Building a Collaborative DNA Barcode Library for the Diptera of Churchill, Canada: A Resource for Northern Research

DNA Barcode Library for Subarctic Diptera

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Abstract

Churchill, Manitoba, Canada is a diverse Subarctic region that has been the focus of DNA barcoding efforts and research for decades. Despite this effort, there are still taxa in this region that are underrepresented in public databases, particularly hyperdiverse insect groups. We present a collaborative large-scale DNA barcode reference library for Diptera based on molecular data and expert taxonomic identification. The reference library contains 16786 specimens sampled around Churchill from 2005 to 2011. Specimens were identified by morphological means, sequenced for the animal barcode marker, and sorted into 2235 Molecular Operational Taxonomic Units (using Barcode Index Numbers: BINs) representing 68 families and 1211 named species. Eighty-four species shared a BIN with another species, 98 species were split across several BINs, and 1028 species were assigned to a unique BIN. The intraspecific and nearest neighbour distances varied across families, but most species can be easily distinguished from their nearest neighbours. By combining molecular data with taxonomic expertise, the barcode library provides species-level information for 12612 specimens, supplying future researchers with detailed taxonomic information and the opportunity to perform barcode-based specimen identifications for large-scale studies through sequence matching. Comparison of the Diptera composition of Churchill to other geographic regions reflected likely postglacial colonization northwards as well as a Beringian component. This study provides a publicly available, detailed reference library for use in future research, as well as new insights into the current diversity of Diptera present among the diverse habitats in this Subarctic region.

Key Words: Subarctic, Cytochrome c oxidase subunit 1 (CO1), Boreal habitats, Biodiversity

Introduction

DNA barcoding, the process by which standardized gene regions are used to identify, discover, and delineate species, is a useful tool that can allow us to document further the diversity of different regions and ecosystems (Hebert et al., 2003). These barcodes also enable further analysis and research, allowing us to study biodiversity and temporal trends in community composition.

There has been a concentrated effort to barcode species in various areas globally, including Costa Rica (Janzen & Hallwachs, 2011, Janzen et al., 2009), Finland (Pohjoismäki et al., 2016, Roslin et al., 2021), Greenland (Wirta et al., 2016), and Nunavut (deWaard, 2023, Pentinsaari et al., 2020). Regional barcode studies and libraries provide a valuable resource and allow for the diversity of regions to be elucidated, as well as provide the opportunity for further research. Wirta et al. (2016), for example, provided a barcode library including over 403 terrestrial animal species and 160 vascular plant species for the Zackenberg Valley in Northeast Greenland. This library has been used for a variety of novel studies, including to determine the prey of spiders and investigate niche overlap (Wirta et al., 2015b), to elucidate Arctic food webs and interactions between predators and prey (Wirta et al., 2015a), and to investigate the role of flies in the pollination of Arctic plant species (Tiusanen et al., 2016). By continuing to barcode specimens and build these regional libraries, we can expand our knowledge of the communities and ecological processes within these regions. Across geographic regions, DNA barcoding has been shown to be a helpful and successful approach for Diptera, and it is a useful tool for identifying and delineating species within this group (Adeniran et al., 2019, Banerjee et al., 2015, Brodin et al., 2012, Changbunjong et al., 2018, Cywinska et al., 2010, de Souza Pinto, 2023, Machado et al., 2017, Posada-López et al., 2023, Rivera & Currie, 2009, Votýpka et al., 2019).

Since the 1800s, the northern regions of Canada have been the focus of various biological surveys and sampling events. One notable sampling event that spanned several insect orders was the

Canadian Northern Insect Survey, conducted from 1947 – 1962 (Danks, 1981, Freeman, 1958, Huckett et al., 1965a, McAlpine, 1965). In 2010-2011, the Northern Biodiversity Program resampled the sites of the Northern Insect Survey with the goal of comparing their more recent survey to the historical one (Northern Biodiversity Program, n.d.).

A taxon that has been of special research interest, both in historical and recent studies, is Diptera (true flies), particularly biting flies and those that act as vectors of disease. Diptera is a diverse and ecologically important group that contributes to ecosystem functioning, and it has a major impact on agriculture and public health (Adler & Courtney, 2019, Bedini et al., 2017, Bragard et al., 2020, Mesmin et al., 2019, Molinatto et al., 2020, Olafson et al., 2021, Shamshad, 2010, Wearing et al., 2013). Flies act as useful bioindicators (Czechowski et al., 2020, Hamerlík & Bitušík, 2009, Medeiros et al., 2020, Rossaro et al., 2022) and, in the Arctic particularly, act as an important pollinator group (Adler & Courtney, 2019, Cirtwill et al., 2022, Tiusanen et al., 2016). Flies are also vectors for several important, widespread diseases, including Malaria, Zika virus, Dengue, Chikungunya virus, and West Nile virus (Bonizzoni et al., 2013, World Health Organization, 2018, World Health Organization, 2020). There have been Zika virus outbreaks in over 30 countries, and *Aedes aegypti* (Linnaeus), the principal vector of this virus, as well as Dengue, Chikungunya, and yellow fever, is a highly adaptive and widespread species (World Health Organization, 2018). Similarly, *Aedes albopictus* (Skuse), a vector of Dengue and Chikungunya, is an extremely invasive species affecting an increasing proportion of the world (Bonizzoni et al., 2013). Developing a capacity for monitoring potential vectors of disease is important, as species may shift their geographic distributions in response to climate change. Non-biting fly species can also be a public health concern, transmitting microorganisms through their mouthparts, feces, and body surface, and acting as vectors of parasites, disease, and food-borne pathogens (Förster et al., 2007, Graczyk et al., 2001, Pava-Ripoll et al., 2012).

Observations on Diptera diversity have been recorded in northern North America since the 1800s. Huckett et al. (1965a) provided a study of Muscidae (Diptera) specimens from Arctic and Subarctic regions of North America, Greenland, and Europe, and described the historical collection events and expeditions that have occurred in these regions. Various historical collection events, occurring from 1835 – 1954, recorded Muscidae flies in northern regions such as the Hudson Bay Territory, Alaska, the Bering Sea Region, Northwest Territories, Ellesmere Island, Yukon territory and the Labrador coast (Aldrich, 1918, Coquillett, 1900, Huckett et al., 1965a, James and Huckett, 1952, Johnson, 1929, Malloch 1919, 1920a, 1921, 1923, Stein, 1898, 1920, Walker, 1849, Weber, 1950, 1954).

Another region that has been the focus of past sampling is Churchill, Manitoba, Canada. Churchill is a diverse Subarctic region containing a wide range of wildlife and plant species. Churchill is in the Coastal Hudson Bay Lowland ecoregion (Smith et al., 1998) (Fig 1a), an area characterized by short, cool summers; long, very cold winters; and widespread permafrost (Smith et al., 1998). This region contains a wide range of habitat types, including peat bogs, fens, tundra, wetlands, and boreal forests, and is bordered by Hudson Bay where sandy beaches and rocky outcrops can be found (Smith et al., 1998). The Churchill River is also an important water feature, in addition to several small, unconnected lakes throughout the region (Smith et al., 1998). The diversity of habitats within this region makes it a place of interest for investigating the diversity of Subarctic species and for research into northern ecology and climate change. The establishment of the Churchill Northern Studies Centre in 1976 facilitated the usage of the Churchill area as a hub for northern research (Churchill Northern Studies Centre, 2022). There have been several historical studies and surveys that included Churchill fly species, such as the Biting Fly Survey and Experimental Control Program and the Canadian Northern Insect Survey (Hocking et al., 1950, Miller, 1951, Twinn et al., 1948, Danks, 1981, Freeman, 1958, Huckett et al., 1965a).

Despite this past sampling, work is still needed to fully understand the diversity of species present in Churchill and other northern regions. By continuing sampling efforts, we can develop a solid baseline which can be used by future researchers. Historical sampling events and present-day efforts provide information on Diptera diversity across northern regions and the opportunity to see how diversity changes over time. Renaud et al. (2012a) compared the historical surveys conducted during the Canadian Northern Insect Survey (1947 – 1962) to a more recent Diptera survey conducted in 2007. The sampling methods differed between these time periods, as historical samples were primarily collected using a sweep net, but the 2007 survey was done using Malaise traps and pan traps, in addition to sweep nets (Renaud et al., 2012a). This survey compared 10294 specimens representing 155 species of Muscidae from the 2007 survey to 2881 historical specimens representing 105 species (Renaud et al., 2012a). Eighty-seven percent of the species found in the historical records were rediscovered in the 2007 survey, and *Spilogona* Schnabl remained the most diverse genus in both time periods, with 38 species as of 1965 and 51 species as of 2007 (Renaud et al., 2012a). With consistent sampling over time, we can document how diversity changes and continue to add to our knowledge and number of known species.

Renaud et al. (2012b) added to our knowledge of Diptera diversity in Churchill by conducting a large-scale barcoding study of Muscidae that included 1114 individuals and 160 species. In addition, Churchill has been the focus of many barcoding studies for arthropod taxa such as Coleoptera (Woodcock et al. 2013), Ephemeroptera, Plecoptera, Trichoptera (Zhou et al. 2009, 2010), Hymenoptera (Fernandez-Triana et al., 2011, Stahlhut et al. 2013), Collembola (Porco et al. 2013), Araneae (Blagoev et al. 2013), Ostracoda (Laforest et al. 2013), Crustacea (Jeffery et al. 2011), and Acari (Young et al. 2012).

To add to this barcoding effort, we conducted a large-scale barcoding study of Diptera species in Churchill. Despite previous surveys, there are still gaps in our understanding of Diptera diversity, and there are many families which have not yet been the focus of dedicated barcoding efforts. Srivathsan et

al. (2023) found that 20 insect families, 10 of which were from Diptera, made up 50% of the local diversity of flying insects at their sites worldwide. They also found that some of the most abundant taxa were still very poorly known (Srivathsan et al., 2023). In this paper, we present a large-scale barcoding study of Diptera including 16786 specimens and 2235 Barcode Index Numbers (BINs; Ratnasingham and Hebert, 2013) representing 68 families and 1211 species. Through a collaborative approach, we have conducted extensive field collections and created a taxonomically linked reference library that includes molecular data and expert taxonomic identifications. This approach is necessary due to the complex patterns of DNA sequence divergence within Diptera. For example, Lin et al. (2015) determined that a divergence threshold of 4–5% was suitable to delineate species within Chironomidae, reflecting the high genetic diversity within species of the family. However, Renaud et al. (2012b) found that a divergence threshold of 1.2% was more suitable for Muscidae, to optimize between cases of taxonomic lumping vs. over-splitting. Due to these complex and differing patterns of divergence and genetic diversity within Diptera, it is valuable to construct an extensive DNA barcode library so that new sequences can be linked with named species and their associated information. In this study we provide a taxonomically linked reference library, outline the diversity of Diptera currently present in Churchill across diverse habitat types, and contribute barcodes for thousands of individuals to the Barcode of Life Data (BOLD) Systems (https://boldsystems.org/; Ratnasingham & Hebert, 2007).

Materials and Methods

Study Site

Samples were collected from sites around Churchill, Manitoba (Latitude: 58.7, Longitude: -94) (Fig.1b) from 2005 to 2011. Sampling intensity varied across years, with the most sampling occurring in 2010 and the least occurring in 2011 (supplementary file 1). Sampling was conducted across several

areas within the Churchill region, and areas closer to the town of Churchill and the Churchill Northern Studies Centre were sampled more frequently. Two hundred and sixty-seven sites were sampled, based on unique pairs of latitude and longitude coordinates. Sites were chosen to represent a wide variety of habitat types in the Churchill area, and accessibility of the sites was also considered.

Sampling Methods

Specimens were collected using various methods, including baited traps, Berlese traps, dip nets, fan traps, hand collection, intercept traps, light traps, Malaise traps, pan traps, pitfall traps, sweep nets and UV light traps. The sampling method used can result in a bias as to what types of species are collected. By using different trap types and sampling methods, a wider variety of species present in the community should be detected. Most specimens were caught using Malaise traps (Fig. 2). There were two main approaches used for sampling. The first is general collection, using a wide variety of methods and conducted by a variety of investigators, towards the reference library. The collector and other metadata are available through BOLD. The second is sampling conducted by Malaise traps by a single investigator (J.J. Wang) to obtain detailed data from five of the sites. These sites were repeatedly sampled throughout a single summer (2010). This subset of the data could be queried for a temporal analysis over a summer season by future researchers.

Barcoding Methods

DNA barcoding was conducted at the Canadian Centre for DNA Barcoding (https://ccdb.ca/) located at the University of Guelph. Three individuals per morphospecies per collecting event were generally selected for molecular analysis. DNA was extracted from each adult specimen using a single leg, and from each larvae using a section of the specimen. For some small-bodied specimens, the protocol from Porco et al. (2010) was used to extract DNA from the whole specimen. The protocol from Ivanova et al. (2006) was used for DNA extraction, and the final elution volume was 50 µl. The primers

used for the amplification of the DNA barcode region of COI are included in supplementary file 2. The PCR reaction volume was 12.5 µl, and included 1.25 µl of 10x reaction buffer, 1.25 pmol of primer, 0.3U of Platinum Taq DNA polymerase, 5% trehalose, 2.5 mM of $MgCl_2$, 50 um of dNTP, and 2 µl of genomic DNA. The E-gel 96 System (Invitrogen, Carlsbad, Ca, USA) was used to visualize the PCR products on 2% agarose gels. The PCR products were bidirectionally sequenced using the protocols from Hajibabaei et al. (2005) and BigDye v3.1 on an ABI 3730xl DNA Analyzer. Using CodonCode Aligner v.3.0.2 (CodonCode Corporation), the forward and reverse sequences were used to assemble consensus sequences for each individual.

Specimen Identification

Specimens were identified using morphological characteristics to the family level (McAlpine, 1981). After sequencing, 1-5 specimens from each BIN were identified to species level for 19 of the families based on the specimen's morphology. BINs are clusters of barcode sequences similar to species and are the operational taxonomic units (OTUs) used by BOLD (Ratnasingham and Hebert, 2013). The goal was to identify a minimum of three specimens per BIN and per species, but some BINs were singletons or doubletons. Of the 16786 records, the taxonomy of 9159 records were assigned using morphological identification. The remaining 7303 records were identified using barcode matching to BINs on the BOLD database. The reference materials used to identify the specimens are included in table 1.

Specimen and Data Storage

The specimens are stored in 95% ethanol, pinned or slide mounted material in various institutions including the Centre for Biodiversity Genomics (13222 specimens), the Wallis Roughley Museum of Entomology at the University of Manitoba (92 specimens), the Carnegie Museum of Natural History (251 specimens), the Insect Collection at Bishop's University (868 specimens), the Canadian

National Collection of Insects, Arachnids and Nematodes (1312 specimens), and the Department of Natural History at NTNU University Museum (1041 specimens).

The specimens were each assigned a unique specimen ID and photographed before being uploaded to BOLD. The data are available in the dataset DS-CDIPTERA (dx.doi.org/10.5883/DS-CDIPTERA) (Majoros et al., 2023) and are also available on GenBank. A portion of the data has been previously published in association with Renaud et al. (2012b). A list of process IDs and GenBank accessions included in the dataset is available in supplementary file 3.

Quality Checking, Analyses and Summary Statistics

A phenogram was created using the Neighbor-Joining method (Saitou & Nei, 1987) for species in the dataset using the Taxon ID Tree tool available on BOLD version 4.0 (Ratnasingham & Hebert, 2007). For the phenogram, Kimura 2 parameter was used as the model for calculating distances between DNA sequences, and pairwise deletion was used for ambiguous base/gap handling. The sequences were aligned using the BOLD aligner (amino acid based hidden Markov model (HMM) (Eddy, 1998)). Known contaminants, records with stop codons, and flagged sequences were excluded, and the phenogram was coloured by family. This phenogram was used to look for any misplaced sequences at the family level, and any long branches not matching the typical patterns of genetic diversity in the families. It is not equivalent or intended as a phylogeny. The mean and maximum intraspecific distances for each species and nearest neighbour distances (the shortest distance between a sequence in one species to a sequence in another species) were calculated using the Barcode Gap Analysis tool on BOLD. For these distance measures per species, pairwise distance (p-distance) was used as the sequence distance measure for comparative purposes, as this method is used by the BIN algorithm (Ratnasingham & Hebert, 2013). Discordant BINs (BINs where more than one taxonomic name was provided for the included records, at any taxonomic rank) were found using the BIN Discordance tool on BOLD, using

records with a sequence length over 100 base pairs and excluding known contaminants, records with stop codons, and flagged records.

Other analyses and all summary statistics were completed in the R programming language version 4.3.0 (R Core Team, 2023). Various plots were created using the package ggplot2 version 3.4.2 (Wickham, 2016), including bar plots and scatterplots, to summarize and investigate different aspects of the dataset. Using the categories outlined in Ratnasingham and Hebert (2013), we determined the number of matches (species assigned to a unique BIN), splits (a single species split across several BINs), and merges (species that share a BIN with another species) in the dataset. A linear regression was performed to determine if there is a relationship between the number of specimens sequenced of each species and the number of BINs associated with that species. Only species with two or more specimens were included in the regression. Species and BIN accumulation curves were created using the iNEXT function, and Chao1 estimators (Chao, 1984) were calculated using the ChaoRichness function from the iNEXT package version 3.0.0 (Chao et al., 2014). As only 1-5 specimens per morphospecies were selected during sampling, there is a bias in analyses that rely on abundance data. To account for this, both abundance and incidence-based richness were calculated. For the iNEXT function, datatype was set to "abundance" and nboot was set to 1000. For the ChaoRichness function, datatype was set to "abundance" to determine abundance-based richness, and to "incidence_freq" to determine incidence-based richness.

To compare the intraspecific distances across families and species, a Maximum Likelihood (ML) tree was created using the 50 species with the most records. The ML tree used in this study is not intended to be equivalent to a phylogeny but to provide a visualization tool showing the intraspecific distances across families. The sequences were filtered to remove those with indels and stop codons using the R package Coil version 1.2.3 (Nugent et al., 2020). To ensure a consistent length among the sequences, only those with 669 base pairs were included as this was the most common sequence length

in the dataset. A centroid sequence (the sequence with the minimum average distance to others in the species) was chosen for each species. These sequences were aligned using DECIPHER version 2.28.0, and followed its recommended steps (Wright, 2016). The AlignSeqs function uses the profile-to-profile method to align the sequences following a guide tree. The profile-to-profile method merges profiles along a guide tree until all the sequences in the set are aligned. As no guide tree was provided and the default parameters were used, the sequences were aligned first using a single-linkage guide tree based on a distance matrix of shared k-mers. The initial alignment was then re-aligned using an UPGMA guide tree. Finally, the alignment subsets were re-aligned to the rest of the alignment. Two alignments were created by this method, and the best was selected by the function using the sum-of-pairs score. The maximum likelihood tree was created using phangorn version 2.11.1 (Schliep, 2011). Models were tested using the ModelTest function from phangorn and the best model of molecular evolution for the data was determined to be TPM2u+G(4)+I, as this model had the lowest score for both the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC). The intraspecific distances were mapped to the ML tree using the species names and the function full_join from the package dplyr version 1.1.2 (Wickham et al. 2023). Upon constructing the tree and observing long branch lengths, several steps were taken to further investigate the sequences. The quality of the alignment created using DECIPHER was checked by viewing it in MEGA-X: Molecular Evolutionary Genetics Analysis version 10.2.0 (Tamura et al., 2021). The identities of the sequences were confirmed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). We compared these sequences to others of the same species available on BOLD and constructed trees using other sequences to check for long branches lengths. We also constructed trees based on the first, second and third codon positions, as well as an amino acid tree, in MEGA-X.

The dataset was compared to data from other northern and southern regions to determine how many BINs were shared. Data from Canadian provinces and territories, as well as Alaska, Greenland,

Finland, and Norway, were pulled directly into the R environment using BOLD's application programming interface (API). Data was downloaded on Oct 2nd and 3rd, 2023. The number of shared BINs between each geographic region and Churchill was determined. The alpha diversity was calculated for each region using the Shannon Index and the Simpson Index, and the beta diversity between the regions and Churchill was calculated using the Bray-Curtis Index. Alpha diversity was obtained using the function diversity from the R package vegan version 2.6.4. (Oksanen et al., 2022) and beta diversity was obtained using the function vegdist from the same package. A list of BINs shared between Churchill and each region is available in supplementary file 4.

Land-use classification, visualization, and taxonomic breakdown by land-use class

We determined the family-level taxonomic composition across the varied landscape of Churchill based on the most common sequences encountered in different land-use and land-type categories, such as wetlands and coniferous forest. Land-use classification for the area surrounding Churchill was determined by extracting the Earth Observation for Sustainable Development (EOSD) categories for each unique set of GPS coordinates from the LUC_EOSD_Northeast dataset from the provincial government of Manitoba Conservation and Water Stewardship website (Manitoba Land Initiative, accessed June 16th 2023). Briefly, the "GCS_North_American_1983" Geographic Coordinate System was selected to ensure accurate spatial reference. Next, the unique GPS coordinates were inputted into ArcGIS, and the "Longitude" and "Latitude" attributes were defined (Environmental Systems Research Institute, 2015). The "Spatial Analyst" toolbar was then accessed, and the "Extract Values to Points" tool was selected from the "Extraction" toolset. The shapefile served as the input point layer, while the raster layer was chosen as the input raster. To store the extracted land-classification values, specific output fields were specified and extracted. Classification data were then joined to the rest of the metadata for the unique sites using tidyverse v. 1.3.2 (Wickham et al., 2019). Definitions for various land-use categories can be found in supplementary file 5. The map itself (see Figure 1) was generated in

mapview v. 2.11 in R (Appelhans et al., 2023). For the visualization, specimens lacking GPS coordinates were filtered out, along with seven sites that had <3 specimens and were geographically distant from the primary cluster of sampling sites closest to Churchill itself.

The specimen count by family and for each unique sampling site was determined by grouping data in R using the tidyverse v. 1.3.2 (Wickham et al., 2019) and janitor v. 2.2 (Firke, 2023) suite of data manipulation and data cleaning packages. A table containing the specimen count/site along with their respective EOSD classification is included in supplementary file 6. Pie charts for visualization were also created in R using ggplot2 v. 3.4.1 (Wickham, 2016) and viridis v. 0.6.3 (Garnier et al., 2023). To illustrate the phylogenetic relationships between different families, taxa within the same infraorder or superfamily (as determined by the phylogeny in Wiegmann et al (2011)), were assigned different shades of a similar colour using hex codes in R.

Results

Dataset Characteristics

We created a reference library containing 16786 specimens and 2235 BINs representing 68 families and 1211 species. Not all BINs were identified to the species level, and some species were split across multiple BINs. A phenogram showing the relationship between the taxa collected in this study shows that COI sequences within families generally group together for the Churchill area (supplementary file 7). However, the families of Tipuloidea were separated on the phenogram, as well as species within the family Limoniidae, despite being closely related. As this is not a phylogeny and Churchill is a small northern region with lower species richness, these results are not unusual. Muscidae was the most abundant family in regard to specimen number, with 2478 specimens collected, while Chironomidae had the highest species richness, with 208 species recorded (Fig. 3). A total of 12612

specimens have been identified to species level. Species such as *Aedes nigripes* (Zetterstedt), *Aedes communis* (De Geer), *Aedes excrucians* (Walker), *Aedes campestris* Dyar & Knab, *Aedes flavescens* (Muller), *Aedes cinereus* (Meigen), *Chrysops nigripes* Zetterstedt, *Chrysops mitis* Osten Sacken, *Hybomitra affinis* (Kirby), *Hybomitra zonalis* (Kirby), and *Hybomitra hearlei* (Philip) have been previously sampled in historical studies (Hocking et al. 1950, Miller 1951, Twinn et al. 1948) and have been sampled again in our 2005-2011 survey.

Using the categories outlined in Ratnasingham and Hebert (2013), 1028 matches (species assigned to a unique BIN), 98 splits (a single species split across several BINs), and 84 merges (species that share a BIN with another species) were recorded. Out of the matches, 737 matches were for species represented by more than one sequence. Most species were assigned to a unique BIN. There was a significant relationship between the number of specimens sequenced of each species and the number of BINs associated with that species (p-value = 3.74×10^{-6} $R^2 = 0.02$), and species with more specimens appear to be more likely to be split across BINs (Fig. 4). This relationship persists even when excluding the smaller sample of species with a higher specimen count. A total of 948 BINs did not have a species-level identification. Of these, 491 have a genus level identification, and all have a family level identification. The majority of BINs are not unique to this study, but contain sequences from BINs already on BOLD that were found in previous works and other geographic regions (Fig. 5). Our study contains 142 unique BINs representing 33 species across 25 families. The families Cecidomyiidae and Chironomidae had the most unique BINs, with 38 unique BINs and 29 unique BINs, respectively. Thirty-nine discordant BINs were found, most of which appear to contain two species within the same genus.

Completeness of Sampling

A species accumulation curve and a BIN accumulation curve were created, and Chao1 estimators were calculated (Fig.6). Both curves appear to be approaching an asymptote. For species, 79.6% of the

expected value was observed, and for BINs, 67.02% of the expected value was observed. These results were consistent for both abundance-based and incidence-based richness. As mentioned in the methods, only 1 -5 specimens per morphospecies were selected, so the species accumulation curve is overly conservative due to the sampling bias. These results indicate that the region is reasonably well sampled, though further work is still required to capture the full species richness.

Patterns of Sequence Divergence

The distance to nearest neighbour, and maximum and mean intraspecific distances were investigated for each family. The species within Coelopidae had the largest nearest neighbour distances, while Calliphoridae had the lowest (Fig. 7). For the mean intraspecific distances, the value for Canacidae was the highest, while Ulidiidae, Lonchopteridae, Chamaemyiidae, and Bolitophilidae all had mean intraspecific distances of zero. There is little overlap between the nearest neighbour distances and mean intraspecific distances, with most mean intraspecific distances being below 5%, and most nearest neighbour distances being above 5%. The max intraspecific distances ranged from 0–16.06% and the nearest neighbour distances ranged from 0–16.23%. Most species appear to be easily distinguished from their nearest neighbours, with 1148 out of 1211 species having a greater interspecific distance than intraspecific (Fig. 8). In Figure 9, we present maximum likelihood trees showing the maximum and mean intraspecific distances for the 50 best-sampled species. Genetic distances vary across the tree. The species with higher maximum intraspecific distance (highlighted green on the figure) are candidates for examination for potential cryptic species. The full results of the barcode gap analysis are available in supplementary file 8.

Comparing BIN Composition Across Geographic Regions

The BINs included in this study were also shared across several other northern and southern regions (Fig. 10). When looking at total counts, most BINs were shared with Alberta and the Northwest

Territories, with 1258 BINs and 1046 BINs being shared, respectively. For Alpha diversity, Norway was the most diverse when using the Shannon Index and Simpson Index. For beta diversity, Churchill was most similar to the Northwest Territories, followed by Saskatchewan.

Taxonomic Composition by Habitat

The densest sampling was in wetlands, with a total of 6785 specimens and 62 families represented with at least one specimen. This was followed by water sites (n=5165), exposed/barren areas (n=3917) which were composed primarily of human adjacent/impacted areas and rock pool bluffs overlooking Hudson Bay, and coniferous (n=714) sites. Muscidae specimens were in the top 10 most sampled families by specimen number of all land-use areas, and the most abundant in two of the areas (wetlands, water), with Chironomidae being the second most abundant in water, wetland, and the most abundant in exposed/barren and bryoid sites (Fig. 11). This trend continued with Dolichopodidae, Syrphidae, Chloropidae, and Anthomyiidae found in four of the five areas. Bryoids were less densely sampled (n=198), with Chironomidae and Muscidae dominating as well.

Discussion

New Reference Library for Subarctic Diptera

In this study we present a DNA barcode reference library containing 16786 records for 1211 named species and 2235 BINs of Diptera collected near Churchill, Manitoba. The library includes representatives of 68 families with Muscidae being most abundant. All sequences are easily accessed as they are assembled into a single dataset on BOLD (DS-CDIPTERA, dx.doi.org/10.5883/DS-CDIPTERA). Through this study we show the current diversity of Diptera within Churchill and provide opportunities for farther research into this community using the barcode information provided.

One hundred and forty-two BINs were unique to this project. Most unique BINs showed low abundance in this dataset, containing fewer than 10 records, except for one BIN identified only to the family level as Ephydridae, which contained 32 records, and the BIN identified as *Stictochironomus unguiculatus*, which contained 25 records. The unique BINs represented 25 families, and within these, species of the families Cecidomyiidae and Chironomidae were most abundant, with 38 and 29 unique BINs recorded. Almost all of the Cecidomyiidae BINs were only identified to family and reflected the taxonomic challenge that this family represents and the limited expertise available. Cecidomyiidae and Chironomidae differ in habitat, with Cecidomyiidae having mostly terrestrial larvae, and Chironomidae mostly aquatic (Gagné, 1981, Oliver, 1981). In regard to diet, Cecidomyiidae larvae are often phytophagous or mycophagous, and frequently form galls on plants, while Chironomidae larvae are mostly microphagous, feeding on small particles of detritus, plants, and microorganisms (Gagné, 1981, Oliver, 1981). Chironomidae was the best-sampled family in our study, and it is possible that with increased sampling and research, more unique BINs could be discovered for other families.

Geographic Affiliation of Churchill Diptera

Most of the BINs were not unique to this study but contained members from other studies and geographic regions. Many of the BINs found in this study are shared with other regions, both northern and southern. Most BINs were shared with Alberta and the Northwest Territories when looking at total counts; and when calculating beta diversity, Churchill BIN composition was most similar to the Northwest Territories and Saskatchewan. Many BINs were also shared with the northern Palearctic region, such as Finland and Norway, reflecting broader polar distributions. Churchill shared more BINs with southern regions, but a high northern affinity was observed when calculating beta diversity. The beta diversity results show that Churchill is most similar to other northern regions in Canada, as well as nearby southern regions, and is more similar to distant northern regions such as Finland and Norway, than some Canadian southern regions such as Ontario. The similarity to other northern regions, such as

the Northwest Territories, may reflect post glacial dispersal from the Beringia glacial refuge, while similarity to nearby southern regions may reflect northern and southern dispersal. The similarity in BIN composition across northern regions has been documented in other studies, such as Ekrem et al. (2018), who compared the species and BIN composition of Greenland Chironomidae to other Nearctic and Palearctic regions. They found that most taxa in Greenland were shared with at least one other geographic region, and that, when looking at taxon counts, Greenland was more similar to North America (Ekrem et al., 2018). While Greenland was one of the least similar regions compared to Churchill in our study, 91 BINs were still shared between them, and the overall sharing of dipteran BINs across Arctic regions here augments the findings for chironomids from Ekrem et al. (2018).

Genetic Divergence Patterns across Studies and Taxa

Based on the intraspecific distances, most species can be easily distinguished from their nearest neighbours using COI. For the dataset, across all Diptera families, the maximum intraspecific distances ranged from 0-16.06% and the nearest neighbour distances ranged from 0–16%. The high distance values, particularly those over 5% on the basis of other studies (e.g. Lin et al., 2015), are candidates for examination for cryptic species, particularly in understudied taxa. These distance values could also be high due to the biology of the species. The distances within individual families varied. For example, the mean nearest neighbour distance in Tachinidae was 6.97%. This value is comparable to previous research by Pohjioismaki et al. (2016), who determined that the mean nearest neighbour distance within the family Tachinidae was 5.52%. The maximum intraspecific distance for Drosophilidae ranged from 0–2.47%, similar to Machado et al. (2017) who found that the intraspecific distances ranged from 0–5.3%. The maximum intraspecific distances for Muscidae ranged from 0–4.25% here, similar to the results of Renaud et al. (2012b), who found distances of 0–3.01% for Churchill Muscidae, though there is some dataset overlap between our studies.

Some of the differences between our study and literature could be due to the geographic scope of the studies. The intraspecific divergence range can differ based on the area included in the study. Lin et al. (2015) found an average intraspecific divergence of 2.14% in a global scale study of Chironomidae, with maximum divergences reaching 21.1% due to cryptic species complexes. This is similar to our study, which found a maximum intraspecific divergence of 16.06%, but a much lower average of 1.56%. When focusing on the Baltic Sea region, Brodin et al. (2012) found that Chironomidae had intraspecific distances ranging from 0– 3.15%. For Psychodidae, the distances ranged from 0– 4.9% within the family in Brazil, and increased to 0– 8.32% when including multiple countries in the Neotropical region (de Souza Pinto et al., 2022, Posada-Lopez et al., 2023). When focusing only on Mexico, Adeniran et al. (2019) found intraspecific distances of 0.12–1.22%. This range increased to 0–9.48% when including other sequences from North and South America (Adeniran et al., 2019). The maximum intraspecific distance was 1.07% for Psychodidae in our study, which is similar to other studies that focused on small geographic regions. Tabanidae has also been studied across several different countries with varying intraspecific distances, such as Thailand (0–4.4%), Africa (0–6.5%), India (0–6.34%), and Canada (0–3.3%) (Banerjee et al., 2015, Changbunjong et al., 2018, Cywinska et al., 2010, Votýpka et al., 2019). Our results were similar to previous Tabanidae results from Canada, with maximum intraspecific distances that ranged from 0–2.5%.

A family where the results differed from the literature was Mycetophilidae, which had intraspecific distances of 0–13.46%. Jürgenstein et al. (2015) conducted a study of *Mycetophila ruficollis* Meigen using 116 specimens collected throughout Europe from 1984 to 2014. Their study revealed intraspecific distances with a range of 0.08–0.8% (Jürgenstein et al., 2015), which is much lower than that of our study. The average intraspecific distance from our study, 0.7%, aligned more with Jürgenstein et al. (2015). Similarly, Kjærandsen (2022) found a mean intraspecific distance of 0.81% for

Sciaroidea. This suggests that while the average genetic diversity in this family is low, there are some potential cryptic species to consider.

Nearest neighbour distances and intraspecific distances vary across insect orders. Lepidoptera has been shown to have lower levels of intraspecific variation than Diptera, with average intraspecific distances of 1.08% being found in species from Finland and Austria (Huemer et al., 2014), divergences ranging from 0-5.92% found in species from Nigeria (Nneji et al., 2020), and a threshold of <2% being found appropriate to differentiate most Lepidoptera species in Canada, though 7.7% of species had deeper divergences (Zahiri et al., 2014). The mean divergence between species of Lepidoptera was reported as 5.73% (Pentinsaari, 2016) and between Coleoptera was 11.99% (Pentinsaari et al., 2014). The mean intraspecific divergence and mean nearest neighbour distance reported in this study was 0.91% and 7.22% respectively. Though Lepidoptera has been reported to be lower than Diptera in the literature, the mean intraspecific distance in our study is comparable to that seen in Lepidoptera. The mean nearest neighbour distance, however, appears to be lower in Lepidoptera. Both nearest neighbour and intraspecific distances appear to be higher in Coleoptera than Diptera, which aligns with what has been previously seen in the literature (Pentinsaari et al., 2014).

Taxonomic Composition by Habitat

We found a strong presence of families associated with aquatic habitats in the top 10 families for each land type classification, from aquatic larvae (e.g. Chironomidae, Culicidae, Simuliidae, Tipulidae) to predatory adults feeding near water (e.g. Dolichopodidae, Empididae) (Cook, 1981, Oliver, 1981, Stone, 1981, Peterson, 1981, Alexander & Byers, 1981, Robinson & Vockeroth, 1981, Steyskal & Knutson, 1981). This is not surprising considering that Churchill is located on the Hudson's Bay coast, and surrounded by diverse water body types like the Churchill River and a plethora of lakes. Despite the high occurrence of water in the area, shore flies (Ephydridae), which can be found around coastal areas,

maritime marshes and even salt lakes, only made the top 10 in the wetlands. Furthermore, fungus gnats (Mycetophilidae, Sciaridae) were only abundant in the coniferous sites. As their larvae are typically fungivores, we can suspect a moist forest with abundant bracket fungi among others. Overall, the top 10 families are similar across the different habitats (with the exception of bryoids), which can suggest habitat fragmentation and edge effect uniformizing the family composition of each. A closer look at the species composition across habitats could provide more insight.

Findings for Further Investigation

Several of the findings outlined above could be elaborated upon in future studies, such as the comparison between Churchill and other geographic regions or the differences in Diptera community composition across different habitat types in the Churchill region. Significant differences in composition across land use cannot be evaluated, as samples were collected over many seasons by a variety of people using different sampling methods and biases for what was sampled and later sequenced. We therefore used a graphical approach to explore the biodiversity across Churchill habitats. Future research could focus on comparing these different Churchill habitats with more consistent methods and timespan to determine if there are any significant differences in community composition.

A wide variety of collection methods were used to target a wider diversity of taxa and to obtain the specimens for this reference library. This resulted in a stronger reference library, and the benefits of using different trap types have been discussed in various studies (Ernst et al., 2015, Pentinsaari et al., 2020). However, we acknowledge there could still be biases and groups potentially missed or underrepresented. Different families are caught most often using different methods. For example, hand netting usually results in more specimens of Limoniidae being collected, while larger crane flies, like Tipulidae, are able to be caught in Malaise traps more often. As dipteran are strong flyers, most specimens were caught using Malaise traps. Hence, they are commonly used to collect these taxa, but

there can be some biases depending on the type of Malaise trap used, the material used to make the trap, and the type of attractant used (Betts, 2010, Martinez et al., 2020, Skvarla et al., 2020, Uhler et al., 2022). Despite the potential limitations of each, we believe that combining a variety of trap types and sampling methods allowed us to construct a robust reference library for the region. Further research could be done to determine the effectiveness of trap type and their effect on species composition.

While many specimens were collected, there are still groups missing from the barcode data. About 20% of the known Churchill species of Empidoidea were not accounted for in the barcode data. It is possible that the habitats associated with these groups, such as running water habitats, were not well sampled, likely resulting in some species being missed. Future research could focus on the continued sampling of Churchill and other northern regions to further expand the DNA barcode data available to other researchers.

Further research could also be done regarding the long branch lengths observed in the ML tree. After following the steps outlined in the methods, we determined no issues with the quality or identification of the sequences. These sequences, when BLASTed, returned the expected results and were similar to other sequences within the species. For *Tetanocera fuscinervis* (Zetterstedt), all codon positions showed the long branch length, but for *Bradysiopsis vittigera* (Zetterstedt), the long branch length only appeared when using the third codon position and could suggest a more rapid rate of evolution. Overall, the branch lengths do not appear to be caused by an issue with the sequence or alignment, but instead are something to do with the species itself. *Tetanocera fuscinervis* is a member of the family Sciomyzidae and is a predator of snails on shorelines (Foote, 1996). There is little information available regarding *Bradysiopsis vittigera*, but this species is a member of the family Sciaridae, where many larvae are saprophagous (Scudder & Cannings, 2006, Steffan, 1981). These species are the only members of their families on the tree. There is possibly something about these species and families that results in the long branch lengths and genetic differences. Further research could look into the reasons

for these branch lengths and the relationships between these species and other Diptera. Further work could also be done to sequence the genomes of Diptera species. To look more closely at the phylogenetic relationships between the taxa recorded in Churchill, a range of nuclear and mitochondrial markers would need to be combined with morphological characters in order to get a more accurate picture, such as the phylogeny presented in Wiegmann et al. (2011), which uses nuclear loci, mitochondrial genomes, and morphological characters.

Opportunities for Using New Library

As outlined earlier in the discussion, there are differences across and within insect orders regarding the intraspecific divergence. The families within Diptera differed in their intraspecific distances, and these differences may be considered when clustering specimens into species. A single clustering threshold is not optimal for all of Diptera when performing *de novo* clustering of sequences into taxonomic units as close to species as possible, but rather this threshold should differ based on the genetic diversity present in the family. If a threshold is used that is too large, this could result in closely related species being grouped together, or if one is too small, this could result in species being split across operational taxonomic units. These differences can impact estimates of species richness. However, through this collaboration between molecular analysts and taxonomic experts, researchers are not solely reliant on specific divergence thresholds. By using well-populated reference libraries, future researchers can perform identifications despite variation in genetic diversity within and among species. When sorting sequenced specimens into species, we recommend accessing reference libraries when possible.

References libraries are valuable resources that can help researchers more accurately identify and delineate species and provide opportunities for future research. The barcode library presented in this study reflects the current diversity of Diptera in Churchill, and the barcode sequences are publicly

available on BOLD and accompanied by their voucher, taxonomic, and collection information. This library is an important resource for any researcher in need of species-level information. Taxonomic experts identified the specimens, and 12612 specimens were assigned species-level identification. While molecular information and BINs are an important resource, BINs do not always correspond to species. By combining molecular data with taxonomic identification, we can reduce the number of specimens that are misidentified and provide more accurate and detailed information to future researchers. Trait information is usually associated with species names, so having this taxonomic information available allows future researchers to make use of the molecular data to a greater extent. Future research could include comparing our reference library to past Churchill studies and future studies to see how these communities are changing. This region can also be compared to other northern regions, such as those mentioned above. Overall, this barcode library can be applied to a wide range of studies investigating this northern region and the ecology and evolution of Diptera. However, further work is needed, and future studies could focus on a larger geographic scale, and include other northern regions, as well as temperate and tropical regions, so we can further our knowledge of Diptera diversity and barcode variability at a global scale.

Conclusion

In conclusion, we present a large-scale barcoding study of Diptera from Churchill and contribute to the growing amount of barcode data available for research. We provide a detailed reference library, making use of molecular data and taxonomic knowledge, and make this library publicly available for future research. Our work demonstrates the utility of broad, multi-year collecting programs as well as collaborations to build well-populated DNA barcode reference libraries that will enable identification at the species level even for hyper-diverse arthropod groups. We outline the diversity of Diptera and contribute to the knowledge of this ecologically important group, including taxa that interact closely with humans and those that can be used for freshwater ecosystem health monitoring. By studying

northern Diptera communities and understanding current diversity, we can study the change over time, especially as northern regions are impacted by the changing climate. By collecting and releasing barcode libraries we open the door for future research and allow other researchers to build off our work and further investigate taxa and regions of interest.

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Data and Accessibility and Benefit-Sharing Section

Data Accessibility Statement

The DNA barcode reference library is publicly available on the Barcode of Life Data Systems in the dataset DS-CDIPTERA (dx.doi.org/10.5883/DS-CDIPTERA) (Majoros et al., 2023). The sequences are also available on GenBank. A list of process IDs and GenBank accessions included in the dataset is available in supplementary file 3.

Benefit-Sharing Statement

Benefits from this research accrue from the public sharing of the DNA barcode reference library on the Barcode of Life Data Systems.

Author Contributions

All authors reviewed and edited the manuscript. SEM, TAE and SJA wrote the manuscript. SEM and TAE ran analyses and created figures. FB and CY identified crane flies from the families Cylindrotomidae, Limoniidae, Pediciidae and Tipulidae. JW conducted field work, identified specimens, processed the specimens for barcoding, did family-level determinations, and species-level determinations for Chloropidae. VLB did family-level determinations, co-ordination of sending representative vouchers to collaborators for determination, and contributed to manuscript writing. CJB identified the Mycetophilidae specimens. SEB, JMC and BJS identified the Empidoidea (Empididae and Dolichopodidae) specimens. SB identified the Agromyzidae specimens. DCC and PS did collecting and determinations for Simuliidae, Culicidae, and Tabanidae. TE and ES did collecting and determinations for Chironomidae. PDNH and RER contributed to the collecting. JS did Muscidae determinations for the 2010 clusters and the Anthomyiidae determinations. JHS did Syrphidae and Pipunculidae collecting and determinations. AMS conducted field work and specimen identifications. TAW did the Chloropidae, Piophilidae and Heleomyzidae identifications. TSW contributed to collecting and sorting the malaise traps to family. ADY identified Syrphidae specimens.

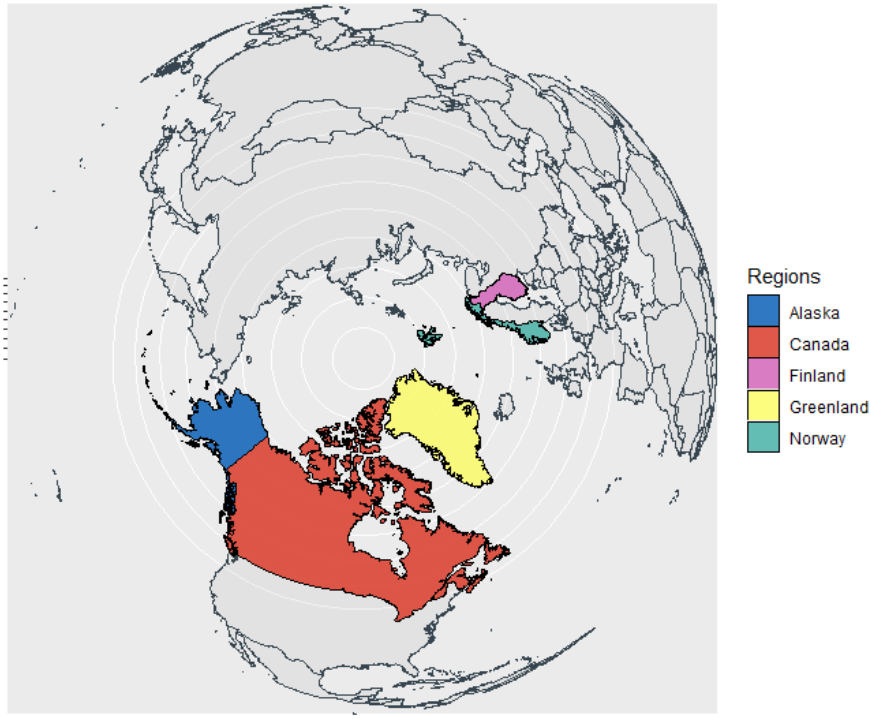
Tables and Figures

Taxa	Reference Material
Muscidae	Arntfield (1975), Collin (1930), Gregor et al. (2002), Hennig (1955-1964), Huckett (1932, 1934a, 1934b, 1936, 1954, 1965a, 1965b), Huckett and Vockeroth, (1987), Malloch (1918, 1919, 1920b, 1923), Michelsen (2006), Pont (1984, 1986, 2011), Savage (2003), Snow (1891), and Snyder (1949a, 1949b, 1954)
Empidoidea (Empididae and Hybotidae)	Comparisons were made with the Canadian National Collection of Insects (CNC) and the following publications: Barták (2002), Chillcott (1962), MacDonald (1994), Sinclair (2008), and Sinclair et al. (2011).
Tipuloidea (Cylindrotomidae, Limoniidae, Pediciidae and Tipulidae)	Alexander (1943), Brodo (1967, 1987, 2017), Byers (1961), Oosterbroek (1984), Starý and Brodo (2009), Tangelder (1983). Identifications were checked by comparing with specimens in the CNC and in the private collection of F. Brodo.
Syrphidae	Vockeroth (1992), Thompson (1991), Thompson (unpublished manuscript keys), and Vockeroth and Thompson (1987). Specimens were then compared to reference material in the University of Guelph Insect collection (DEBU) and to specimens in the CNC.
Pipunculidae	Albrecht (1990), De Meyer (1989), Hardy (1943), Skevington and Marshall (1998) and Skevington (2005) and compared to specimens in the CNC.
Agromyzidae	Boucher (2002, 2008, 2012a, 2012b), Griffiths (1964), Spencer (1969, 1976), Spencer & Steyskal (1986) and Zlobin (1995).
Chloropidae	Fedoseeva (1971, 2003), Sabrosky (1936, 1938, 1940, 1943, 1948, 1950, 1951, Sabrosky and Valley (1987), and Wheeler (1994 and unpublished keys). Specimens were then compared to reference material in the Lyman Entomological Museum (LEMQ) and specimens in the CNC and the Smithsonian Museum of Natural History (USNM).
Chironomidae	Chironomidae: Brundin (1949), Langton & Pinder (2007), Sæther (1977) and Wiederholm (1989). Tanypodinae: Fittkau (1962), Murray (1987), Murray & Fittkau (1989), Roback (1971) and Stur et al. (2019). Diamesinae & Podonominae: Brundin (1989) and Oliver (1989). Orthocladiinae: Brundin (1947), Cranston et al. (1989b), Cranston

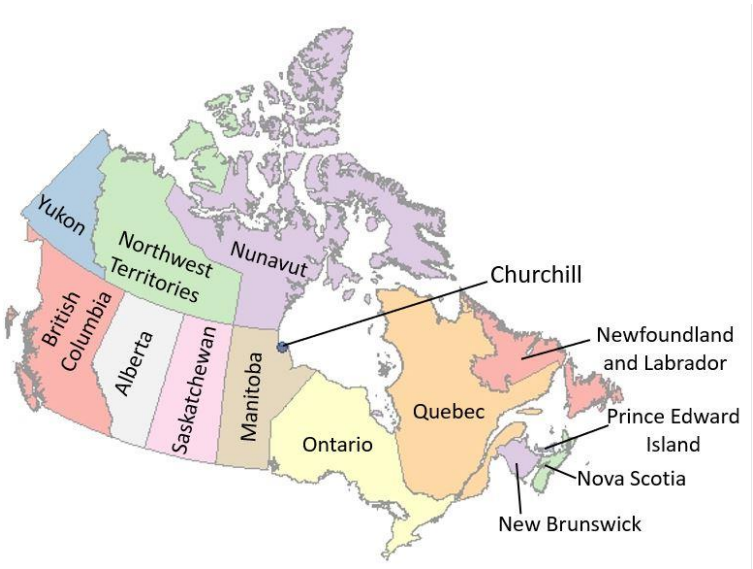
	& Oliver (1988), Ferrington & Sæther (2011), Fittkau & Lehmann (1970), Fu & Sæther (2012), Gowin & Thienemann (1942), Hestenes & Sæther (2000), Hirvenoja (1973), Hirvenoja & Hirvenoja (1988), Langton (1985), Lehmann (1972), Liu & Wang (2005), Oliver & Roussel (1983), Sæther (1969, 1975a, 1975b, 1986, 1989, 1990, 1995, 2004a, 2004b), Sæther & Halvorsen (1981), Sæther & Sublette (1983), Sæther & Wang (1995), Schlee (1968), Soponis (1977, 1990), Sublette (1967a, 1967b, 1970), Tuiskunen (1986), Wülker (1956) and Zelentsov (1985). Chironominae: Anderson et al. (2013), Boesel (1985), Cranston et al. (1989a), Ekrem (2007), Ekrem et al. (2003), Epler (1987), Giłka (2001), Grodhaus (1987), Hirvenoja (1962), Jackson (1977), Kyerematen et al. (2000), Lehmann (1970a, 1970b), Lin et al. (2018), Lindeberg (1967), Lindeberg & Wiederholm (1979), Malloch (1915), Oliver & Dillon (1994), Reiss & Fittkau (1971), Reiss & Säwedal (1981), Sæther (1977, 2009, 2010), Sæther & Oyewo (2008), Säwedal (1981), Sublette (1964), Townes (1945), Wülker et al. (1981) and Zorina (2004). Specimens were identified morphologically, and slide mounts were made to look at structures with a compound microscope.
Anthomyiidae	Griffiths (1991a, 1991b, 1991c, 1993a, 1993b)

Table 1: The reference materials used to identify specimens for each Diptera family.

a)


Regions
Alaska
Canada
Finland
Greenland
Norway

b)


Yukon
Northwest Territories
Nunavut
British Columbia
Alberta
Saskatchewan
Manitoba
Churchill
Ontario
Quebec
Newfoundland and Labrador
Prince Edward Island
Nova Scotia
New Brunswick

c)

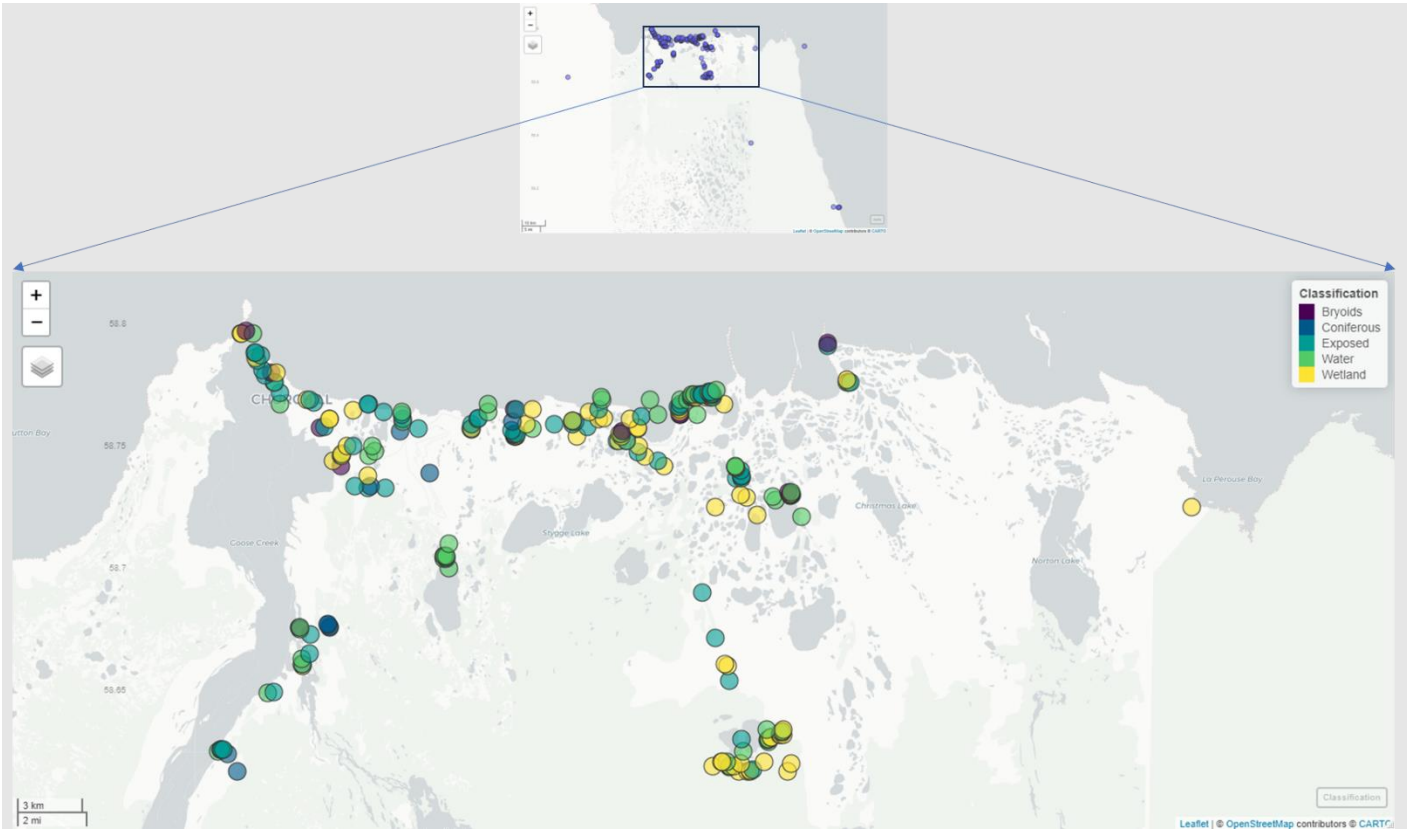


Figure 1: The location of Churchill, Manitoba, and the study sites within the region. a) shows the placement of Canada in relation to other northern countries and regions. b) shows the location of Churchill within Canada. Churchill is located on the coast of Hudson Bay in northern Manitoba. It is a Subarctic region located within the Coastal Hudson Bay Lowland ecoregion. c) shows the locations where samples were collected in the Churchill region and the land-use classification categories of sampling sites, with the most samples inferred using the EOSD classifications from satellite data. For details on the classification status for each site, please refer to Supplementary File 6.

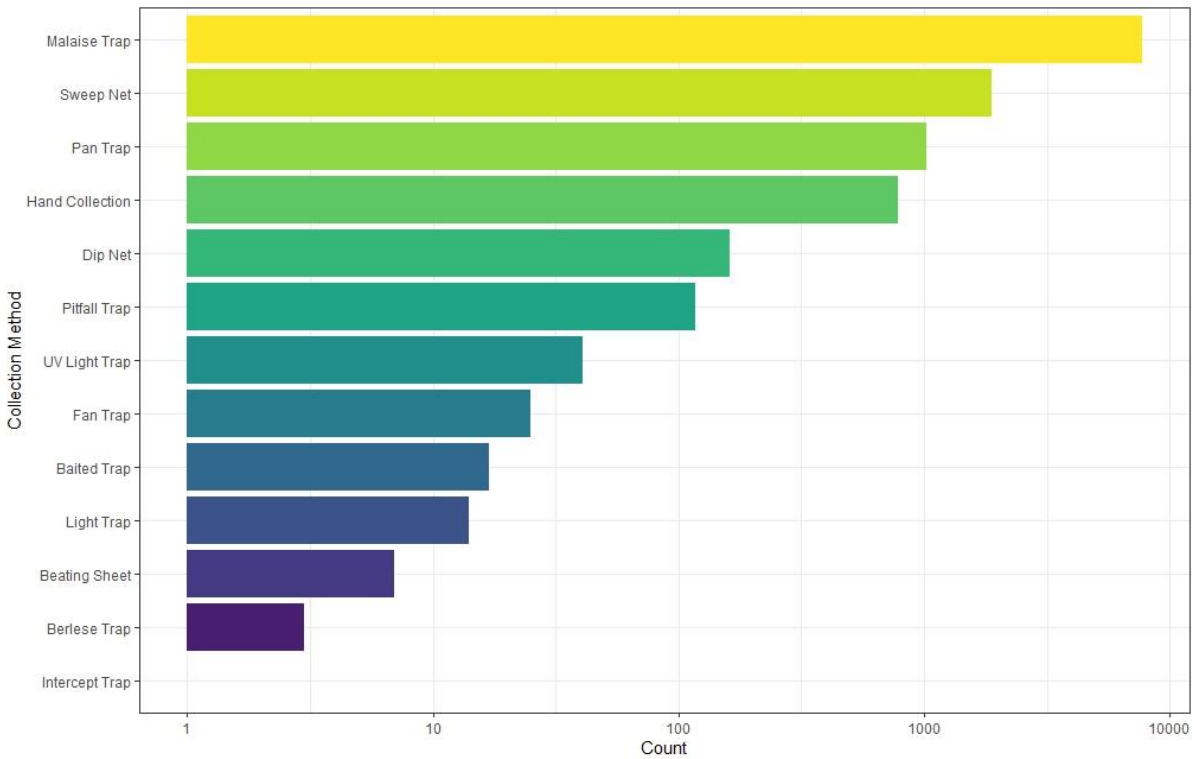
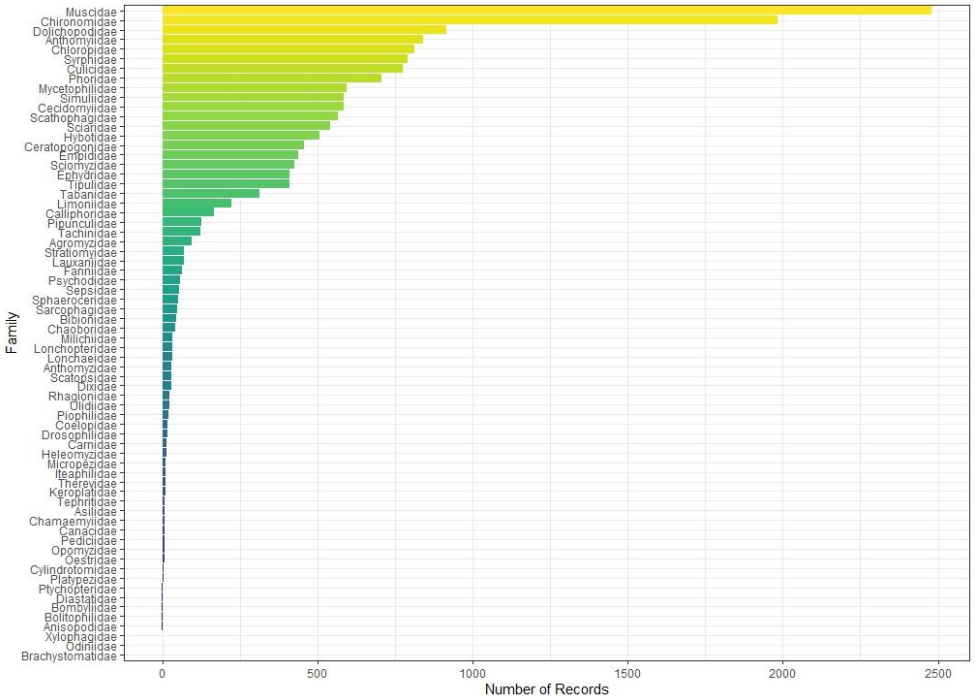


Figure 2: The number of Diptera specimens sampled with each collection method. The majority of specimens were caught using Malaise traps.

a)



b)

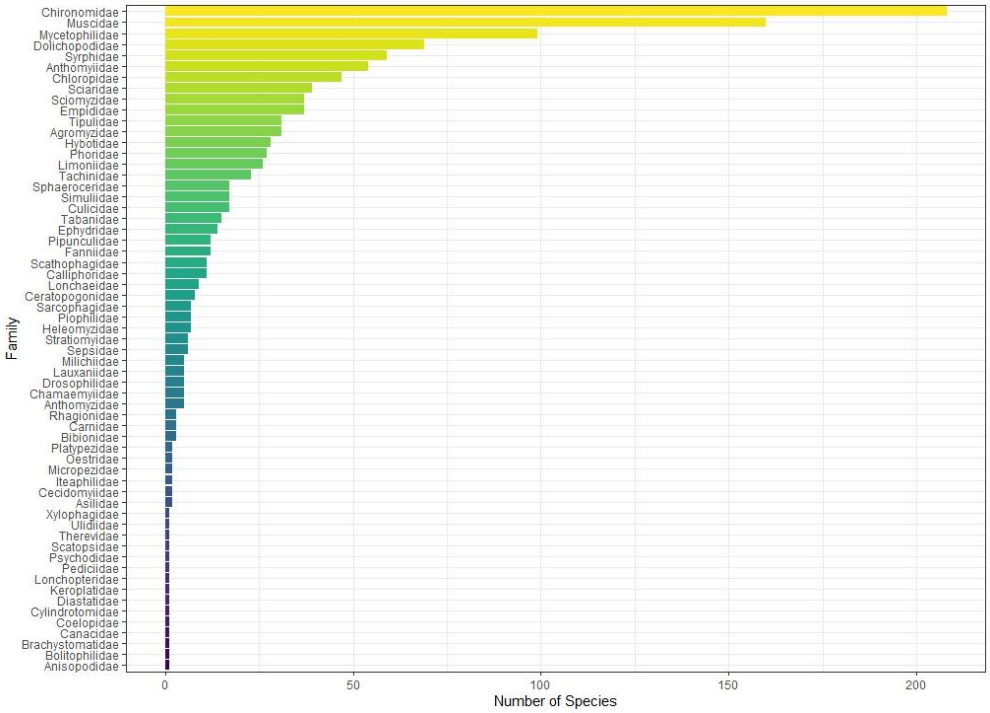


Figure 3: The number of a) records and b) named species collected for each family. Muscidae was the most abundant family in regard to specimens, with 2478 specimens collected, while Chironomidae had the highest species richness, with 208 named species recorded.

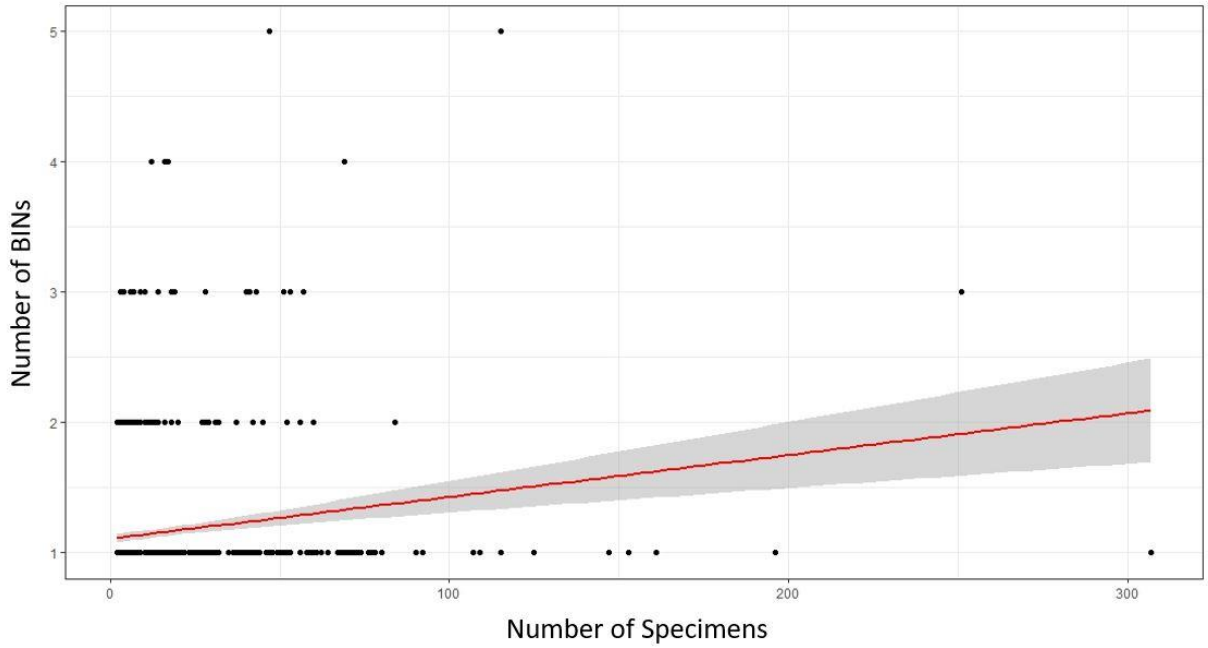


Figure 4: The relationship between the number of specimens of each species and the number of BINs associated with each species. Each dot represents a species. Considering only those species with 2 or more specimens, there was a significant relationship between these factors (p-value = 3.74×10^{-6}, R^2 = 0.02), and species with more specimens are more likely to be split across BINs. This relationship persists even when excluding the smaller sample of species with a higher specimen count.

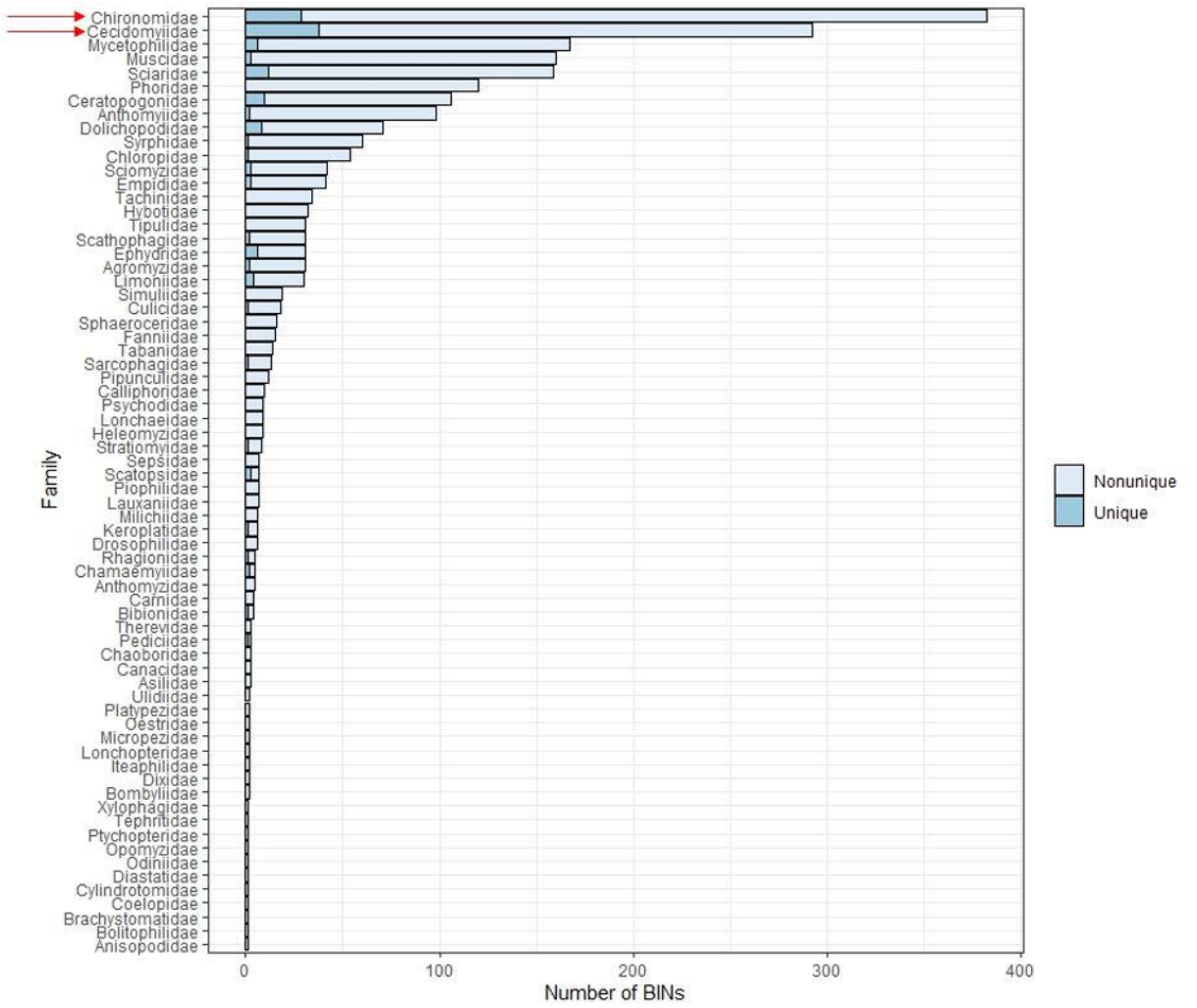


Figure 5: The number of BINs unique to the project on BOLD and the number of nonunique BINs for each family. The families Cecidomyiidae and Chironomidae (indicated with red arrows) had the most unique BINs, with 38 and 29 respectively.

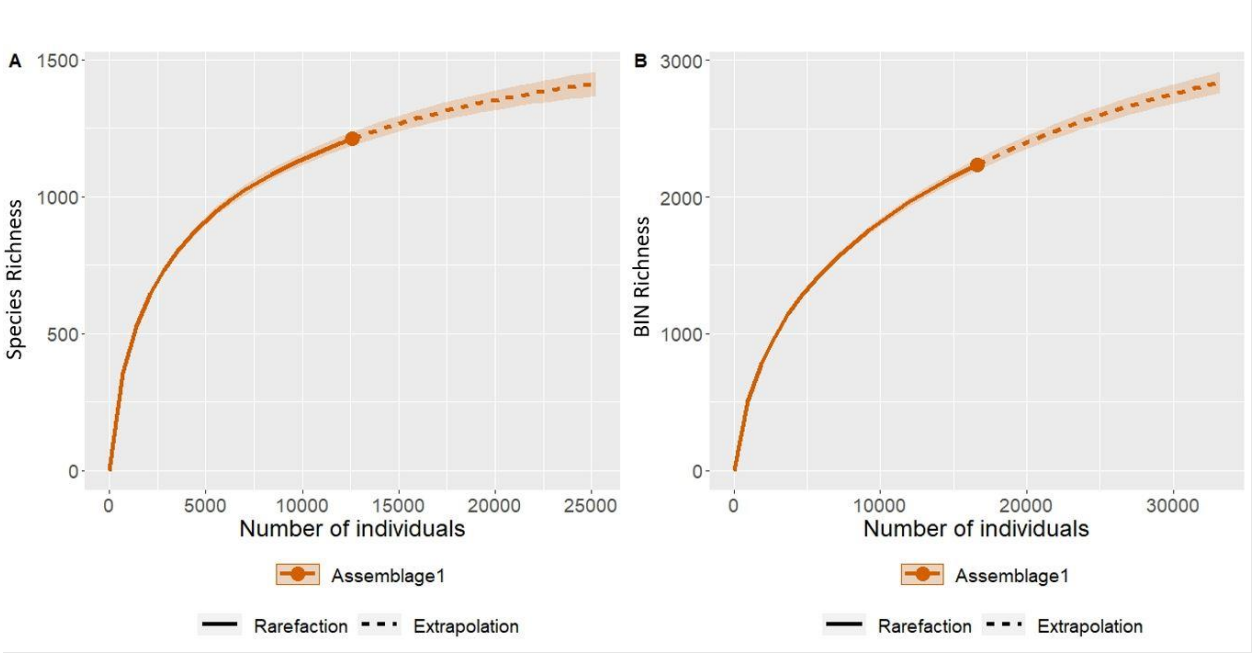


Figure 6: Accumulation curves for the sampled Churchill region. Both curves appear to be approaching an asymptote. A) shows the named species richness. The observed value was 1211 and the estimated value was 1521. B) shows the BIN richness. The observed value was 2235 and the estimated value was 3335. These results indicate that the region is reasonably well sampled.

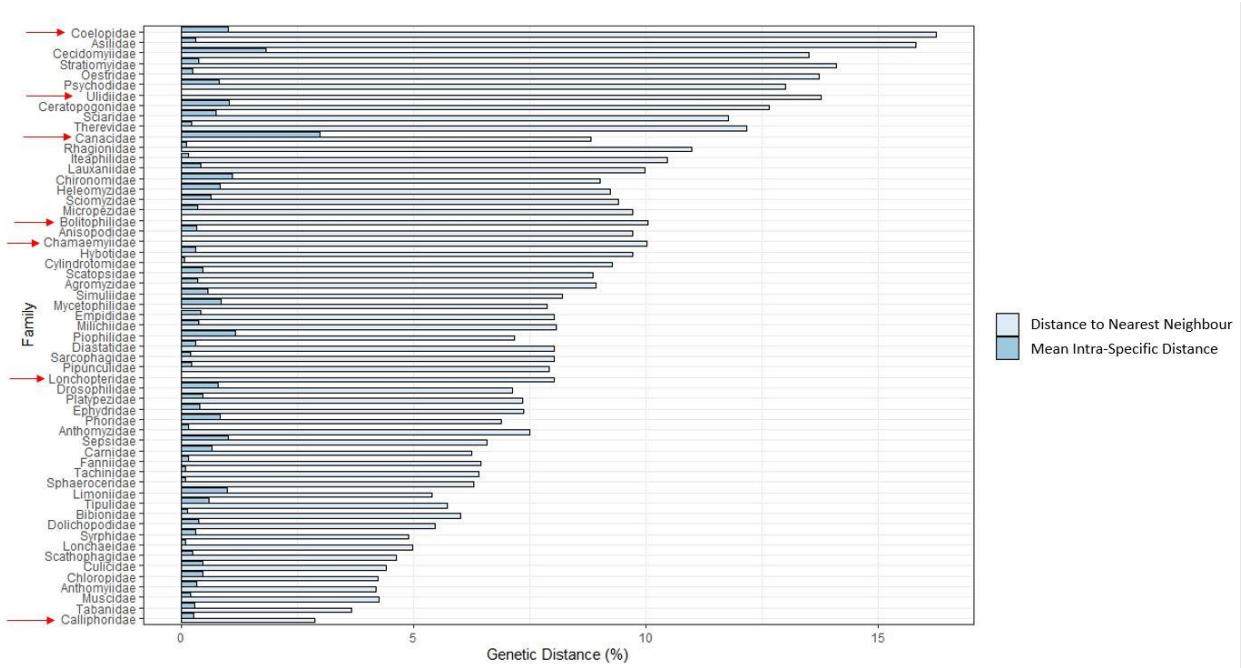


Figure 7: The mean distance to nearest neighbour and mean intraspecific distance for species within each family. The species within Coelopidae had the largest nearest neighbour distances, while Calliphoridae had the lowest. For the intraspecific distances, the value for Canacidae was the highest, while Ulidiidae, Lonchopteridae, Chamaemyiidae, and Bolitophilidae all had mean intraspecific distances of zero. The families mentioned are indicated with red arrows.

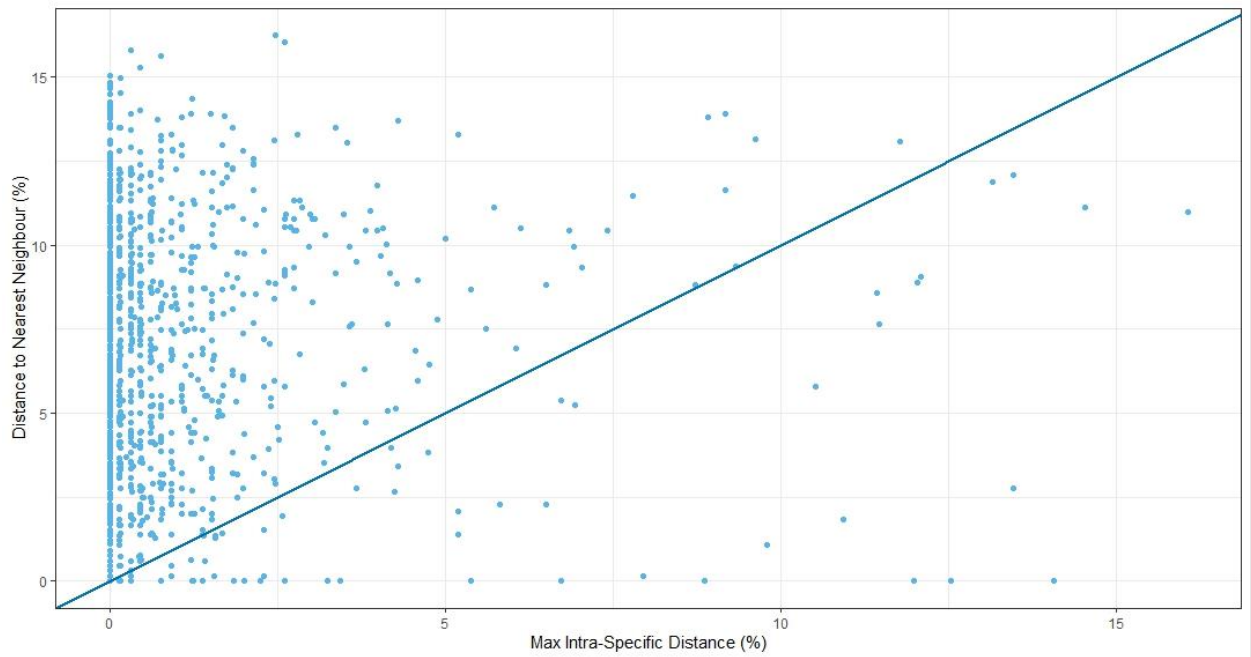
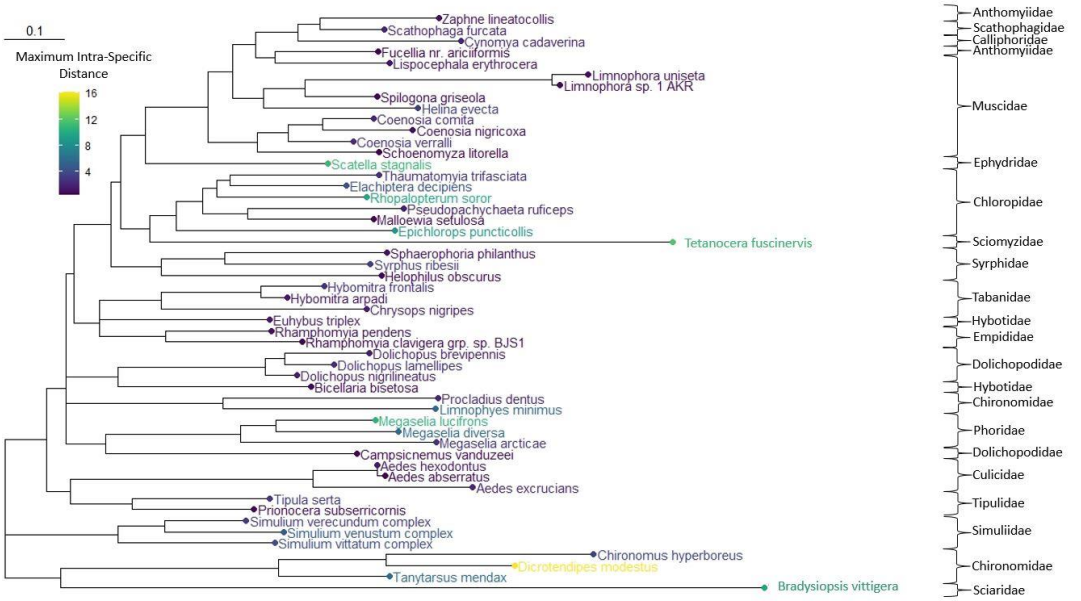


Figure 8: The overlap of the maximum intraspecific distances and the distances to nearest neighbours. Each dot represents a species. Out of the total sample size of 1211 species, 1148 lie above the line, and 63 lie below the line. This indicates that most species have a larger interspecific distance than intraspecific distance, and most species can be easily distinguished from their nearest neighbour.

a)



b)

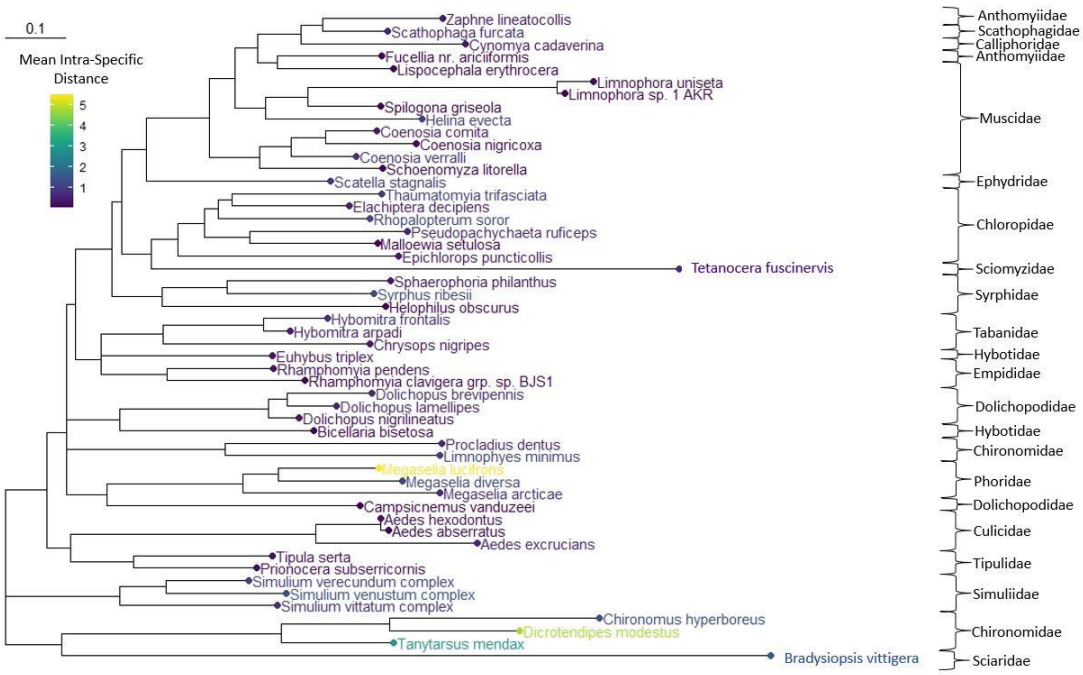
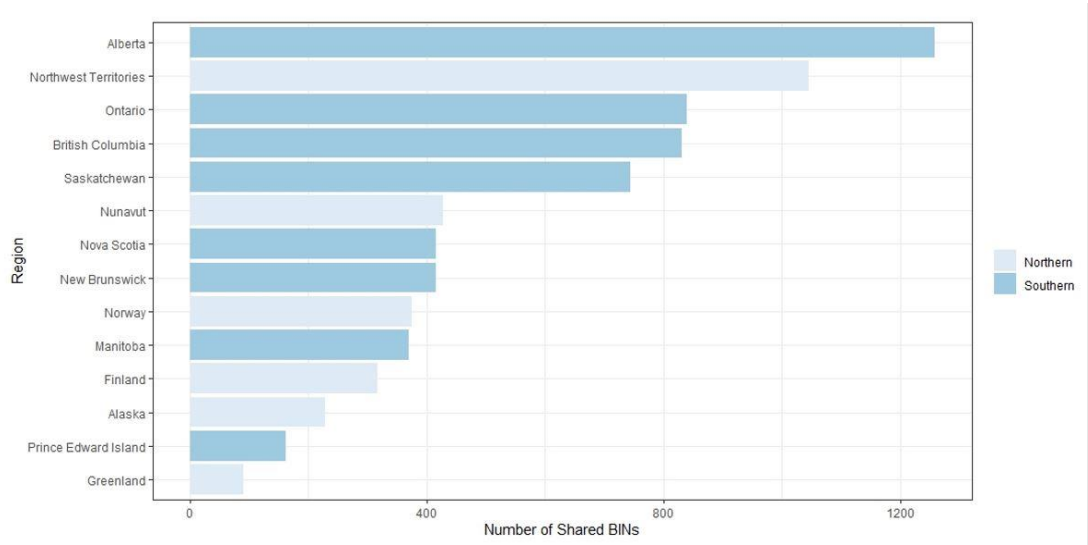


Figure 9: Maximum Likelihood tree of the 50 best-sampled species with an assignment to a Linnean species in the dataset. The a) maximum intraspecific distance (p-distance in %) and b) mean intraspecific distance (p-distance in %) for each species is shown on the tips using the colour scale. The sequences were aligned using DECIPHER version 2.28.0 (Wright, 2016) and the maximum likelihood tree was created using phangorn version 2.11.1 (Schliep, 2011). Higher divergence values were present in scattered locations on the tree.

a)



b)

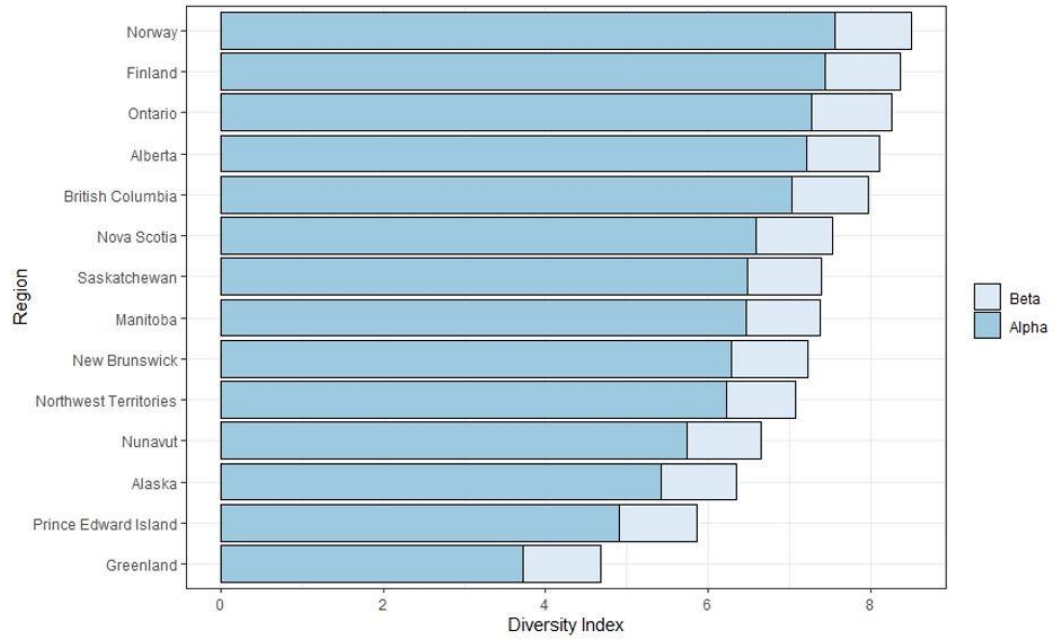


Figure 10: The number of BINs shared between our Churchill barcode library and other geographic regions. a) shows the total count of shared BINs between the regions, while b) shows the alpha diversity of the geographic regions and the beta diversity between the region and Churchill. For a), most BINs were shared with Alberta, with 1258 BINs being shared. b) indicates that Churchill is most similar to the Northwest Territories with a beta diversity of 0.855. Norway and Finland had the highest alpha diversity. Churchill shared BINs with both northern and southern regions. For total counts, Churchill shared more with southern regions, but when calculating beta diversity, Churchill was more similar to other northern regions.

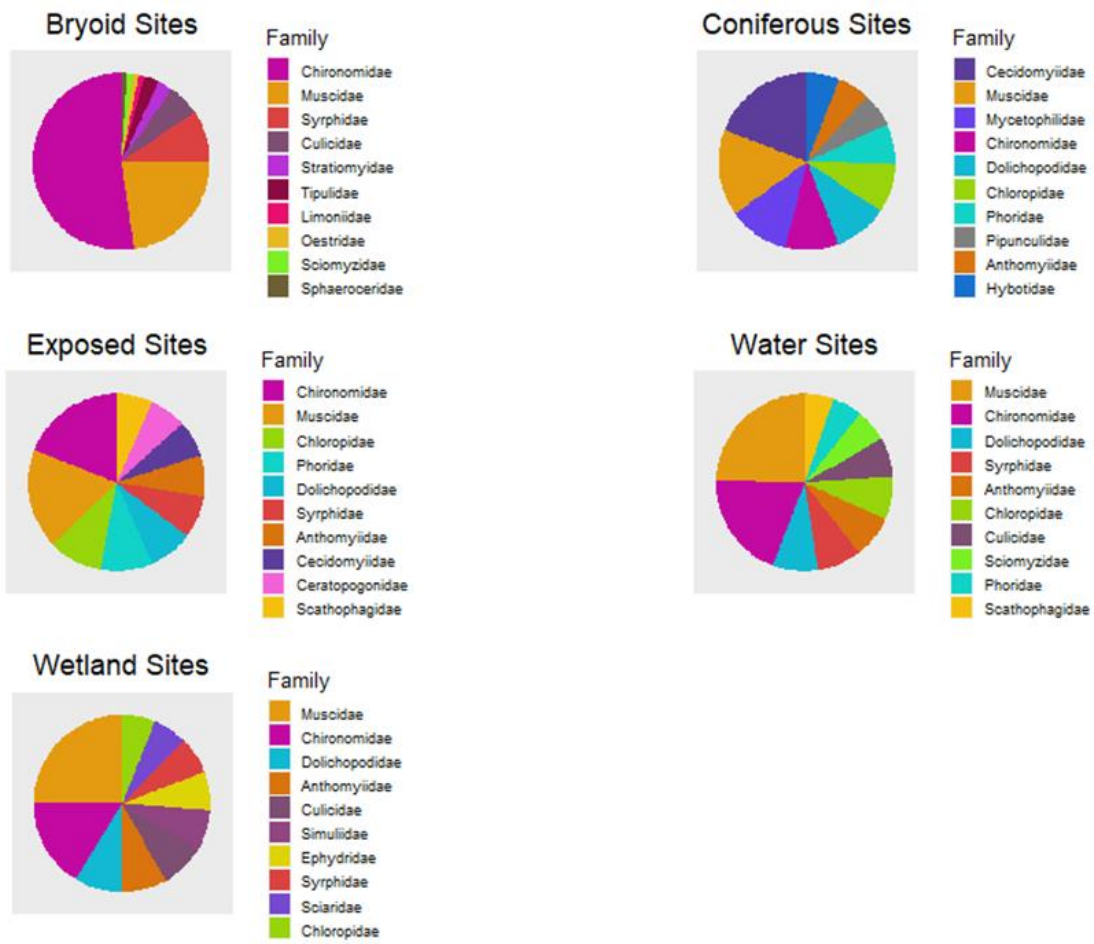


Figure 11. The top 10 families for each land type classification. Each pie diagram depicts the number of specimens in the top 10 most abundant families in that site classification, and the order of the associated legend proceeds from the most to least abundant. Portions appear in each pie diagram from most to least abundant in a counter-clockwise manner.