity, BOLD offers new prospects for linking tick specimen identification with the identification of the tick's host. In 2012, a study by Gariepy et al. validated a method to use DNA barcoding to identify the host species through tick bloodmeals. When both tick and host identifications are linked on BOLD, this becomes a powerful tool for ecologists.

B. burgdorferi Testing

While midgut or whole specimen testing is the norm when screening for *B. burgdorferi*, systemic testing was used in this study for three reasons (Persing et al. 1990). 1) A leg DNA extract, which was initially used for barcoding, was easily screened for *B. burgdorferi* without further harming the morphological voucher specimen. 2) Very few studies have examined the rate of systemic infections, especially in less common vector ticks, such as *I. muris*. It is also known that systemic infection varies by species (Crippa et al. 2002). 3) No known studies have attempted to test tick exoskeletons for the presence of *B. burgdorferi*.

It is important to note that performing a systemic test for *B. burgdorferi* does not rule out the possibility that a particular tick harbours a midgut infection. Therefore, additional Lyme-competent specimens included in this study may be infected in their midgut, but not exhibiting a systemic infection, suggesting that our results represent a conservative estimate of infection rate. While individuals from 7 Lyme-competent species were tested, only *I. scapularis* ticks were positive in this study. Notably, this study is the first to detect *B. burgdorferi* from an exoskeleton. This finding may provide a method of investigating the infection status of morphologically important voucher specimens, without sacrificing the specimen or distorting any anatomical part of the newly-molted tick. Further research should investigate the frequency of exoskeleton infection compared to midgut infection. As well, future studies should verify that the same abdominal-derived extract might be used for both host identification and infection status determination.

The ability to both identify specimens to species level and to screen for important pathogens from the same whole genomic extract allows for expanded applications of DNA barcoding for public health

monitoring. Real-time quantitative PCR (qPCR) has been shown to be a fast and highly accurate technique for use in the detection and quantification of bacterial pathogens present in municipal water sources to screen for potential risks of pathogen exposure (Shannon et al. 2007). A duplexed qPCR assay designed to be able to simultaneously identify if a specimen is an important Lyme-competent vector with species-specific accuracy (for example, *I. scapularis*) and identify the presence of *B. burgdorferi* within a specimen (for example, by detecting the *OspA* gene) within a single DNA extract could allow for the rapid and accurate screening of potential tick-related health risks. The ability to identify host identities as well as information about pathogen presence is valuable, as it can allow for improved risk assessments. Being able to determine if you have the right host species as well as being able to determine if the particular specimen poses a risk of pathogen transmission to the public simultaneously can allow for more rapid and more informed risk-prevention decisions. Further study into the suitability of assays like real-time qPCR for the simultaneous identification of pathogen host and pathogen presence for the screening of Lyme-competent ticks is recommended.

Conclusion

This study significantly increases the barcode reference sequence library for Canadian ticks. It also demonstrates the presence of a “barcode gap,” with the distance between nearest neighbour species being 8x greater than the mean maximum intraspecific variation, suggesting that barcodes are highly effective for discriminating tick species. Importantly, it extends our ability to confidently identify immature stages, damaged specimens, and fragmentary remains that are often encountered in a clinical setting. This study also reveals two putative cryptic species based on genetic divergence and BIN analysis originally identified as *Ixodes texanus* and *Ixodes soricis*. Furthermore, a PCR-based test determined that 18% of Lyme-competent ticks in this study were positive for systemic *B. burgdorferi* infection. We provide the first-ever report of a *B. burgdorferi* positive exoskeleton from a molted tick. Future work should focus on increasing coverage for these taxa and ideally involve comparisons with type specimens as part of a comprehensive taxonomic revision, as well as further exploring the potential for assays to be designed

that allow for simultaneous specimen detection and *B. burgdorferi* infection detection. Based on the results of the 26 species of ixodid ticks examined, DNA barcoding provided a rapid, accurate, and cost-effective DNA-based method to enhance the identification of tick specimens.

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Tables

Table 1. Primer sequences used in PCR for DNA barcoding.

Order	Primer	Sequence	Reference
1	LCO1490	5'-ggccaacaaatcataaagatattgg-3'	Folmer et al. 1994
	HCO2198	5'-taaacttcagggtgacaaaaaatca-3'	
2	LepF1	5'-attcaaccaatcataaagatat-3'	Hebert et al. 2004
	LepR1	5'-taaacttctggatgtccaaaa-3'	
3	LCO1490	5'-ggccaacaaatcataaagatattgg-3'	Folmer et al. 1994 and Hebert et al. 2004
	HCO2198	5'-taaacttcagggtgacaaaaaatca-3'	
	LepF1	5'-attcaaccaatcataaagatat-3'	
	LepR1	5'-taaacttctggatgtccaaaa-3'	
4	LCO1490	5'-ggccaacaaatcataaagatattgg-3'	Folmer et al. 1994, Hebert et al. 2004, Simon et al. 1994, and Smith et al. 2005
	LepF1	5'-attcaaccaatcataaagatat-3'	
	RonIdeg_R	5'-ggrggrrarayagttcatccwgtwcc-3'	
	AMR1deg_R	5'-cawccwgtwccrmnccwkat-3'	
5	MLepF1	5'-gctttccacgaataaataata-3'	Hajibabaei et al 2006, Folmer et al 1994, and Hebert et al. 2004
	HCO2198	5'-taaacttcagggtgacaaaaaatca-3'	
	LepR1	5'-taaacttctggatgtccaaaa-3'	

Table 2. OspA primer sequences used to detect *B. burgdorferi*

Outer/ Inner	Position	Sequence	Reference
Outer	169-194	GGTCTAATATTAGCCTTAATAGGCATG	Matuschka et al. 1996
Outer	665-643	TCAGCAGCTAGAGTTCCTTCAAG	Matuschka et al. 1996
Inner	191-214	CATGTAAGCAAAATGTTAGCAGCC	Matuschka et al. 1996
Inner	589-568	CTGTGTATTCAAGTCTGGTTCC	Matuschka et al. 1996

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Table 3. Collection sites and sources of 22 genomic DNA samples tested for infection by *B. burgdorferi*

Tick species	Part of tick tested	Location collected	Presence of <i>B. burgdorferi</i> ?
<i>Ixodes scapularis</i>	Leg	Turkey Point, ON	Yes
<i>I. scapularis</i>	Exoskeleton	Toronto, ON	Yes
<i>I. scapularis</i>	Exoskeleton	Sibley Peninsula, ON	Yes
<i>I. scapularis</i>	Exoskeleton	Sibley Peninsula, ON	Yes
<i>I. affinis</i>	Leg	Georgia, USA	No
<i>I. affinis</i>	Leg	United States	No
<i>I. dentatus</i>	Leg	Long Point, ON	No
<i>I. dentatus</i>	Leg	Long Point, ON	No
<i>I. muris</i>	Leg	New Brunswick, Canada	No
<i>I. muris</i>	Leg	Nova Scotia, Canada	No
<i>I. muris</i>	Leg	McGill Bird Observatory, QB	No
<i>I. baergi</i>	Leg	Elora, ON	No
<i>I. pacificus</i>	Leg	United States	No
<i>I. pacificus</i>	Leg	Victoria, BC	No
<i>I. pacificus</i>	Leg	California, USA	No
<i>I. spinipalpus</i>	Leg	Cypress Hills, ON	No
<i>I. spinipalpus</i>	Leg	Ontario, Canada	No
<i>I. spinipalpus</i>	Leg	Vancouver Island, BC	No
<i>I. scapularis</i>	Exoskeleton	N/A	No
<i>I. scapularis</i>	Exoskeleton	N/A	No
<i>I. scapularis</i>	Leg	Canada	No
<i>I. scapularis</i>	Leg	Canada	No

Table 4. Summary of divergences observed within genera produced by the barcode gap analysis on BOLD.

Genus	Number of Species	Mean Maximum Intraspecific Divergence (%)	Range of Maximum Intraspecific Divergences	Mean Distance to Nearest Neighbour (%)	Range of Mean Distances to Nearest Neighbour
			(Min% - Max%)		(Min% - Max%)
<i>Amblyomma</i>	6	0.77	0.00 - 2.57	16.87	16.32 - 17.17
<i>Dermacentor</i>	2	2.51	1.73 - 3.29	10.80	10.80 - 10.80
<i>Haemaphysalis</i>	1	0.46	-	16.18	-
<i>Ixodes</i>	16	1.87	0.00 - 14.50	11.03	3.60 - 17.94
<i>Rhipicephalus</i>	1	0.49	-	16.18	-

Figure Captions

Figure 1. Collection sites for ixodid tick specimens acquired between 1993 and 2012 from Canada, the United States, Mexico, and Costa Rica. Map generated using SimpleMappr.

Figure 2. Collection sites for ixodid tick specimens acquired between 1993 and 2012 from Ontario and Quebec. Map generated using SimpleMappr.

Figure 3. Neighbour joining tree analysis of 96 tick sequences from 5 genera, including 26 different species. Analysis was conducted using a Kimura 2 Parameter (K2P) distance model with branch lengths drawn to scale. The terminal node of each haplogroup was collapsed, with the height of the terminal node proportional to the number of sequences and depth proportional to sequence divergence within a haplogroup. Analysis shows ixodid tick species distinguish into distinct haplogroups or “barcode clusters”, with the exception of *I. texanus* (clusters A and B), and *I. soricis* (clusters C and D) with each distinguishing into two haplogroups each (indicated as Cluster A-D). Evolutionary analysis and tree construction were performed using MEGA 6.06.

Figure 4. Barcode gap analysis comparing the maximum intraspecific distances with the nearest neighbor distances (a metric of interspecific distance) using a Kimura 2 Parameter distance model and a MUSCLE alignment. All non-singleton species of ixodid ticks with DNA barcodes of ≥ 500 bp length successfully generated in this study were included in the analysis. The majority of species showed low maximum intraspecific distances and large nearest neighbor distances (solid black dots), indicating the presence of a “barcode gap”. Two notable outliers were observed: 1) *Ixodes soricis* (solid white dot) was found to have a greater nearest neighbor distance than maximum intraspecific distance, and 2) *I. texanus* (white dot with cross) was found to have much larger divergences than all other included species, with a 9.1x greater maximum intraspecific distance than the mean maximum intraspecific divergence observed.

Figure 1
228x114mm (300 x 300 DPI)

Figure 2
228x114mm (300 x 300 DPI)

Draft

Figure 3
50x53mm (300 x 300 DPI)

Figure 4
113x79mm (300 x 300 DPI)

Supplemental Data 1. Specimen Information Table

Sample ID	BOLD Process ID	GenBank Accession Number	Species
DO-006	TODO001-11	KX360354	<i>Haemaphysalis leporispalustris</i>
DO-007	TODO002-11	KX360417	<i>Ixodes cookei</i>
DO-023	TODO003-11	KX360416	<i>Ixodes scapularis</i>
DO-028	TODO004-11	KX360382	<i>Ixodes scapularis</i>
DO-030	TODO005-11	KX360339	<i>Dermacentor variabilis</i>
DO-035	TODO006-11	KX360407	<i>Amblyomma americanum</i>
DO-039	TODO007-11	KX360331	<i>Ixodes scapularis</i>
DO-042	TODO008-11	KX360377	<i>Ixodes scapularis</i>
DO-048	TODO009-11	KX360340	<i>Ixodes cookei</i>
DO-050	TODO040-11	KX360412	<i>Ixodes texanus</i>
DO-056	TODO043-11	KX360399	<i>Ixodes muris</i>
DO-059	TODO044-11	KX360379	<i>Amblyomma maculatum</i>
DO-062	TODO046-11	KX360372	<i>Ixodes muris</i>
DO-063	TODO047-11	KX360390	<i>Ixodes texanus</i>
DO-071	TODO067-11	KX360342	<i>Ixodes texanus</i>
DO-074	TODO070-11	KX360385	<i>Ixodes spinipalpis</i>
DO-075	TODO108-11	KX360366	<i>Ixodes marxi</i>
DO-081	TODO109-11	KX360408	<i>Ixodes pacificus</i>
DO-085	TODO105-11	KX360343	<i>Ixodes pacificus</i>
DO-089	TODO083-11	KX360328	<i>Ixodes affinis</i>

DO-091	TODO085-11	KX360360	<i>Ixodes kingi</i>
DO-092	TODO086-11	KX360380	<i>Ixodes kingi</i>
DO-093	TODO087-11	KX360400	<i>Ixodes brunneus</i>
DO-095	TODO089-11	KX360422	<i>Ixodes affinis</i>
DO-099	TODO091-11	KX360351	<i>Amblyomma imitator</i>
DO-100	TODO092-11	KX360341	<i>Amblyomma imitator</i>
DO-101	TODO093-11	KX360364	<i>Ixodes brunneus</i>
DO-103	TODO095-11	KX360396	<i>Ixodes muris</i>
DO-104	TODO096-11	KX360344	<i>Amblyomma maculatum</i>
DO-105	TODO097-11	KX360356	<i>Amblyomma longirostre</i>
DO-106	TODO098-11	KX360336	<i>Rhipicephalus sanguineus</i>
DO-107	TODO099-11	KX360329	<i>Amblyomma longirostre</i>
DO-109	TODO107-11	KX360371	<i>Amblyomma tuberculatum</i>
DO-111	TODO100-11	KX360363	<i>Amblyomma longirostre</i>
DO-112	TODO101-11	KX360370	<i>Amblyomma tapirellum</i>
DO-117	TODO106-11	KX360376	<i>Amblyomma imitator</i>
DO-118	TODO103-11	KX360388	<i>Ixodes baergi</i>
DO-123	TODO104-11	KX360373	<i>Ixodes soricis</i>
DO-145	TODO142-12	KX360402	<i>Ixodes texanus</i>
DO-147	TODO140-12	KX360368	<i>Ixodes banksi</i>
DO-149	TODO144-12	KX360383	<i>Ixodes texanus</i>
DO-151	TODO143-12	KX360404	<i>Ixodes texanus</i>

DO-152	TODO145-12	KX360421	<i>Ixodes pacificus</i>
DO-153	TODO146-12	KX360375	<i>Ixodes spinipalpis</i>
DO-159	TODO147-12	KX360374	<i>Ixodes dentatus</i>
DO-160	TODO148-12	KX360409	<i>Ixodes dentatus</i>
DO-161	TODO141-12	KX360369	<i>Ixodes banksi</i>
DO-168	TODO149-12	KX360381	<i>Dermacentor andersoni</i>
DO-169	TODO150-12	KX360395	<i>Dermacentor andersoni</i>
DO-170	TODO151-12	KX360398	<i>Dermacentor andersoni</i>
DO-200	TODO011-11	KX360393	<i>Ixodes cookei</i>
DO-201	TODO012-11	KX360361	<i>Dermacentor variabilis</i>
DO-203	TODO013-11	KX360353	<i>Ixodes cookei</i>
DO-205	TODO014-11	KX360387	<i>Dermacentor variabilis</i>
DO-206	TODO015-11	KX360384	<i>Dermacentor variabilis</i>
DO-207	TODO016-11	KX360391	<i>Haemaphysalis leporispalustris</i>
DO-208	TODO017-11	KX360359	<i>Ixodes cookei</i>
DO-211	TODO019-11	KX360337	<i>Haemaphysalis leporispalustris</i>
DO-212	TODO020-11	KX360346	<i>Dermacentor variabilis</i>
DO-213	TODO021-11	KX360365	<i>Ixodes cookei</i>
DO-218	TODO025-11	KX360358	<i>Ixodes cookei</i>
DO-219	TODO026-11	KX360378	<i>Ixodes cookei</i>
DO-221	TODO028-11	KX360333	<i>Dermacentor variabilis</i>
DO-222	TODO029-11	KX360405	<i>Haemaphysalis leporispalustris</i>

DO-223	TODO030-11	KX360330	<i>Ixodes cookei</i>
DO-225	TODO032-11	KX360413	<i>Ixodes cookei</i>
DO-226	TODO033-11	KX360415	<i>Dermacentor variabilis</i>
DO-230	TODO036-11	KX360332	<i>Dermacentor variabilis</i>
DO-306	TODO055-11	KX360338	<i>Rhipicephalus sanguineus</i>
DO-309	TODO056-11	KX360403	<i>Rhipicephalus sanguineus</i>
DO-311	TODO057-11	KX360367	<i>Rhipicephalus sanguineus</i>
10AVB-PAR0064	CSBMP064-10	HM398837	<i>Ixodes soricis</i>
10AVB-PAR0065	CSBMP065-10	KX360410	<i>Ixodes soricis</i>
10AVB-PAR0191	TGBT001-10	KX360420	<i>Amblyomma americanum</i>
10AVB-PAR0192	TGBT002-10	KX360347	<i>Amblyomma americanum</i>
10AVB-PAR0193	TGBT003-10	KX360414	<i>Amblyomma americanum</i>
10AVB-PAR0194	TGBT004-10	KX360392	<i>Dermacentor variabilis</i>
10AVB-PAR0195	TGBT005-10	KX360418	<i>Dermacentor variabilis</i>
10AVB-PAR0196	TGBT006-10	KX360389	<i>Dermacentor variabilis</i>
10AVB-PAR0198	TGBT007-10	KX360386	<i>Dermacentor variabilis</i>
10AVB-PAR0199	TGBT008-10	KX360334	<i>Amblyomma americanum</i>
10AVB-PAR0200	TGBT009-10	KX360362	<i>Amblyomma americanum</i>
10AVB-PAR0201	TGBT010-10	KX360352	<i>Amblyomma americanum</i>
10AVB-PAR0202	TGBT011-10	KX360335	<i>Amblyomma americanum</i>
10AVB-PAR0203	TGBT012-10	KX360419	<i>Amblyomma americanum</i>
10AVB-PAR0204	TGBT013-10	KX360350	<i>Amblyomma americanum</i>

10AVB-PAR0205	TGBT014-10	KX360401	Amblyomma americanum
KCM025	PAVOC027-13	KX360355	Ixodes spinipalpis
KCM032	PAVOC033-13	KX360397	Ixodes angustus
KCM033	PAVOC034-13	KX360406	Ixodes angustus
KCM034	PAVOC035-13	KX360411	Ixodes angustus
KCM037	PAVOC038-13	KX360345	Ixodes uriae
KCM039	PAVOC040-13	KX360357	Ixodes angustus
KM028	PAVOC029-13	KX360349	Dermacentor variabilis
KM029	PAVOC030-13	KX360394	Dermacentor variabilis
KM030	PAVOC031-13	KX360348	Dermacentor variabilis

Life stage	Collection Date	Country	State/ Province	Region	Latitude
nymph	10-Sep-09	Canada	ON	Long Point	42.592
female	20-Mar-10	Canada	ON	Toronto	43.761
nymph	N/A	Canada	ON	Turkey Point	42.698
nymph	20-Oct-09	Canada	ON	Turkey Point	42.698
nymph	N/A	Canada	ON	Welland	42.993
female	18-May-04	Canada	ON	Wellesley	43.477
female	18-Apr-08	Canada	ON	Turkey Point	42.698
adult	7-Jun-07	Canada	ON	Toronto	43.653
female	25-Jul-07	Canada	ON	Muskoka	44.902
female	3-Jun-02	Canada	ON	Burlington	43.326
larva	1-Jul-01	Canada	NB	St. Andrews	45.559
adult	3-Apr-02	Canada	ON	Stoney Creek	43.219
female	25-Jul-01	Canada	NB	St. Andrews	45.558
female	3-Jun-02	Canada	ON	Georgetown	43.65
female	21-May-02	Canada	ON	Chesterville	45.103
male	N/A	Canada	BC	Victoria	48.429
female	19-May-08	Canada	ON	Highland	47.6
male	N/A	United States	CA	Willits	39.55
female	N/A	Canada	BC	Hope	49.385
female	N/A	Canada	MB	Delta Beach	50.187

nymph		21-Jun-00	Canada	SK	Acerage	52.134
nymph		21-Jun-00	Canada	SK	Acerage	52.134
female		16-Apr-98	Canada	ON	Long Point	42.592
male	N/A		United States	GA	Geogian Southern University	32.419
nymph		18-May-99	Canada	ON	Long Point	42.592
nymph		5-Jun-07	Canada	ON	Toronto	43.653
female		25-May-04	Canada	YT	Watson Lake	60.065
nymph		27-May-08	Canada	QC	Ste-Anne-de-Bellevue	45.404
male	N/A		Canada	ON	Bobcageon	44.545
nymph	N/A		Canada	ON	Bobcageon	42.592
nymph		22-Sep-04	Mexico		Ekbalam	20.887
nymph		24-Apr-01	Canada	MB	Delta Beach	50.187
larva		9-Jan-01	Canada	ON	Mount Albert	45.603
nymph		26-May-04	Canada	ON	Long Point	42.592
nymph		9-Jan-97	Costa Rica		Tarcoles	9.773
nymph	N/A		Canada	ON	Long Point	42.592
gravid female		14-Jun-99	Canada	ON	Elora	43.683
female		2-Sep-11	Canada	BC	Rocky Point	48.429
nymph		19-Aug-94	Canada	ON	Fort Erie	42.902
nymph	N/A		Canada	ON	Kenora	49.767
female		12-Aug-94	Canada	ON	Halton Hills	43.642
nymph		19-Aug-94	Canada	ON	Fort Erie	42.902

larva	14-Oct-08	Canada	BC	Victoria	48.429
female	15-May-10	Canada	AB	Cypress Hills	49.64
nymph	27-Apr-07	Canada	ON	Long Point	42.597
nymph	27-Apr-07	Canada	ON	Long Point	42.597
nymph	22-May-97	Canada	ON	Kenora	49.767
female	18-Apr-12	Canada	BC	Kelowna	49.953
male	11-May-12	Canada	BC	Kelowna	49.953
female	16-May-12	Canada	BC	Kelowna	49.953
female	N/A	Canada	ON	Sarnia	42.985
male	10-Aug-92	Canada	ON	Sarnia	42.985
adult	13-Jul-93	Canada	ON	Kincardine	44.175
male	25-May-93	Canada	ON	Rondeau	42.293
male	N/A	Canada	ON	Keewatin	49.767
nymph	12-Jul-94	Canada	ON	Kenora	49.767
adult	23-Jun-94	Canada	ON	Morrisburg	44.901
female	9-Jun-95	Canada	ON	Kenora	49.767
male	27-May-96	Canada	ON	Thunder Bay	48.416
adult	30-May-96	Canada	ON	Mount St. Marie	46.813
adult	25-May-98	Canada	ON	Kingsville	49.767
adult	7-May-98	Canada	ON	Hamilton	43.244
adult	11-Jun-99	Canada	ON	Hensall	43.434
adult	23-Jun-99	Canada	ON	Timmins	48.476

adult	26-May-99	Canada	ON	Londesborough	43.729
adult	25-Sep-00	Canada	ON	Maidstone	42.209
adult	24-May-00	Canada	ON	Wainfleet	42.922
gravid female	20-Jul-02	Canada	ON	Fort Erie	42.902
adult	20-Dec-02	Canada	ON	Toronto	43.653
nymp	9-Sep-02	Canada	ON	Kincardine	44.175
adult	13-Sep-02	United States	KY	Lebanon	37.555
Immature	20-Aug-08	Canada	AB	Kananaskis	50.7649
Immature	20-Aug-08	Canada	AB	Kananaskis	50.7649
Immature	30-Mar-10	United States	FL	Tarkiln Bayou SP	30.367
Immature	28-Mar-10	United States	FL	Ocala Nat Forest	30.3154
Immature	28-Mar-10	United States	FL	Ocala Nat Forest	30.3154
Adult	17-May-10	United States	CO	N/A	35.6937
Adult	17-May-10	United States	CO	N/A	35.6937
Adult	17-May-10	United States	CO	N/A	35.6937
Adult	17-May-10	United States	AR	N/A	35.3666
Adult	26-Mar-10	United States	FL	Ocala Nat Forest	29.2631
Adult	26-Mar-10	United States	FL	Ocala Nat Forest	29.2631
Adult	27-Mar-10	United States	FL	Ocala Nat Forest	29.1824
Adult	27-Mar-10	United States	FL	Ocala Nat Forest	29.1824
Adult	27-Mar-10	United States	FL	Ocala Nat Forest	29.1824
Adult	27-Mar-10	United States	FL	Ocala Nat Forest	29.1824

Immature	27-Mar-10	United States	FL	Ocala Nat Forest	29.1824
N/A	7-Feb-13	Canada	BC	Vancouver Island	48.4544
Nymph	N/A	Canada	BC	N/A	48.3667
Adult	N/A	Canada	BC	N/A	48.3667
Adult	N/A	Canada	BC	N/A	48.3667
Adult	2-Aug-13	Canada	NL	N/A	53.934
Nymph	N/A	Canada	BC	N/A	48.3667
Adult	29-Jun-13	Canada	MB	Riding Mountain National Park	50.7251
N/A	29-Jun-13	Canada	MB	Riding Mountain National Park	50.7251
Adult	28-Jun-13	Canada	MB	Riding Mountain National Park	50.6793

Longitude	Accuracy (km)
-80.447	1
-80.447	1
-80.331	25
-80.331	1
-79.239	25
-80.764	25
-80.331	1
-79.383	1
-79.576	50
-79.799	25
-61.89	25
-79.766	25
-61.89	25
-79.904	25
-75.23	25
-123.366	25
-79.816	25
-123.438	100
-121.441	25
-98.32	1

Draft

-106.648	25
-106.648	25
-80.447	1
-81.787	1
-80.447	1
-79.383	25
-128.703	25
-73.95	25
-78.541	25
-80.447	1
-88.137	25
-98.32	1
-75.835	25
-80.447	1
-84.63	25
-80.447	1
-80.431	25
-123.366	25
-78.972	25
-94.489	100
-79.932	25
-78.972	25

Draft

-123.366	25
-110.19	1
-80.45	1
-80.45	1
-94.489	25
-119.375	50
-119.375	50
-119.375	50
-82.36	25
-82.36	25
-81.636	25
-82.134	1
-94.55	25
-94.489	25
-75.185	25
-94.489	25
-89.267	100
-71.176	25
-94.489	25
-79.889	25
-81.503	25
-81.33	25

Draft

-81.465	50
-82.888	25
-79.375	25
-78.972	25
-79.383	25
-81.636	25
-85.391	25
-115.143	N/A
-115.143	N/A
-87.4071	N/A
-87.4214	N/A
-87.4214	N/A
-101.573	N/A
-101.573	N/A
-101.573	N/A
-93.344	N/A
-81.8552	N/A
-81.8552	N/A
-81.7133	N/A
-81.7133	N/A
-81.7133	N/A
-81.7133	N/A

Draft

-81.7133 N/A	
-123.458	25
-123.707	
-123.707	25
-123.707	
-123.707	25
-56.505	
-123.707	
-99.5553 N/A	
-99.5553 N/A	
-99.8942 N/A	

Draft