Phylogeographic Analysis of an Arctic Rabies Host Species,the Red (*VulpesVulpes*) and Arctic (*Vulpes Lagopus*) Fox, in the Eastern Subarctic Region of Canada

by

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Abstract

Rabies is a fatal disease that raises public health concerns in the Canadian Eastern Subarctic region, however, the origins and the spread of epizootics of this lethal zoonotic disease are little understood. Coloured or red (*Vulpes vulpes*) and arctic (*Vulpes lagopus*) foxes across northern Canada are considered to be the principal maintenance or reservoir hosts of rabies virus. Therefore, I studied the phylogeography of one host, the red fox, to better understand the movement of the host and compare it with virus variant distribution across the landscape. Many studies confirm the impacts of environmental changes, particularly climate change, on the migratory behaviors of both fox and rabies virus variants. Also, different geographical areas have different population densities of red and arctic foxes. This study focused on red foxes sampled from the areas of Montreal, Abitibi-Témiscamingue, James Bay, Kuujjuarapik, Umiujaq, Inukjuak, and Kuujjuaq in Quebec, and Labrador City, North West River, Port Hope Simpson and Cartwright in Labrador. A series of 15-loci microsatellite profiles were used to genetically characterize 396 foxes and determine their phylogeographic relationships with respect to the landscape. Microsatellite markers were assessed using Micro-Checker to test for null alleles, stuttering or large allele dropout. Linkage disequilibrium among all pairs of loci was evaluated with GenePop. Genetic diversity and F-statistics was measured by using Arlequin in both loci and populations. Population structure was investigated with pairwise F_{ST} measures, Analysis of Molecular Variance, and individual clustering methods such as STRUCTURE and Geneland. F_{IT} was significant at four loci and F_{ST} was significant at all loci indicating that the loci selected are suitable for analysis. F_{IS} was not significant in any populations. The conclusions from the phylogeographic analysis were that there are four genetic groups of red foxes in this region of Canada, consisting of one in the Montreal area, one in James Bay, and two segregating in

northern Quebec and Labrador. The Abitibi region was a mixture of the Montreal and the James Bay clusters. When a northwestern locality, Churchill NB, was included and 9-locus genotypes used, there were three distinct clusters, with Churchill dominated by one, Montreal by another and a third northeastern group prevalent in northern Quebec and Labrador. James Bay was a mixture of the Churchill cluster and the northeastern one, Abitibi- Témiscamingue was a mixture of the Montreal and the Churchill clusters, and the Churchill cluster also spread into northern Quebec and Labrador. These results suggest routes by which rabies virus could be spread via red fox movement patterns, such as along the coast, or from north to south, but also suggests a possible barrier to movement further south than Montreal. This study is important because the rabies virus is still a relevant public health concern for Canadians and, as such, research contributing to its effective eradication and control is of great importance. The results from this study will be used to inform theoretical models to help predict future rabies virus transmission and spread.

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List of abbreviations and symbols

AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
BIC	Bayesian Information Criterion
bp	Base pairs
CREAIT	Core Research Equipment and Training Network
DAPC	Discriminant Analysis of Principal Components
DNA	Deoxyribonucleic acid
F_{CT}	Proportion of total variation among population
F_{IS}	Inbreeding coefficient
F_{ST}	Proportion of total within subpopulation
R_{ST}	Between population component of variation taking into account allele size
H_E	Expected heterozygosity
H_O	Observed heterozygosity
K	Allelic richness
K	The number of genetics clutches inferred by the program STRUCTURE
LD	Linkage disequilibrium
mL	Millilitre
mm	Millimetre
mM	Millimolar
mtDNA	Mitochondrial deoxyribonucleic acid
nM	Nanomolar
N_A	Number of alleles

Ng	Nanogram
PCR	Polymorphism chain reaction
SSR	Simple sequence repeat
STR	Simple tandem repeat
RFLP	Restriction fragment length polymorphism
^{o}C	Degrees Celsius
μL	Microliter

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Chapter 1-Introduction

Genetic mutation of bacteria and viruses represent a serious health concern and new interventions must be brought to shed light on new variants of diseases such as the rabies virus, as often the variants are resistant to existing drugs. One example is the presence of rabies variants identified in fox populations, which are the primary reservoir of the arctic lineage of the rabies virus (Mansfield et al., 2006). As such, an understanding of fox population demographics, dynamics and phylogeography is essential in the prevention of rabies virus transmission, which is a concern considering that the infection leads to neurological dysfunction and death. In efforts to eradicate the virus, Vos et al. (2004) indicated that oral vaccination campaigns of the red fox in Europe against the classical rabies virus were quite successful; however, mutations of the classic strain appeared and lead to a resurgence in identified cases within animal and human populations. Bernardi et al. (2005) report that the United States and Canada took great efforts to control the virus during the 1940s and the 1950s, but it still remains a present significant public health concern. This is because the virus remains hosted in various terrestrial and aerial animals, especially dogs, foxesand bats, through which the rabies virus is transmitted to human populations.Phylogeographic analysis of the rabies virus in both red and arctic foxes across northern Canada, including the Eastern Subarctic regions of Nunavik and Labrador, reveals predominance of the arctic rabies virus lineage A3 with diversification and spread of new subtypes in recent years (Nadin-Davis et al., in preparation, 2019). The goal of this work is to characterize genetic diversity and phylogeography of one of the two host species of the arctic viral lineage, the coloured (red) fox (*Vulpes vulpes*) to contribute knowledge to understanding the spread of rabies virus variants in the Eastern Subarctic region of Canada.

Red fox: host distribution and lifestyle

The red or colouredfox (*Vulpes vulpes*) is identified as the most widely distributed terrestrial carnivore in the *Canidae* family present in Canada, USA, Europe, Asia, North Africa and Australia (Yan et al., 2015; Statham et al., 2011; Harris and Rayner, 1986). Red foxes are larger in stature than their arctic counterpart weighing between 3.6-6.8 kg and measuring 90-112 cm in length (Canadian Wildlife Federation, 1993). In the wild, their diet can consist of voles, birds, eggs, insects, berries and fruits (Jędrzejewski&Jędrzejewska, 1992, and Elmhagen et al., 2002). They are not only found in the wild but also in rural and urban areas where they rely on food resources provided by human populations. They have also been shown living in spatial groups with a single breeding pair and a number of barren females, which implies that in the population females are greater in number than male foxes (Gachot-Neveu et al., 2009; Canadian Wildlife Federation, 1993). The male foxes move from one habitat to another for mating which increases the potential for spreading of viruses. Pagh (2008) indicates that rural foxes migrate into urban areas where they intrude on human settlements due to the increase in rural population density, or because they find adequate or suboptimal habitats and food resources. As such, the foxes adapt to urban conditions, which offer special hiding places as dens for breeding while food is obtained through scavenging. However, their range of movement is influenced by availability of food resources and shelter, as well as territory defense costs. Human populations in different geographical areas are tolerant of foxes to a greater or lesser degree (Lovari, 1996).

Besides food availability, familial relationships are another factor driving fox movement and dispersal as indicated by Whiteside et al. (2011) in their research on juvenile red fox dispersal among urban foxes across the United Kingdom. Using microsatellite markers and long-term dispersal data, the researchers found that juveniles tend to follow their mothers, not fathers;

male foxes with dominant mothers dispersed more often than those of subordinate mothers, while dispersing female foxes were more likely to have subordinate mothers (Whiteside et al., 2011). Also, a study by Gachot-Neveu et al. (2009) implicated female red foxes in long-distance dispersal rather than males in rural France, a situation which may be linked to a variety of factors, including human population density and differences in habitat. Social organization and the defining mating system of the red fox dictate that subordinate females venture out into new territories in search of mates, which may explain the long-distance dispersal of the female red fox. Moreover, as the behaviors of dominant females drive them away, they move to other places to get more attention.

Different geographical areas have different population densities of red and arctic foxes; the dynamics within eastern Canada can be understood from data amassed from areas including northern Quebec (Nunavik), Newfoundland and Labrador, and Prince Edward Island, among others. For example, population dynamics of the red fox on Prince Edward Island revealedthat a high number of harvested individuals were juveniles,which wasattributed to increases in food supply supporting survival(Wapenaar et al., 2012). More importantly, the authors indicate that the harvested red foxes no longer fear people as they are fed in most urban areas, increasing the health risks to humans as the animals may be rabies carriers.

Farm-bred silver foxes, coat color variants of the red fox, identified as originating from Prince Edward Island in eastern Canada, are distinctly different from the native red fox populations in Europe, the USA, Alaska and Asia. For example,the Russian silver fox has been tied to eastern Canada through phylogenetic analysis: specifically, four of the seven combined cytochrome b/D-loop haplotypes were previously identified in red foxes in Eastern Canada (Statham et al., 2011).

Arctic fox: host distribution and lifestyle

In general, the arctic fox is a circumpolar specialist predator with a tendency to venture long distances in search of food (Berteaux et al., 2017; Norén et al., 2005). According to Mansfield et al. (2006), the arctic fox (*Vulpes lagopus*) lives in small groups with territories spanning between 8.5 to 72.8 square kilometers in one of the most extremely frigid environments in the world. The foxes' territorial span depends on the abundance of available food resources, which include rodents such as voles and lemmings, fish, and carrion. Eide et al. (2012) confirmed that large local arctic fox populations are sustained by seasonal access to seabirds and other prey including reindeer carcasses, which explains elements like high juvenile survival rates and large litter sizes when prey is abundant. The territories are expanded during harsh winters when food resources decline, which force the foxes to venture further out into new territories in search of food. Decline in food resources is associated with small litter sizes and low juvenile survival rates, as well as a lack unoccupied dens considering the harsh climate conditions (Eide et al., 2012). This was affirmed by Giroux et al. (2012) whose research showed that the arctic fox population in Nunavut exhibits similar population dynamics, with food resource availability influencing habitat locations and den occupancy, as well as home range size. Scarcity in food resources stimulates long distance migration, which expands arctic fox territories as they search for food and habitats for breeding; successful growth of cubs relies on adequate food supplies.

Research by Norén et al. (2005) identified two types of arctic foxes in Scandinavia, the domesticated or farmed and the wild, where the latter is identified as highly migratory and faces potential extinction due to a variety of factors including hybridization with the domesticated farmed fox. The study used microsatellite variation and mitochondrial control region sequences

to identify farm and wild arctic foxes; the method was highly useful in differentiating between the two populations.

Virus strain types and distribution

Classical rabies virus (RABV) is identified by Mansfield et al. (2006) as one of the seven genotypes of the genus *Lyssavirus* within the family *Rhabdoviridae* and detected in wild animals like the raccoon dog and seals, as well as domestic animals such as sheep among others. The virus is generally transmitted through the bite of a rabid animal or through saliva infected with the rabies virus when it comes into contact with fresh wounds or mucous membranes. Differences in the viral strains can be defined by their rate of infection, where the range can span from a few days to weeks for the effects of the virus to manifest as it attacks the nervous system. More specifically, the attack on the nervous system is identified primarily as fatal encephalomyelitis, which leads to neurological dysfunction and death if not treated early enough before the virus establishes itself in the central nervous system (Nadin Davis, Sheen and Wandeler, 2012; Nadin- Davis, Casey and Wandeler, 1994).

Through gene sequencing, Mansfield et al. (2006) identified Arctic and Arctic-like virus isolates of rabies from foxes, including two lineages in the Arctic group, Arctic 1 and 2, where Arctic 1 mainly consisted of Canadian isolates. Divided into sub-lineages 2a and 2b, Arctic 2 isolates hail from Alaska and Siberia, while the arctic-like group isolates are from countries such as Pakistan, Japan and India. Other rabies virus isolates distinct from the arctic variants originate from countries in northern Europe and North America, as well as the Russian steppes. Nadin-Davis, Sheen, and Wandeler (2012) confirm the presence of the current arctic rabies virus lineage in central and northern Asia, particularly India, tracing back to a common ancestor 40

years ago, while Arctic variants overall are identified as emerging within the last 200 years when the Arctic clade entered Canada. Nadin-Davis et al. (2008) indicates that the virus is also present in neighboring Greenland via fox movement across pack ice. These studies show that the arctic rabies virus lineage is widely spread in many distant areas which can be traced back to Canada.

A study by Nadin-Davis et al. (1999) identified four major viral types (N T1-4) and a small group of intermediate type (N T2/4) in the N and G non-contiguous regions of the viral genome linked to the original rabies virus that invaded Ontario in the 1950s. The identified viral types have many variants with some of them topographically restricted to specific geographical areas like the infestation of T1 rabies variants in eastern Ontario and the T4 variants in the area between Lake Simcoe and Huron, as well as the Severn River and Georgian Bay. The low number of mostly synonymous differences defining the variants are hypothesized as resulting from high level of adaptation necessary for the survival of the virus variants in fox host populations, where viral genetic variation is limited by selective pressures.

Nadin-Davis, Muldoon, and Wandeler (2006) reported that the arctic fox strain of rabies virus remains endemic in Ontario but that the identified variants in the region differed from the ARC.T5 variant circulating in the northern regions of Quebec, Newfoundland, and the Arctic zones. The fifth N variant or ARC.T5 is implicated in the recurrence of the 2001 rabies virus in northern Ontario and parts of Quebec. The ARC.T5 is spread through infected red foxes and other wild hosts, such as skunks, considering that the rabies virus can infect a myriad of mammals. Given that the arctic fox is the primary reservoir of the arctic and arctic-like rabies virus, the transmission of the arctic virus lineage through the red fox implies interaction between the two foxes. This is consistent with research on home range size and movement patterns of

arctic foxes, which are identified as moving long distances away from their normal territory range in search of food, especially during harsh winters (Eide et al., 2012).

Evidently, new rabies variants still manage to cross over to other geographical areas and their movement is not solely contingent on the dispersal patterns of the aforementioned fox species. However, effective adaptation to specific areas with adequate food resources may enhance genetic isolation with time as gene flow between foxes in two geographical areas (like urban and rural) is limited, inhibiting the transmission of rabies across geographies. This is affirmed in that the spread of the identified arctic fox rabies virus variants to specific geographical areas, in terms of landscape topographies, displays a high correlation to fox movement restriction (Nadin-Davis, Muldoon, and Wandeler, 2006). Topographical restriction is also identified in genetic differentiation defining the red fox in Hokkaido Island in Japan where geographical isolation due to land shape limits the movement of the red fox (Oishi et al., 2011). The transmission of rabies across geographies can also be explained through transmission to other animals like skunks and raccoons which migrate to other areas including urban sites (MacInnes, 2001).

The prevalence of the arctic fox rabies virus was investigated by Nadin-Davis et al. (2008) whose study on the 2003 outbreak of the rabies virus in Newfoundland revealed similarity to the genetic variants identified in Labrador. The Labrador variant is also acknowledged as having spread to many areas of northern Quebec, indicating that similar virus variants are present in the Eastern Subarctic regions. However, the authors of the study further acknowledge the presence of unique variants, apart from the 2003 outbreak, in Newfoundland that are largely different from those identified in other regions such as Labrador and northern Quebec (Nadin-Davis et al., 2008). Nadin-Davis et al. (2012) speculated that the spread of the rabies virus

infection stimulates emergence of new variants, and those that are strongest manage to replace previous viral variants as they spread across wide geographical areas. This is supported by Deviatkin et al. (2017) whose research was conducted in the Russian Federation and confirmed arctic viral spread into the northern part of European Russia and the Franz Josef Land. The spread was tracked through gene sequencing, and showed that rabid foxes and raccoon dogs are central to the spread of the virus, with various native animal populations being infected after which it rapidly disperses into other regions.

Rabies in the mainland region of Labrador is considered endemic in both the red and arctic foxes where rabies cases vary in accordance with changes in fox population density; this is heavily influenced by food resources and habitat availability. For the Island of Newfoundland, Nadin-Davis et al. (2008) identify four recorded instances of rabies: including single isolated cases in both 1955 and 1989, an arctic fox rabies virus outbreak in 1988 with five confirmed cases and the previously mentioned outbreak in 2003. Of particular interest is this most recent 5–month outbreak in 2003 with 21 confirmed cases where a cat, three sheep, and 17 red foxes were infected. Mansfield et al. (2006) stressed that the distribution of the rabies virus, particularly through epidemics, is tied to increases in the population, as well as migratory behavior of the arctic fox; the animal is considered to be the major reservoir for the arctic rabies virus lineage. Essentially, the arctic fox transmits the virus to other animals, including the red fox and domestic dogs, which not only represents a major risk to human health but also to that of other wildlife (Berteaux et al., 2015).

Climate and environmental change

Many research studies including one by Kim et al. (2014) confirm the impact of environmental changes, especially the climate, on the migratory behavior of fox populations and

their transmission of the rabies virus as they venture into new environments in search of food. The author indicates that both the red and arctic foxes are carriers of the rabies virus even though arctic foxes are disproportionately affected by climate changes when compared to red foxes. In general, rabid fox numbers are shown to peak during winter and spring seasons, especially at high latitudes, while rising temperatures may lead to a decrease in reported rabid arctic foxes. The influence of season change on the movement of the arctic fox is affirmed by Frafjord and Prestrud (1992) who indicate that limited food availability, during winter and autumn seasons in Svalbard, forces the animals to roam over wide areas in search of food. These two periods are also characterized by strong winds and low temperatures, which complicate hunting even though ice enhances their movement during winter. Factors that influence the range of movement of the foxes are generally identified as habitat richness by Lovari (1996) whose study on red foxes confirms the influence of similar elements, including food resources, shelter and territory defense costs. Moreover, the authors indicated that shelter and movement are restricted to peripheral areas where humans do not tolerate foxes.

More extreme climate changes, identified as climatic oscillations by Hewitt (2004), have been identified as potent influences on species divergence and biodiversity, when considering changes that accompany extinction-level events. Further, research by Fontanella et al. (2012) indicated that climatic changes do impact migration of species, which are forced to adapt to different environments. Focusing on the Patagonian lizard, the authors found population increases in the lizards that diverged to the South as their environment expanded, while the population of the northern group was stable even though the environment and its offerings had shrunk. Morphometric analyses presented post-isolation demographic change, which, with findings from ecological niche modeling (ENM), suggests different adaptive responses to climate

change. The changes were identified as imposing strong selection pressures for fitness traits, especially body size, which should allow for better chances of survival.

Genetic markers as tools for understanding host movement and virus distribution

Information on population dynamics is quite well gleaned from genetic markers and the associated commonalities in their patterns across different regions. Hewitt (2004) suggested mitochondrial DNA (mtDNA), chloroplast DNA (cpDNA) and non-coding nuclear regions as some of the DNA sequences sought in identifying and tracing ancient genealogies; hypervariable markers for studying more recent events include microsatellites and Amplified Fragment Length Polymorphisms (AFLPs). Additionally, phylogenetic analysis of gene sequences helps to link the various rabies strains to specific geographical areas, thus providing a clear picture of virus strain distribution (Mansfield et al., 2006). As Nadin-Davis et al. (2008) asserts,"the application of phylogenetic methods to the study of rabies viruses is not only an important epidemiological tool to discriminate between viral strains but allows exploration of viral variation within an individual strain" (p. 86-7). Some researchers showed that the spread of rabies is restricted to specific geographical areas in spite of environmental pressures that reflects migration of the host. One such study is by Goldsmith et al. (2016) who show that the population structure of arctic foxes, considered highly mobile, maintains rabies strains within specific geographical areas in Alaska. Using mitochondrial DNA (mtDNA) sequencing and nine microsatellites markers, the authors found that the distribution of the rabies virus variants in Alaska was mostly driven by the Arctic fox. An assessment of fox population genetics is shown to be vital in the determination of roles played by different foxes in rabies transmission, even though this strategy must be supported by other techniques. Still, the restriction of viral strains to specific geographical areas may be

nullified by the spread of the virus to other animal populations as indicated by viral transmission of rabies between the Arctic and red foxes (Kim et al., 2014).

Further, through gene sequencing, the study by Carnieli Jr. et al. (2013) traced genetic lineage of the rabies virus in Brazil to wild dogs and the crab-eating fox where the virus diversified and dispersed to other locations in the north and south. The rate identified by the authors is 30.5 kilometers per year for the dog-associated lineage and 9.5 kilometers per year for the fox, given that their dispersion is tied to human activities where dogs follow human and foxes choose peripheral home territories and protect them. Like Carnieli Jr. et al. (2013), Drygala et al. (2016) recognize the importance of knowledge on the movement of animals, such as rabies-carrying foxes, especially in terms of epidemiology. The authors analyzed the genetic structure of the European raccoon dog which, given their susceptibility as secondary hosts to the rabies virus and their dispersal capabilities as their population expands, could complicate management of a potential rabies epidemic. Microsatellites markers were used to identify genetic collections of the population and its dispersal in different environments after introduction. Gene sequencing also aided in identifying re-emergence of the arctic rabies virus lineage in central and northern Asia (particularly India) where the Indian and Arctic variants are identified as emerging within the last 40 and 200 years, respectively (Nadin-Davis et al., 2012).Through microsatellite genotyping, Dalén (2005) was able to distinguish four genetically different populations of the arctic fox in Scandanavia while affirming their migratory behavior over long distances in search of prey resources.

Through microsatellite investigation, a study of genetic variation in the rural and urban fox populations in Zurich, Switzerland, revealed high urban-rural gene flow even though increased rural and urban fox migration are expected to erode genetic variation over time

(Wandeler et al., 2003). This particular study highlights the impacts of environmental change through human activity on the adaptive behavior of animal populations and their migratory patterns across geographies where migration of rural foxes creates new dispersers of the rabies virus.

Microsatellite markers

Hypervariable markers such as AFLPs and microsatellites are best utilized in studies of comparatively recent events. Indeed, microsatellites have been identified as providing efficient and cost-effective genetic markers highly useful in molecular ecology, as well as the assessment of genetic structure in widespread and mobile species like the red and arctic foxes (Basto et al., 2016).

Fundamentally, microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are genetic markers utilized in the measurement of genetic variation, where genetic information is traced to specific locations within a genome. They can provide highly specific information about the genetic relatedness of populations and subpopulations (Vieira et al., 2016), in part due to their abundance and uniform distribution in the genome. Locus-specificity enabled by species-specific primers is another of the reasons why microsatellites are valuable as genetic markers besides the expression of co-dominance and hypervariability, which allows focused identification of several alleles as pointers of genetic differentiation in a given population (Mittal and Dubey, 2009).

Other useful information gleaned from microsatellites includes assessment of demographic histories, inbreeding levels, gene flow variables and population size, especially when populations are highly dispersed such as in the fox population. As such, microsatellites can

pinpoint relatedness between individuals and groups in the redand arctic fox populations, provide information on inbreeding levels, as well as genetic differentiation between fox populations in different geographical areas (Gachot- Neveu et al., 2009).

Foxes in the Eastern Subarctic region of Canada

The Eastern Subarctic region of Canada, including northern Quebec (Nunavik) and the mainland Labrador portion of Newfoundland and Labrador, defines the area of interest in this study.

Northern Quebec has a large land mass with numerous lakes and rivers, and is less populated than the rest of Quebec. Nunavik, the northern third of Quebec and the homeland of the Inuit in the province, is marked by boreal forest in the south and tundra in the north. It stretches from the subarctic climate zone into the arctic region where many arctic foxes have habitats (Nuttall & Callaghan, 2000). As such, the northern parts are characterized by harsh winters and relatively cool summers, in which red and arctic foxes have numerous food resources as their prey ventures out in the improved climate conditions (Riouxet al., 2017).

The province of Newfoundland and Labrador is the most easterly Canadian province and is comprised of two distinct geographical areas: the mainland region of Labrador and the Island of Newfoundland. The latter is largely boreal forest, while the north of the mainland is tundra. Like northern Quebec, Labrador is characterized by harsh winters and relatively cool summers with both arctic and subarctic climatic conditions in the north and south, respectively (Roberts at el., 2006). This explains the presence of both coloured and arctic foxes in the mainland of Labrador, as well as the distribution of the rabies virus, while the island of Newfoundland has mostly coloured foxes, consistent with differences in the climatic conditions of the two areas.

Considering that geographical locations in northern Quebec have relatively similar climatic conditions to areas such as Svalbard in Norway, factors defining fox populations and their migratory patterns can be inferred as similar (Eide et al., 2012). Similarly, arctic fox population dynamics in northern Canada were elucidated by Giroux et al. (2012); given that the climatic conditions of Nunavut parallel those of northern Quebec and Nunavik, arctic fox behavior and population dynamics are expected to be close in the two areas. These patterns include elements such as litter size and juvenile survival rates, habitat use and den occupancy, as well as home range size, among others. For example, the arctic fox population, marked by litter size and number of adults, as well as home range size, is influenced by the availability of food resources in that foxes build habitats close to prey resources. However, the lack of relevant research studies targeting specific fox populations in eastern Canada undermines extrapolation of the few identified findings of fox population demographics and dynamics to that geographical region.

Summary

Given the adverse effects of the rabies virus, which primarily include neurological dysfunction and death, an understanding of fox population demographics and dynamics is indeed vital, especially in light of the potential for new lethal mutations of the virus to occur. The coloured and arctic foxes act as carriers of the virus, which means that their distribution can predict potential epidemics even though information relevant to the Eastern Subarctic regions ofnorthern Quebec and Labrador is quite scarce.

Population demographics for the red fox indicate preference of relatively warmer climates, which explains their closeness to human settlements as compared to the arctic fox, well

adapted to the harsh frigid environments (Prestrud, 1991). Given that foxes are reservoirs for the various rabies virus strains, the distribution of the virus in Eastern Canada is tied to population demographics and dynamics of the red and arctic fox population in the region. This means that the influences of climate changes and landscape topography, especially the availability of food resources and migratory behavior, alongside social organization norms, heavily dictate the spread of the rabies virus in those regions.

With regards to the various types of fox rabies virus in eastern Canada, the arctic rabies virus is identified as the primary resident in the eastern subarctic of Canada where there are arctic foxes. Conversely, the other variants are predominant in geographical regions characterized by relatively warmer weather conditions and thus most of the red fox populations reside in these areas. Nevertheless, the arctic rabies virus lineage has been transmitted in the warmer geographical areas through contact between the arctic and the red foxes, as well as other mammalian species through which the virus is transmitted to human populations.

The use of microsatellite markers has been identified as quite useful in population genetic studies where highly specific information like demographic histories, inbreeding levels and gene flow variables can be gleaned.

Objectives of this thesis

The rabies virus is still a relevant public health concern for Canadians and, as such, research contributing to its effective eradication and control is of great importance. Broadly, in this thesis, rabies virus host distribution and phylogeography will be analyzed and compared with viral strain distribution. The area of focus is the Eastern Subarctic region of Canada, defined by parts of northern Quebec and Labrador, while the host analyzed is one of the primary vectors

of the disease: the red or coloured fox. Generally, it is a widely accepted thought that the movements of the host affect the viral strain distribution. Investigation of the host distribution is achieved through the analysis of microsatellite genetic variation and correlating similarities among sampling localities with their geographical locations. When considering previous studies, 9-locus microsatellite have been most commonly employed in foxes, however, this study will expand on that amount and 15 loci will be examined. With regards to examining the rabies virus variants and their distribution, fragments of the virus genome, namely the N and G genes, have been sequenced and a phylogenetic tree has been constructed to describe the variants currently circulating in northern Canada (Nadin Davis et al., in preparation, 2019). Thus, stated more explicitly, the objectives are to answer these questions:

1. What the geographic distribution of genetic groups clusters looks like?
2. What are the factors that contribute to the shaping of this geographic distribution?
3. Can host movement help explain virus spread?

Rabies and fox host distribution has been researched extensively in other areas, for example in European and Asian nations, as well as in southern Ontario and Alaska, but little is known in the eastern subarctic regions mentioned. This thesis will aid to shed light on the relationship of fox hosts and the rabies viral strains in north east Canada which can assist in the efforts to provide the public with an effective vaccination strategy.

Chapter 2- Materials and Methods

Tissues

Coloured fox tissue samples from four different localities in Labrador (Labrador City, North West River, Port Hope Simpson, and Cartwright) were provided by Dr. Hugh Whitney, DVM, former Provincial Veterinarian with the Government of Newfoundland and Labrador. Samples from six localities in Quebec were provided by Ariane Massé, a biologist with the Ministère des Forêts, de la Faune et des Parcs, Gouvernement du Quebec. These included two southern localities (Montreal and Abitibi-Témiscamingue), James Bay, and three Nunavik localities (Kuujjuarapik, Inukjuak, and Kuujjuaq). A sample from the Island of Newfoundland was included in some analyses; these were tissues provided by Dr. Hugh Whitney as part of the rabies surveillance effort during the 2003 outbreak. Data for 49 foxes from Churchill, Manitoba, and two foxes from Nunavut, were included in DNA analyses. Additional localities were represented by 1 to 2 individuals each as shown in Table 1. Tissue samples were from four origins as these were available: the masseter muscle of the jaw, hind leg muscle, an ear clipping, or a portion of salivary glands from where DNA were isolated. There were 23 rabies-positives samples included as shown in Table 1; these were provided as extracted DNA (10 ng/µL) from Dr. Susan Nadin-Davis, PhD, of the Canadian Food Inspection Agency in Ottawa. Sample sizes and codes are given in Table 2.1. All samples were approved for use in this study by the Memorial University Animal Care Committee in compliance with the Canadian Council on Animal Care.In total, 461 samples from 22 localities were provided and analyzed.

DNA extraction

DNA was extracted from fox tissue samples by one of two methods. Both used Qiagen DNeasy Mini Kit (Qiagen Inc., Toronto, Canada). The first method, where the binding, wash and elution steps were done manually, required considerably more time and effort given its repetition of many steps. The second method was an automation of the bind, wash, and elution steps, achieved using a QIACube (Qiagen Inc.). The advantages provided by the QIACube were that it is less labour-intensive and reduces potential human errors such as incorrect pipetting volumes or mixing up of samples. However, the manual method was used for a number of samples in initial stages of the project.

For both approaches, initially 20 mg tissue samples were measured and added to 180 µL of buffer ATL and 20 µL of protein K in 1.5 mL microcentrifuge tubes, then mixed in a vortex and centrifuged briefly. The samples were incubated in a Thermomixer R (Eppendorf AG, Mississauga, Canada) at 56°C overnight to homogenize and lyse the samples. After 24 hours, either the manual or automated method was performed to continue the DNA extraction. In the manual method, the following steps were executed: 200 µL of buffer AL and 200 µL of 100% ethanol were added to the samples and the samples were returned to the Thermomixer for ten minutes. Subsequently, a spin column was used to bind DNA within the sample; the mixture was added to the spin column and centrifuged at 8000 rpm for one minute. The liquid that passed through the column in the previous step was discarded and 500 µLof buffer AW1 was added and again centrifuged for one minute at 8000 rpm. Following this, 500µLof buffer washer AW2 was added then centrifuged at 14000 rpm for three minutes. The DNA was eluted by adding 100µL buffer AE and centrifuged at 8000 rpm for

one minute. This completed the manual method of extraction. DNA samples were stored at -20 °C until use.

For convenience, the aforementioned QIACubewas used to extract DNA for most samples. Using the Qiagen DNeasy kit, the lysed and homogenized samples previously incubated overnight were vortexed and spun down then placed in theQIAcube, 12 samples per run. The QIAcube reagent tube rack was inspected for sufficient amounts of the following: ethanol 100%, and buffers AL, AW1, AW2, and AE. Following the touch screen options, the standard DNeasy protocol was selected and executed. Each run of 12 samples took an hour to complete, and this extracted DNA was stored in a freezer at -20 °C.

By using the spectrophotometer, NanoDrop 1000 (Thermo Fisher ScientificInc., Waltham, USA), the extracted genomic DNA quality was tested and quantified by measuring absorbance values on 2.0 μL of each sample. This was a quality control step providing DNA concentration values in ng/μL and thus provided information on whether the sample was suitable for use and to provide a dilution factor. The DNA samples were diluted to 10 ng/μL in 30 μL volumes using nuclease-free water for standardization of the DNA concentration for polymerase chain reaction (PCR).

PCR amplification

Following the DNA extraction of the fox tissue samples, PCR amplification of microsatellite loci was performed using an EppendorfMastercyclerthermal cycler (Eppendorf AG). All analyses presented here used at least 9 loci while those that were focused on the eastern Subarctic region used an additional six. The first 9 loci were:CPH3,CPH9, CPH15 (Fredholm&Winterø 1995), AHT121, AHTh171, Co4.140, Co1.424,

REN105L03 andREN247M23 (Molecular Ecology Resources PrimerDevelopment Consortium et al. 2010).These 9 loci were divided into three multiplexes on the basis of previous studies (Goldsmith et al. 2016): group A (CO4.140, REN105, AHTh171); group C (CPH3, AHT121, REN247M23); and group D (CPH9, CO1.424, CPH15). Most analyses used15 loci in order to improve fine-scale resolution of population structure. The additional 6 loci were: CXX109, CXX250, CXX172, CXX173, CXX20, and CXX377 (Ostrander, Sprague, and Rine, 1993). For the 15-locus analyses two multiplexes were added: group E (AHT121, CXX109, CXX250) and group F (CXX172, CXX173, CXX20, CXX377).Primers sequences are given in table 2.2. Forward primers were fluorescently labelled with 6-FAM, VIC, NED, or PET. CPH15 did not always produce results when in multiplex D so was amplified and electrophoresed singly in those cases. Note that one locus, AHT121, was included in two separate multiplexes and as well, known positive controls were included in each round of electrophoresis. These were measures taken to ensure consistency of scoring.

For each PCR for multiplexes A, C, and D 12.5 μL Qiagen Type-It Master Mix (Qiagen Inc.), 2.5 μL primer mix and 7 μL RNase- free water were added to 3 μL 10ng/μL DNA. Primer mixes consisted of 200 nM of each primer with the exception that the mix for multiplex C contained 600 nM CPH3F and CPH3R and the mix for multiplex D contained 400 nM CPH15F and CPH15R. For the multiplexes A, C and D, the thermal cycler profile was 95°C for 5 minutes;35 cycles of 95°C for 30 seconds, 60°C for 45 seconds, 72°C for 30 seconds; 60°C for 30 minutes; 12°C hold. For the multiplex E, 2.5 μL Q solution was added to each reaction as well as 12.5 μL Qiagen Type-It Master Mix (Qiagen Inc.), 0.25 μL each of 10 μM AHT121F and R, 0.36 μL each of 10 μM CXX109 F and R, 0.09 μL each of 10 μM CXX350 F and R, 6.58 μL RNase-free water and 3 μL 10ng/μL DNA. For the multiplex E the profile was: 95°C for 5

minutes; 13 cycles of 94°C for 30 seconds, 63°C - 0.8°C per cycle for 90 seconds, 72°C for 60 seconds; 22 cycles of 94°C 30 seconds, 55°C for 90 seconds, 72°C for 60 seconds; 60°C 10 minutes; 12°C hold. For multiplex F the reactions contained 12.5 μL Qiagen Type-It Master Mix (Qiagen Inc.), 0.5 μL of each primer (10 μM), 6.5 μL of RNase-free water, and 3 μL of DNA. In case of Multiplex F, the following profile was used: 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 53°C for 45 seconds, 72°C for 30 seconds; 60°C for 30 minutes; 12°C hold.

The next step was preparing forelectrophoresis. Samples were diluted by adding 39 μL nuclease-free water and 1 μL PCR product followed by vortexing and 5 seconds centrifugations. After that, 0.1 μL Liz Size Standard (Applied Biosystems, Thermo Fisher Inc.) and 8.9μL Hi-Di Formamide were added to 1 μL of the diluted PCR product. The samples were vortexed and centrifuged and then replaced in the thermal cycler to denature for 3 minutes at 95°C. An Applied Biosystems 96-capillary 3730xl DNA Analyzer was used in the Genomics and Proteomics Facility of CREAIT at Memorial University to electrophorese the samples.

Data analysis

Electropherogram traces were scored by two readers using Peak Scanner™ Software v1.0(Applied Biosystems, Thermo Fisher Inc.) to determine the exact length of alleles (bp) for each locus of all samples.Upon the scoring of all samples, they were manually entered into a Microsoft Excel spreadsheet. Following this, the software program CREATE (Coombs et al., 2008) was employed to convert the file into a text format, enabling the data to be converted to input files for many existing genetic software analysis programs.

To assess the quality of the scored data, Micro-Checker (van Oosterhaut et al., 2004) was used, which provides information quantifying genotyping errors and can aid in the adjustment for

such issues. Null alleles, short allele dominance (large allele dropout), typographic errors and stutter peaks can all be determined using the Micro-Checker program (van Oosterhaut et al., 2004). The program employs a Monte Carlo bootstrap method and the Hardy-Weinberg theory to generate expected frequencies of heterozygote and homozygote allele size differences, as well as null allele detection. When researching genotypes with many loci, it is imperative to discriminate between inbreeding, Wahlund effects and deviations from the Hardy Weinberg equation caused by null alleles (van Oosterhaut et al., 2004). Micro-Checker is a program that can provide clarification to these factors and also account for the null allele frequencies by providing an adjustment to the observed allele frequencies allowing for further genetic analysis of otherwise flawed data (van Oosterhaut et al., 2004). Results from the program are produced in the format of graphs displaying two main parameters: homozygote frequencies and allele size differences at each locus for all populations.

The program Arlequin v3.5 was used to provide information on the genetic diversity for both loci and populations. Using the raw data from all 15 of the selected microsatellites the software was used to generate standard indices of diversity, such as the number of allele (N_A) and expected heterozygosity (H_E), Hardy-Weinberg proportions (the inbreeding coefficient (F_{IS}), and measures of population differentiation (F_{ST} and R_{ST}) for both loci and populations. In this study, it was important to test the 15 loci to check for their conformity with the Hardy-Weinberg proportions across all 10 populations, as well as characterize their level of diversity and potential to reveal population structure. In each population understanding the genetic diversity changes can have implications for vector control programs, hence potential information for future rabies vaccination attempts (Kitinic et al., 2013). As well, it is important to know whether a sampling

locality fits Hardy-Weinberg proportions, as this informs about whether it is a randomly mating population.

With regards to characterizing loci, it is important to determine whether pairs of loci are in significant linkage disequilibrium or not. If two loci are physically linked together on a chromosome, they can provide the same information about population structure and diversity which can possibly lead to erroneous inferences. Genepop on the Web v4.2 was used to investigate genotype equilibriums amongst pairs of loci, allowing for the analysis of linkage disequilibrium for pairs of loci.

In this study, FSTAT v1.2 (Goudet 1995) used to calculate k, allelic richness, which is also a measure of genetic diversity, in each population. Allelic richness is a modification of the number of alleles that takes samples size into account (Foulley and Ollivier, 2006), and is not currently an option within Arlequin.

Arlequin also allows for hierarchical Analysis of Molecular Variance (AMOVA) to detect variation association with groups of populations as well as among populations (Excoffier, Smouse& Quattro, 1992). After using Arlequin to calculate pairwise measures of F_{ST} among populations, a dendrogram of these distances was constructed using Molecular Evolutionary Genetic Analysis v7 (MEGA) to show the relationships among sampling localities. Then AMOVA was conducted to determine how well the level of grouping suggested by the dendrogram was supported. The resulting statistic, F_{CT}, indicates the proportion of genetic variance that is apportioned among groups.

STRUCTURE software version 2.3.4 was used in this study to deduce the genetic population structure among and within populations without priori assigning individuals to populations (Pritchard et al. 2000). It is one of the most widely used programs for genetic

analysis that allows the scientist to analyze the patterns of genetic structure within a set of samples (Porras-Hurtado et al, 2013). The program is useful in ascribing individuals to specific sub-populations or groups based on their allele frequency differences and makes use of Bayesian algorithms (Porras-Hurtado et al, 2013). In this study, we used a burn-in period of 50,000 and a MCMC of 500,000 to test K=1-10 clusters with three iterations at each K. According to Structure Harvester, the number of groups was determined using the Evanno method(Evanno et al. 2005). Jombart, Devillard, and Balloux, (2010) and Guillot, Mortier, and Estoup (2005) described methods for two different strategies for the investigation of individual membership in populations are Discriminant Analysis of Principal Components and Geneland v4.08, respectively.

Discriminant Analysis of Principal Components (DAPC) is a statistical approach that was developed by Jombart and colleagues. DAPC is similar to STRUCTURE but can find less differentiated groups. It may help us determine how many groups are in Labrador.It can be used to predict the number of groups of genetically related individuals (Sofia et al., 2018).The difference in samples is defines as either between-groups or within-groups so that the groups can be distinguished easily, whereas the number of groups remains unknown. To predict the number of groups, a transformation is done by principal component analysis and then the clusters are identified using discriminant analysis. The genetic grouping can also be done by using k-means. This helps in finding the number of groups by maximizing the variations between them. The k-means is run subsequently with an increase in value of k and the clustering solutions are compared using a Bayesian Information Criterion (BIC). According to Thibaut & Caitlin (2015) the best clustering solution refers to the one with the lowest BIC value.

Geneland is a computer package that allows you to statistically analyze the genetic data of a population. In this particular package, the multilocus genetic data of an individual is geo-referenced. This program helps in determining the number of populations and their spatial structure by studying the variation in allele frequency. It uses both genetic and geographic information to quantify the spatial organization of the population and predict the number of populations in a dataset (Mafalda et al., 2016). In Geneland, the idea is to help identify geographic barriers to gene flow without putting individuals into samples. The major assumptions of the method include: the total number of populations are unknown, and all the values are treated equally; populations are spread over not known locations in spatial domains; Hardy-Weinberg equilibrium is considered within every population; allele frequencies within each population are considered as unknown and treated as random variable. In the end, the final output of the package, the number of populations and the probabilities of population membership, is predicted from the simulated data set which is also produced by Geneland (Gilles et al., 2005). Geneland is able to deal with all types of markers whether they are dominant or co-dominant. (Gilles et al., 2008).With Geneland, I used two models correlated and uncorrelated. For each model, I ran 100000 iterations with thinning every 100 iterations. I allowed coordinate uncertainty of 6 and I tested number of populations from 1 to 10. Also, I did a burn-in of 20% of the iterations.

STRUCTURE and Geneland were conducted on the 15-locus 396 individual Eastern Subarctic data (Labrador and Quebec). As well, to help interpret the Eastern Subarctic genetic cluster patterns, STRUCTURE was also conducted with the 9-locus complete data set of 461 individuals, including populations in Churchill, Manitoba, and the Island of Newfoundland. STRUCTURE was also used to further test all individuals from Labrador only (15 locus

genotypes). I used DAPC to help determine if there was any finer population structure within Labrador that could not be detected with the other methods.

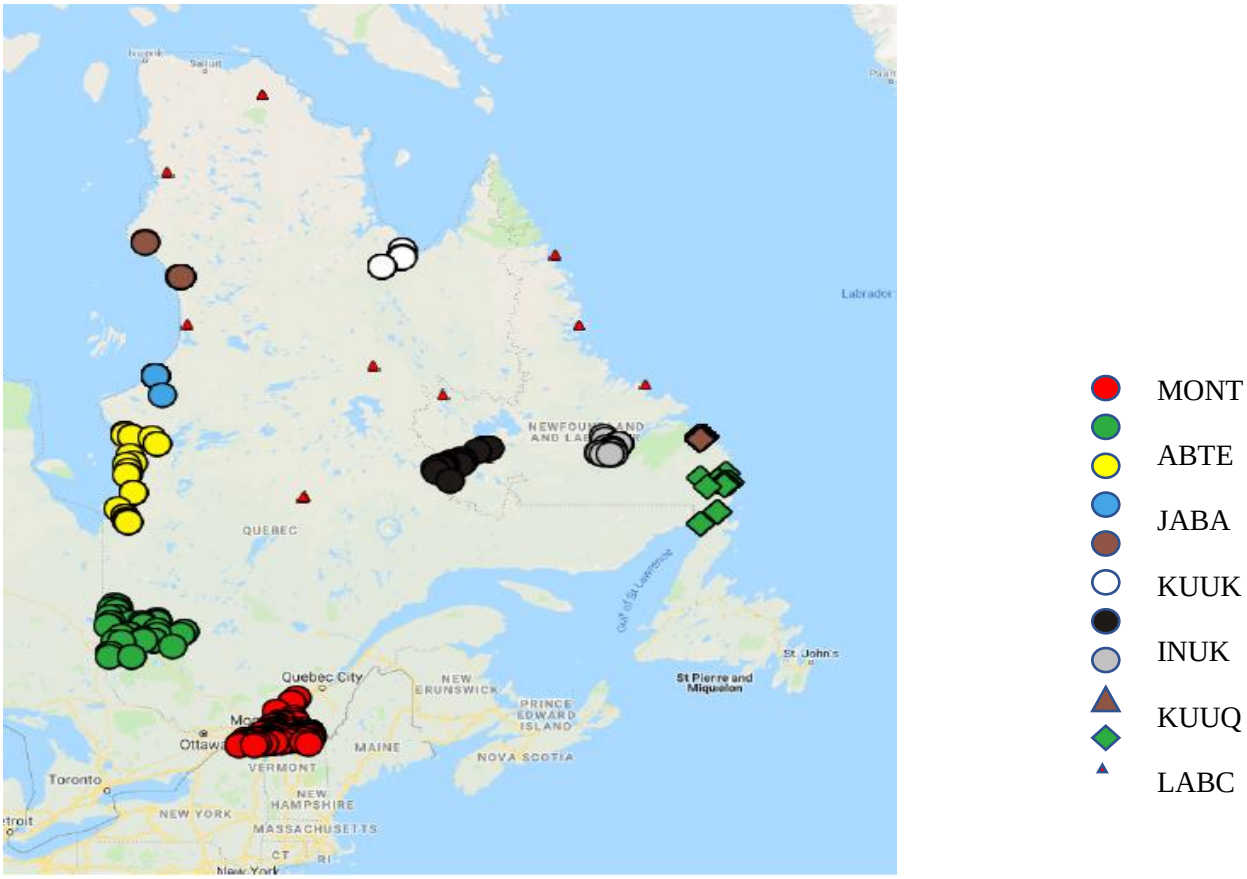


Figure 2.1 Map of sampling localities ofred foxes in or near the Eastern Subarctic region of Canada. Each symbol marks a coordinate, which may be represented by one or more than one individual red fox. Locality codes are as given in Table 2.1.

Table 2.1. Sampling localities, sizes, codes, and sources of red foxes used in this study. Sources include ear clippings (ear), masseter muscle (masseter), Hind leg muscle, DNA, or data provided. The number of rabies-positive foxes is also indicated for each locality. For locations shown in italics, 9-locus genotypes were analyzed.

Locality	**Code**	**Size (N)**	**Source**	**Rabies-positive**
Quebec		**253**		
Montreal	MONT	128	Ear	0
Abitibi-Témiscamingue	ABTE	50	Ear	0
James Bay	JABA	27	Masseter	0
Kuujjuarapik	KUUK	14	Masseter	0
Inukjuak	INUK	11	Masseter/DNA	1
Kuujjuaq	KUUQ	23	Masseter/DNA	1
Labrador		**130**		
Labrador City	LABC	36	Masseter/DNA	13
North West River	NOWR	45	Hind leg muscle	0
Port Hope Simpson	POHS	29	Hind leg muscle	0
Cartwright	CART	20	Hind leg muscle	0
Other locations		**78**		
Island of Newfoundland	NFLD	14	Salivary gland	0
Churchill	CHUR	49	Data provided	0
Nunavut	NU	2	Data provided	1
Umiujaq, QC		1	DNA	1

Purvirnituq, QC	1	DNA	1
Raglan Mine, QC	3	Masseter	0
50 miles from Kuujjuaq	1	Masseter	0
Mistissini, QC	2	Masseter	0
Schefferville, QC	1	DNA	1
Makkovik, NL	1	DNA	1
Hebron, NL	1	DNA	1
Nain, NL	2	DNA	2
Total	**461**		**23**

Table 2.2. Primers for 15 microsatellite loci used in this study. Sequences are given in the 5'-3' orientation.

Locus	**Forward Primer Sequence**	**Fluorescent Dye Label**
Co4.140	Forward –*CAGAGGTGGCATAGGGTGAT*	6FAM
	Reverse –*TCGAAGCCCAGAGAATGACT*	
AHTh171	Forward – *AGGTGCAGAGCACTCACTCA*	VIC
	Reverse –*CCTCAAACCCAGGTGAAGC*	
REN105L03	Forward – *GGTGCCTGACAAGATGGAAT*	PET
	Reverse –*GAGATTGCTGCCCTTTTTACT*	
CPH3	Forward – *CAGGTTCAAATGATGTTTTAAG*	6FAM
	Reverse –*TTGACTGAAGGAGATGTGGTAA*	
AHT121	Forward – *TATTGCGAATGTCACTGCTT*	VIC
	Reverse –*ATAGATACACTCTCTCTCCG*	
REN247M23	Forward – *GACAACACCAAGGCTTTCC*	PET
	Reverse –*AATCCACTCTGGGGATTGAA*	
CPH9	Forward – *CAGAGACTGCCACTTTAAACACAC*	6FAM
	Reverse –*AAAGTTCTCAAATACCATTGTGTTACA*	
CPH15	Forward – *GCCTATATAAAATGCATCTGAGC*	VIC
	Reverse –*CCGTGACTCCTGTCTTCTGAC*	
Co1.424	Forward – *AGCCTAGCTTACTGCCCTGG*	PET
	Reverse –*TCCTTTGGTTTTTAGCAGGG*	
CXX109	Forward – *AACTTTAAGCCACACTTCTGCA*	VIC

	Reverse –*ACTTGCCTCTGGCTTTTAAGC*	
CXX250	Forward – *TTAGTTAACCCAGCTCCCCCA*	6FAM
	Reverse –*TCACCCTGTTAGCTGCTCAA*	
CXX172	Forward – *CCTGTCTCCTGTGGACCAAT*	NED
	Reverse –*ACATGCAAAAGGACACATTACG*	
CXX173	Forward – *ATCCAGGTCTGGAATACCCC*	6FAM
	Reverse –*CCTTTGAATTAGCAACTTGGC*	
CXX20	Forward – *AGCAACCCCTCCCATTTACT*	VIC
	Reverse –*TTGATCTGAATAGTCCTCTGCG*	
CXX377	Forward – *ACGTGTTGATGTACATTCCTG*	PET
	Reverse –*CCACCCAGTCACACAATCAG*	

Chapter 3- RESULTS

Fox Samples

DNA was extracted from 320 fox tissue samples for this study. Appendix A illustrates the NanoDrop spectrophotometry measurements of the concentration of DNA isolated from the tissue samples, as well as absorbance ratios at both 260/280 and 260/230. DNA quality is thus evaluated from these absorbance values (Thermo-Fisher Scientific, 2010). The average DNA concentration was 60.4 ng/µL, with a range from 0 to 820 ng/µL. The large variation in concentration possibly reflects different tissue sources, ages of samples, and storage. Three samples had concentrations of 0 ng/µL and were excluded from further study. With regards to results from the absorbance ratios, the average 260/280 ratio was 1.69 which is a little lower than the 1.8 expected for DNA, perhaps suggesting some protein contamination. The 260/230 ratio average of 0.95 is less than the given range of 1.8-2.2, again suggesting some possible contamination issues, for example carryover or reagents from the DNA extraction kit reagents. Nonetheless, successful microsatellite profiles were obtained from 285 of these samples, after excluding samples missing data at more than 1/3 of the loci.

In an effort to provide greater sample sizes, 9-locus profiles representing multiplexes A,C, and D for 111 samples were provided from a previous study (Nadin-Davis et al. in preparation, 2019) and the additional 5 loci from multiplexes E and F were added to make complete 15-locus genotypes for N=396 individuals across Eastern Subarctic locations, including 10 main samples with N > 10 (Table 2.1).As well 9-locus profiles from the previous study were available for a sample from the Churchill, Manitoba, and the Island of Newfoundland, enabling analysis of a broader geographical range (N=461; Table 2.1).

Locus characterization

Micro-Checker was used to ascertain if there were any null alleles, stuttering, or large allele dropout in any of the tenmain Eastern Subarctic sampling localities at any loci. Each sample (Table 2.1) was analyzed with Micro-Checker software with a repeat motif selection of dinucleotide for all 15 loci. Marker CPH9 had possible null alleles in both Montreal and Abitibi-Témiscamingue, while in the James Bay sample marker REN247M23 displayed evidence indicative of null alleles and stuttering. CXX250 also had possible null alleles present in theKuujjuarapik and Inukjuaksamples, as did CPH3 in Labrador City and Port Hope Simpson. Lastly, Kuujjuaq, North West River, and Cartwright showed no evidence for null alleles, stuttering or large allele drop out. Given that no locus consistently had null alleles across localities, and no localities had null alleles at more than one locus. Micro-Checker results have allowed for the inclusion of all 10 locations and all 15 microsatellite loci for analysis.

GenePop v4.2 was used to determine whether any pairs of loci were in significant linkage disequilibrium. Significance at P=0.05 was altered to be 0.000476 after the Bonferroni correction for multiple tests. This correction factor compensates for large numbers of comparisons by dividing the significance value by the number of comparisons involved, in this case, division by 15 microsatellites resulted in the new value of 0.000476 (Dunn, 1961).

There were a few pairs with significant P values (P<0.000476) as follows: Montreal and Abitibi -Témiscamingue had a linkage disequilibrium between loci CO4.140 and REN105L03 which occur in the same multiplex; Abitibi -Témiscamingue had two additional linkages at REN105L03/CPH9 and AHT121/CXX20 from varying multiplexes; North West River manifested linkages at CPH15/CXX173 and CXXX250/CXX20 from different multiplexes, and Cartwright showed linkage at AHT121/REN247M23 from the same multiplex and at

AHT121/ZXX250 from different multiplexes. Given the large number of comparisons and populations these 8 instances of linkage disequilibrium which are not consistent across loci or sampling localities were considered unlikely to cause errors in interpretation, and further analyses included all loci.

Table 3.1 display the characteristics of the 15 microsatellite loci used in this study. The allelic range was highest at CXX173 with 34 and lowest value was 12 in two loci AHTh171 and CPH9 and the average was 21.7. The number of alleles was highest in AHT121 and CPH15 at N_A=13 while the lowest value was N_A= 6 at the locusAHTh171 and the average was N_A=9.33. The expected heterozygosity was highest at AHT121 with $H_{E=}$ 0.823 and lowest at CXX173 with $H_{E=}$0.229 and the average was $H_{E=}$ 0.678. F_{IT} was positive for all but one locus and significant for four loci. The highest was at CPH3 at F_{IT} =0.190 and lowest at the locus CXX9 with negative value F_{IT} =-0.018 and the average was $F_{IT=}$0.079. Finally, F_{ST} was highest at CXX172 with F_{ST}=0.133 and lowest at AHT121 with F_{ST}=0.013and the average was F_{ST}=0.062. F_{ST} was significant for all loci. These results suggest sufficient diversity and differentiation in this set of loci to detect population structure.

Population diversity and F-statistics

Arlequin and FSTAT were used to calculate various population diversity statistics and F-statistics. Table 3.2 displays results for the 10 main populations quantifying the number of alleles (N_A), allelic richness (k), expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS}). This shows the highest number of alleles occur in the population of Montreal at N_A=7.47 while the lowest was present in Cartwright with N_A=4.33. The average of the number of alleles across all populations was observed to be N_A=5.70. The allelic richness was observed to be the highest in

James Bay population with a value of k=4.81 and the lowest was k=3.71 in Cartwright, while the average of allelic richness across all populations was k=4.33. James Bay had the highest expected heterozygosity at H_E=0.678; Cartwright had the lowest at H_E=0.587 and the average expected heterozygosity was observed at H_E=0.638. The inbreeding coefficient was not significant in any sampling locality, and were generally negative except for Labrador City and Cartwright, with an average of F_{IS}=-0.0430. Thus, there is no indication of inbreeding or undetected population structure in any sampling locality.

Population structure

Arlequin was next used to calculate pairwise measures of population differentiation (F_{ST} and R_{ST}) among the ten sampling localities. Table 3.3 gives these values.

With regards to the F_{ST} pairwise values, a cross-comparison of 10 populations was performed, wherein each of the 10 populations was compared against the remaining 9 andF_{ST}values were generated for each of the pairs. The significance level was adjusted to P=0.00111 to account for multiple tests. These results showed that Montreal was significantly different from all the populations with the highest value observed in comparison to Cartwright (F_{ST} = 0.151 and the lowest in comparison with Abitibi-Témiscamigue (F_{ST} =0.014). Abitibi-Témiscamigue was also significantly different from the other populations, with the highest value observed at F_{ST} =0.109 when compared to Cartwright and the lowest when compared to Montreal. James Bay was significantly different from Montreal, Abitibi-Témiscamigue, Kuujjuaq, Labrador City and Cartwright with the highest value at F_{ST} =0.046 in comparison to Cartwright and the lowest at F_{ST} = .0.003 in comparison with Inukjuak. Among the remaining sampling localities, only Cartwright showed significant difference from other localities, ranging

from F_{ST} = 0.018 compared with Labrador City to F_{ST} = 0.151 to Montreal. Cartwright is significantly different from all localities except Port Hope Simpson.

Overall, Montreal and Abitibi-Témiscamigue were exclusively significantly different when compared to all other populations in terms of F_{ST}. Montreal exhibited the highest level of genetic distance in comparison to the 9 remaining populations. Furthermore, Montreal had the overall highest F_{ST} value of any comparison when paired with Cartwright which resulted in a pairwise difference of F_{ST} =0.151.

While F_{ST} as calculated by Arlequin considers the number of different alleles, R_{ST} considers the sum of squared sized differences (Excoffier, 1995-2010). Due to accounting for the repeat motifs present in the F_{ST} it is postulated that the R_{ST} values are more accurate (Balloux and Lugon-Moulin, 2002). However, this may only hold when a strict stepwise mutation model is appropriate. Furthermore, due to greater variance R_{ST} is less reliable than F_{ST} and so not recommended unless larger than F_{ST} (Miermans and Hedrick, 2011). Here, the R_{ST} values are typically lower than the values of F_{ST} (Table 3.3) but show a similar pattern in that they are highest and more likely to be significant in comparisons with Montreal or Abitibi-Témiscamingue. The highest R_{ST} value was observed between Abitibi-Témiscamingue and Cartwright at R_{ST}=0.075 and the lowest value was observed between James Bay and Kuujjuarapik at R_{ST}=-0.020. Given the arguments above, the R_{ST} results were not explored further in this study.

To further explore population relatedness, a dendrogram was constructed from the F_{ST} matrix using the minimum evolution algorithm in MEGA. This is shown in Figure 3.1. Arlequin was then used to conduct hierarchical Analysis of Molecular Variance to explore differentiation associated with various groupings of sampling localities suggested by the dendrogram. First,

sampling localities were allocated into one of two groups associated with each branch of the dendrogram, and the results shown on the branches of the dendrogram (F_{CT} and P values; Figure 3.1). Thus, there are two main values on which the AMOVA and MEGA results are based. One is F_{CT} (P) value which is calculated by AMOVA on basis of number of localities in groupings according to molecular variance. And the second is the F_{ST} matrix according to which a dendrogram is made. In Figure 3.1, the dendrogram depicts the values of F_{CT}(P), calculated by AMOVA for groupings indicated by that branch. The dendrogram indicates that there are two basic groupings,which can be further subdivided. With F_{CT}=0.047 (P=0.01), Montreal and Abitibi-Témiscamingue foxes showed the highest distance from the other populations showcasing that they had very different alleles from their ancestors. The four Labrador sampling localities (Cartwright, Port Hope Simpson, North West River, and Labrador City) also form a cluster which is significantly diverged from the others. Intermediate in the dendrogram are the northern Quebec localities (James Bay, Kuujjuarapik, Inukjuak, and Kuujjuaq), each of which is successively significantly diverged from the others in a pattern associated with geographic distance.We can also see in the dendrogram the largest difference between two fox populations between Montreal and Cartwright. The dendrogram depicts that Cartwright and Port Hope Simpson are closely-related populations. Although Cartwright is characterized by a long branch compared to all other populations, the F_{CT} is not significant. Thus, Figure 3.1 also indicates that the localities which do not have such a huge difference from others is due to the fact that there might be a higher rate of migration of foxes between these localities which are closer to each other, and lower between distant localities of Cartwright and Montreal. This is consistent with an isolation-by-distance pattern of migration.

Second, different scenarios of K=3-7 groups were tested (Table 3.4). These results show that the highest F_{CT} (P) value is shown when Abitibi- Témiscamingue and Montreal are grouped together, Cartwright is its own group, and all other localities are grouped together, is F_{CT} = 0.082 (P=0.004) and the lowest F_{CT} (P) value is depicted by grouping Abitibi-Témiscamingue with Montreal, North West River, Port Hope Simpson, and Labrador City with each other, and treating each other locality as its own group (F_{CT} = 0.068, P=0.004). The rest of the group assignment schemes fall within the highest and lowest value. However, all values of F_{CT} are similar and significant regardless of differences in the grouping scheme, suggesting that most localities other than those within Labrador are significantly different from each other. This showcases the different sampling localities groupings of colored foxes in the eastern subarctic region of Canada and how the various localities group together.

Number of alleles, a measure of locus diversity, was highest in AHT121 and CPH15 and lowest for AHTh171, on average approximately 9 alleles per locus. Expected heterozygosity ranged from a low of H_E=0.229 to a high of H_E=0.823 for AHT121, with an average of H_E=0.678.

Most analyses supported a pattern of population differentiation. First, pairwise F_{ST} measures were significant and positive in most comparisons except between nearly localities within Quebec or Labrador (Table 3.3). The results of pairwise F_{ST} values yielded a striking distinction between the Montreal and Abitibi-Témiscamingue fox populations and all the other populations. Overall, Montreal exhibited the highest level of genetic distance when compared to the remaining populations. Furthermore, Montreal had the overall highest F_{ST} value in every comparison of Cartwright to other populations. These results are apparent in the dendrogram (Figure 3.1). The fox populations residing in the regions of James Bay, Kuujjuarapik, Inukjuak,

Kuujjuaq, Labrador City, North West River and Port Hope Simpson were more related genetically as was evident from their F_{ST} and R_{ST} values, but still many comparisons were significantly different. James Bay is intermediate in the dendrogram between Abitibi-Témiscamingueand Montreal and northern Labrador and Quebec. However, Montreal, Abitibi-Témiscamingue and Cartwright were genetically distant from each other and distinct to all or most of the other populations in the Eastern Arctic region of Canada in the order of Montreal>Abitibi-Témiscamingue> Cartwright. The AMOVA analysis supported this observation with the highest F_{CT} associated with grouping Cartwright, Abitibi-Témiscamingue and Montreal vs the other localities (|Table 3.4). However other grouping populations tested showed significant F_{CT} consistent with the James Bay sample being different from certain localities.

The primary STRUCTURE result among Quebec and Labrador localities (K=2; Figure 3.3A) reveals two groups, one containing the Montreal and most Abitibi-Témiscamingue individuals and the second containing most James Bay, northern Quebec, and Labrador individuals. James Bay is a mix of the two genetic groups. At K=3&4, many Abitibi-Témiscamingue foxes become part of their own distinct cluster which is intermediate between Montreal and north Quebec and Labrador. Also, James Bay is intermediate between Abitibi-Témiscamingue and north Quebec and Labrador this can be seen visually in Figure 3.3F. Two genetic groups seem to be segregating throughout northern Quebec, and Labrador with one increasing in frequency in an easterly fashion (Figure 3.3F). At a finer scale, analyses also suggest that the Cartwright sampling locality is distinct (STRUCTURE; Figure 3.5 and DAPC; Figure 3.8).

Next, STRUCTURE was used to identify the most likely number of genetically distinct groups (K) among individuals, without *a priori* assignment to sampling locality. This allowed inclusion of individuals collected from other sampling localities in addition to the ten main ones, many of which are the rabies-positive individuals. Three analyses were performed: 1. N=396 individuals from all Quebec and Labrador localities, 15-locus genotypes; 2. N=461 individuals from all localities including Churchill, Island of Newfoundland, and NU, 9-locus genotypes; 3. N=130 individuals from Labrador, 15-locus genotypes. The second analysis was done to help interpret the results of analysis 1, while the third was done to further explore whether there is any finer level structure within Labrador.

For the first analysis, K=1-8 was tested. K=2 was the best supported K by the plot of delta K vs. K (as suggested by Evanno et al. 2006) obtained from Structure Harvester (Figure 3.2A). The log likelihood plot (Figure 3.2. B.) shows that the ln (Probability of the Data) continues to increase at K=3 and K=4, levelling off at K=4. So, results for K=2, 3, and 4 are presented in Figure 3.3. The genetic structure among sampling localities is manifested at all levels of cluster assignment. K=2 (Figure 3.3A) shows Montreal and Abitibi-Témiscamingue in one result, James Bay as a mix of two groups, and the remaining sampling localities from northern Quebec and Labrador dominated by the second cluster.

At K=3 (Figure 3.3B), a third group splits off between 1 and 2 (Figure 3.3C); Montreal is still largely cluster 1, Abitibi-Témiscamingue is now mixed between 1 and 3, James Bay is mixed between 2 and 3, and the remaining Quebec and Labrador populations are still gathered at 2. At K=4 (Figure 3.3D), a fourth group branches off the second cluster (Figure 3.3E), and segregates throughout James Bay, northern Quebec, and Labrador, showing the greatest frequency in the easternmost coastal localities of Port Hope Simpson and Cartwright.The

proportions of each of the four clumps at each of the ten main sampling localities can be seen in Figure 3.3F.

According to figure 3.4, in K=3, total number of genetic groups are three: Montreal being dominated by the first group (red), Churchill is dominated by another (blue), Abitibi-Témiscamingueand James Bay are mixed between two, red and blue for Abitibi-Témiscamingue and blue and green for James Bay. Quebec and Labrador are still presented in the second (green) and third (blue) groups, primarily green. At K=2, Montreal is mostly red while Churchill, Labrador and Quebec arethe green cluster. Churchill is mixed but mostly green. Abitibi-Témiscamingue is mixed between the two. James Bay looks similar to Labrador and Quebec, and Labrador and Quebec are close together relative to Montreal.

Figure 3.5, with K=2 genetic clusters, shows membership of 15-locus genotypes from 130 Labrador foxes. The first includes the samples from Labrador City, Port Hope Simpson, North West River, while the second shows Cartwright is separated and different from the other populations. This isolation of Cartwright could be due to introduction of genetically distinct foxes in the region along with fur farming activity, followed by lack of movement of individuals from Cartwright into the neighboring populations.

Geneland was used to incorporate a spatial component to the analysis of genetic grouping of individuals. Two models were conducted, correlated and uncorrelated, both allowing for coordinate uncertainty. The correlated model analysis shown in Figure 3.6 described four groups, the first group containing Montreal individuals and some individuals from Abitibi-Témiscamnigueand central Quebec. The second group showed mostly Abitibi-Témiscamigue individuals. The third group contained James Bay individuals and some from Abitibi-Témiscamingue. The fourth group was observed in northern Quebec and Labrador localities.

The uncorrelated analysis identified two clusters of individals (Figure 3.7). The first group contained Montreal, Abitibi-Témiscamingue and some James Bay foxes, while the second group contained northern Quebec, Labrador localities and anumber of James Bay individuals.

With regards to DAPC, it identified three collections in Labrador but only Cartwright appears distinct (Figure 3.8).

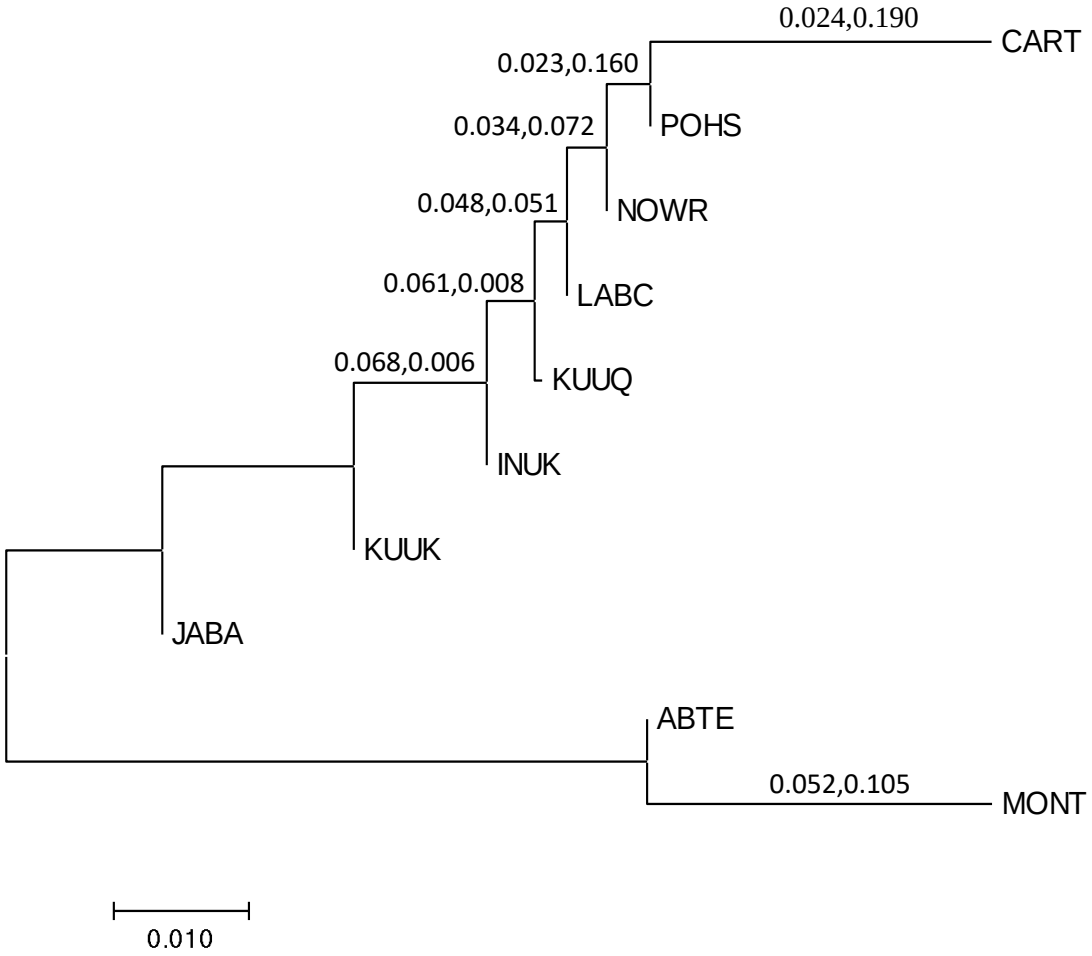
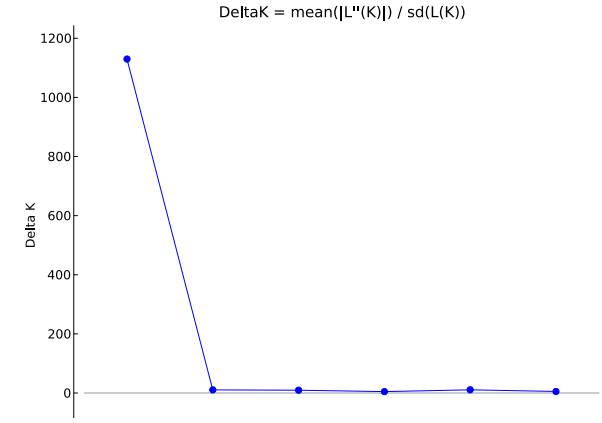


Figure 3.1 Minimum evolution dendrogram constructed from an F_{ST} matrix among sampling localities of red foxes. Locality codes are as given in Table 2.1. The bar indicates distance. Above the branches are the F_{CT} (P) values associated with splitting the sampling localities into two groups at that branch.

A.



B.

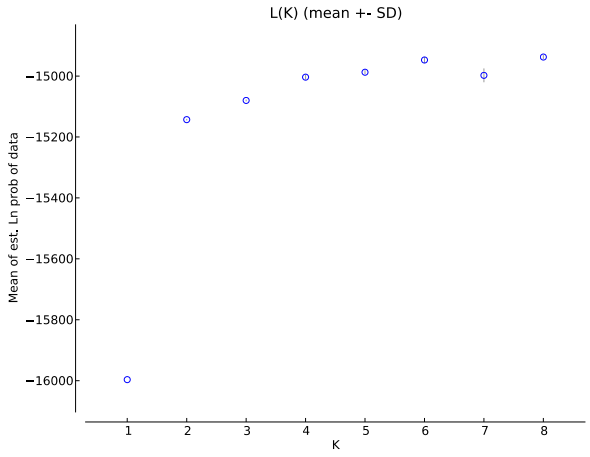
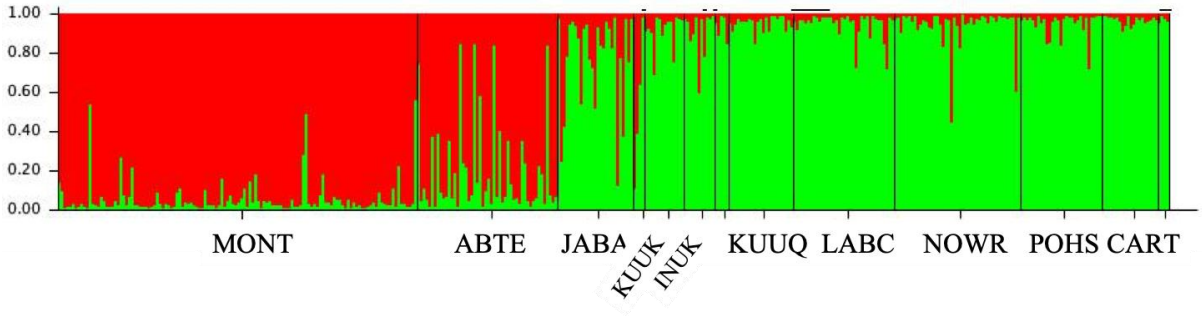


Figure 3.2 Output of Structure Harvester used to determine the most likely number of genetic masses among 396 individual red foxes from the eastern subarctic region of Canada. A. Plot of delta K at each K, as described by Evanno et al. (2006). B. Plot of ln (Probability of Data) at each K (log likelihood plot).

A.


1.00
0.80
0.60
0.40
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0.00
MONT
ABTE
JABA
KUUK
INUK
KUUQ
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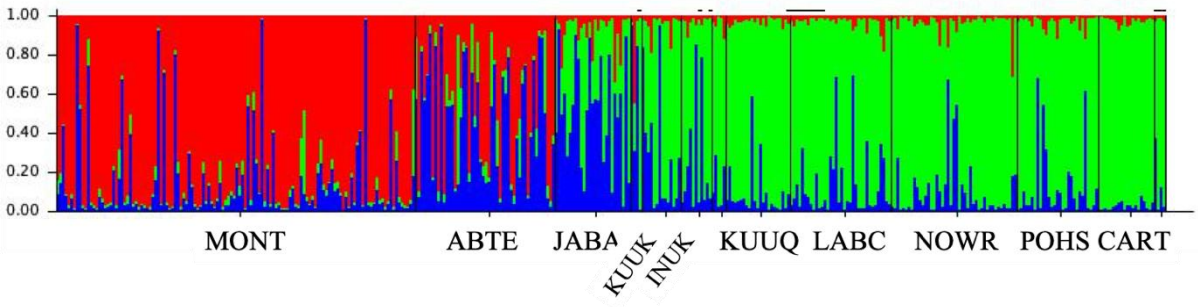
NOWR
POHS
CART

B.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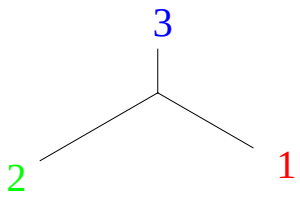
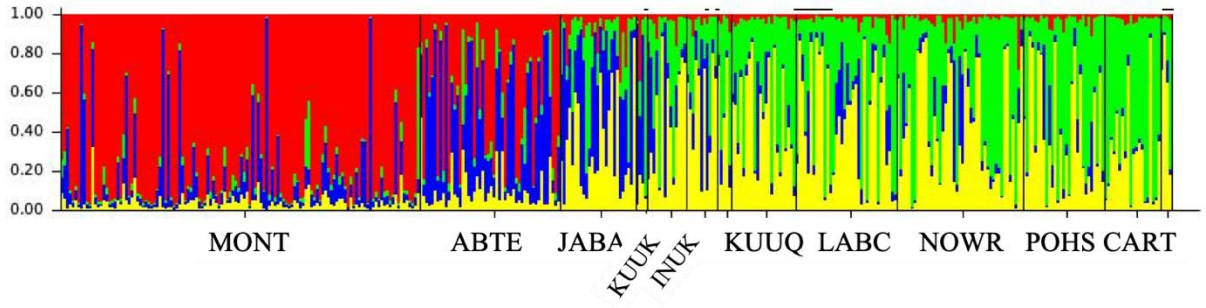
CART

C.


3
2
1

D.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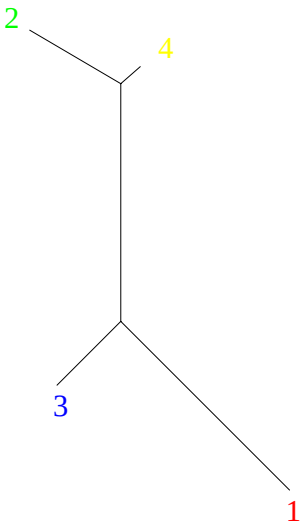
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4
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1

F.

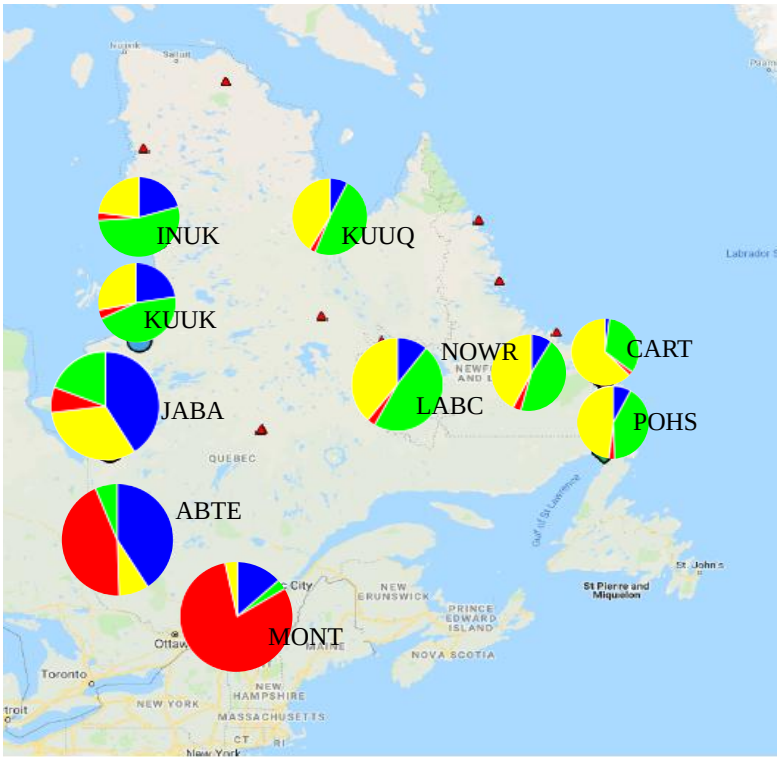

Figure 3.3 STRUCTURE plots showing individual membership of 15-locus genotypes from 396 red foxes to each of A. K=2 genetic clusters. B. K= 3 genetic clusters. D. K=4 genetic clusters. Locality codes show sampling locality, as given in Table 2.1. Bars above individuals indicate rabies-positive foxes. C. Tree plot of the relationships among the K=3 genetic populationfrom B. E. Tree plot of the relationships among the K=4 genetic clustersfrom D. F. Distribution of membership in each of K=4 groups in the ten main sampling localities.

A.

B.

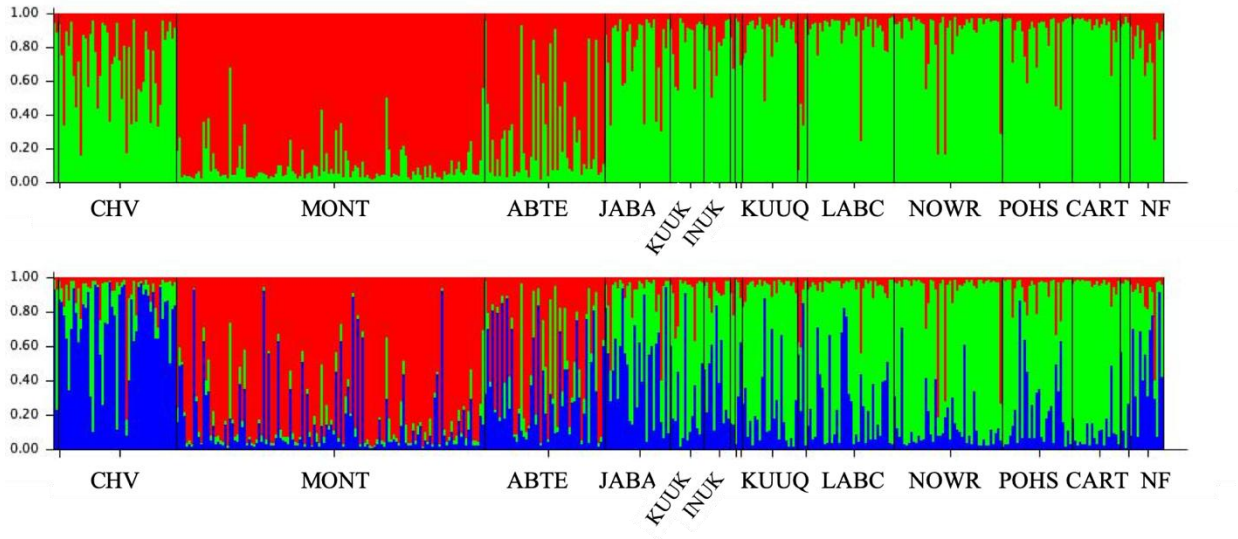

Figure 3.4. STRUCTURE plots showing individual membership of 9-locus genotypes from 461 red foxes from northern Canada to A. K=2 genetic clusters. B. K=3 genetic clusters.

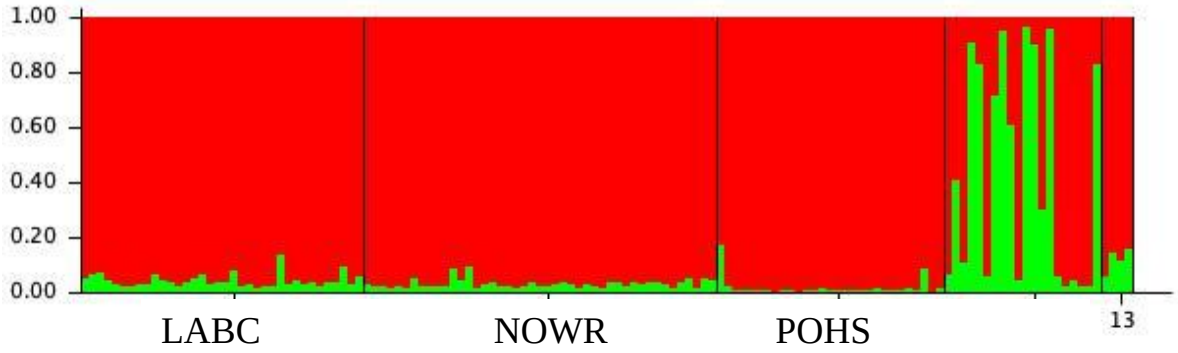

Figure 3.5. STRUCTURE plot showing individual membership of 15-locus genotypes from 130 Labrador red populations to K=2 genetic clusters.

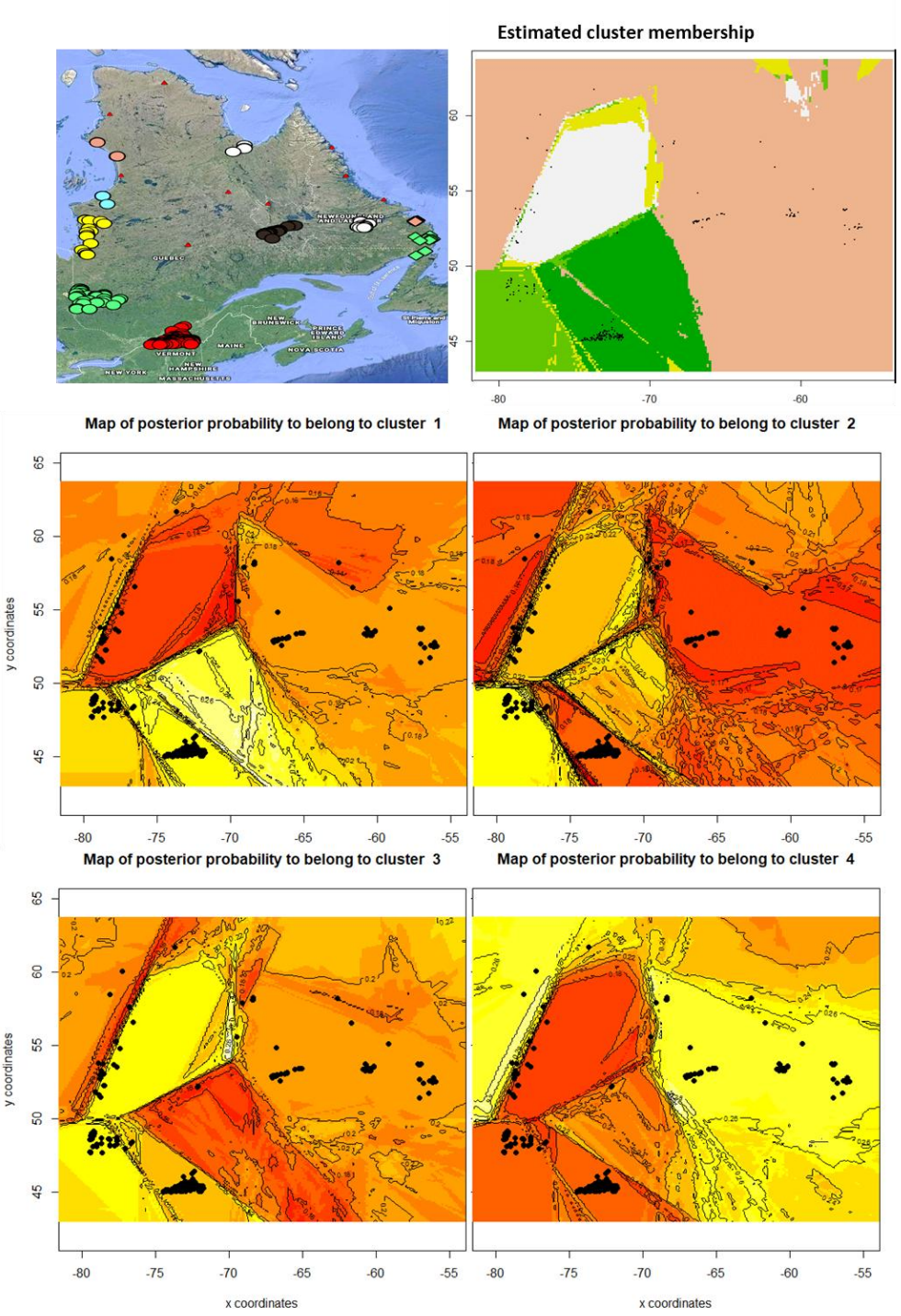
Estimated cluster membership
QUEBEC
NEW BRUNSWICK
PRINCE EDWARD ISLAND
NOVA SCOTIA
MAINE
VERMONT
NEW HAMPSHIRE
NEW YORK
MASSACHUSETTS
Map of posterior probability to belong to cluster 1
Map of posterior probability to belong to cluster 2
Map of posterior probability to belong to cluster 3
Map of posterior probability to belong to cluster 4
y coordinates
x coordinates

Figure 3.6. Spatial genetic analysis using the correlated model with coordinate uncertainty in Geneland. The top left panel shows a satellite map of sampling coordinates. The top right panel shows the estimated result of membership of individuals while the remaining four panels show the posterior probabilities of membership to each of the four members.

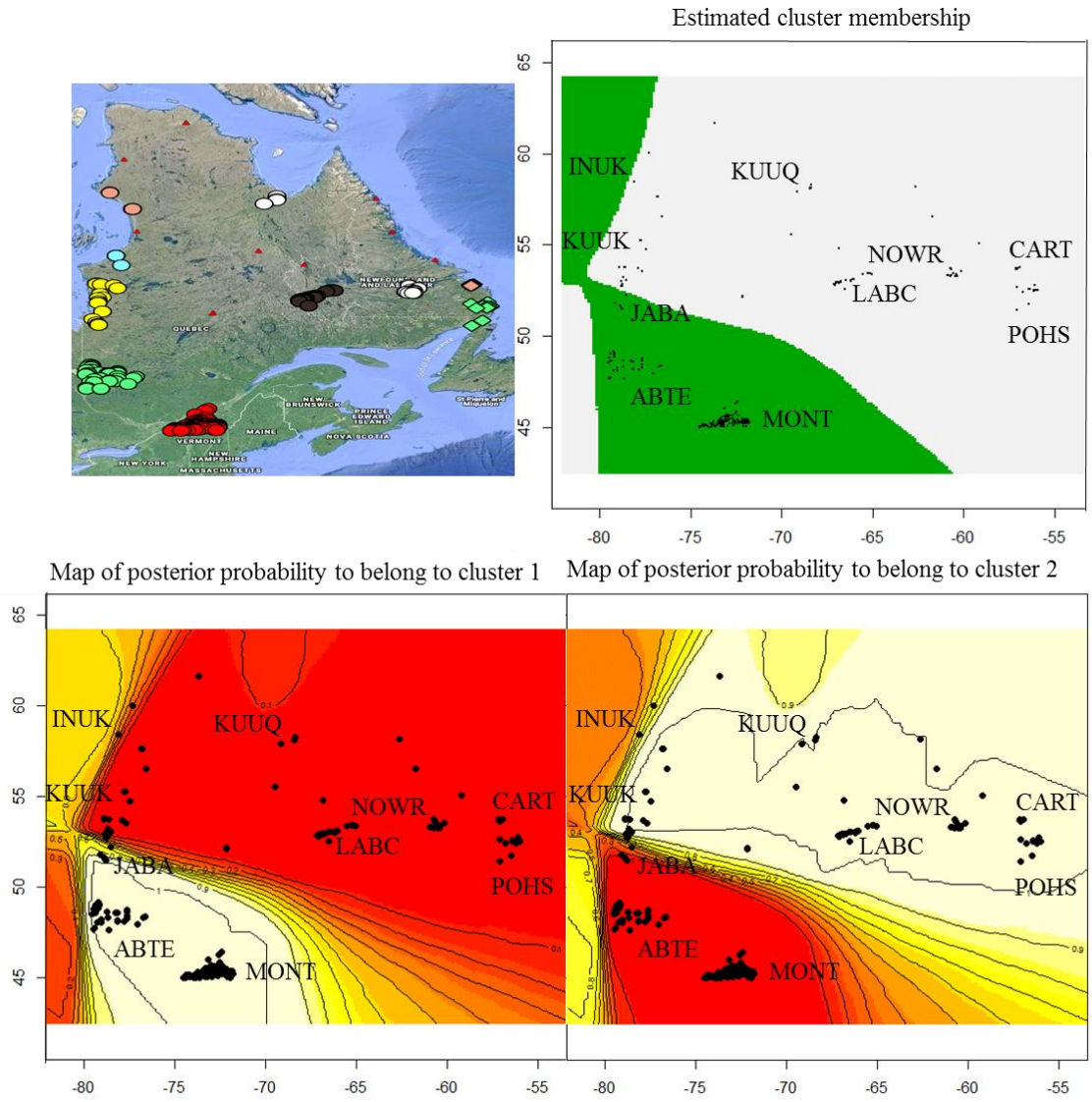

Figure 3.7. Spatial analysis using the uncorrelated model with coordinate uncertainty in Geneland. The top left panel shows a satellite map of sampling coordinates. The top right panel shows the estimated membership of individuals while the two panels show the posterior probabilities of membership to each of the two clusters.

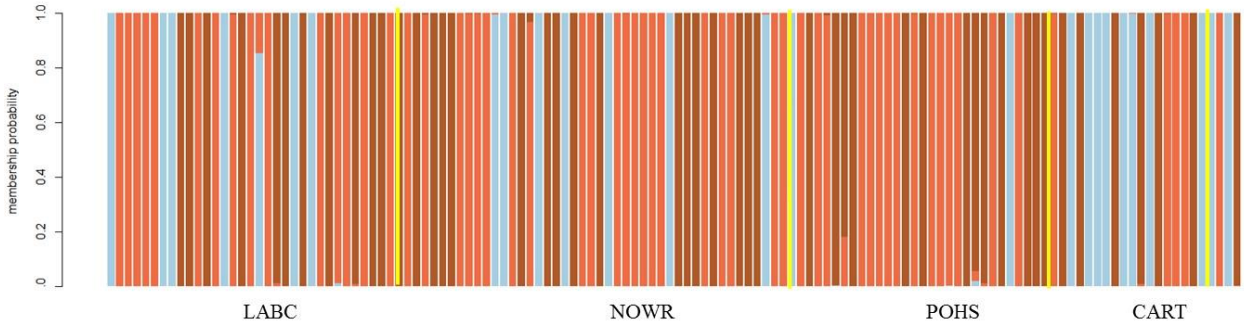

Figure 3.8. Compoplot plot of individual membership to each of three genetic collections identified by DAPC of 134 Labrador red foxes. Locality abbreviations are as given in Table 1.

Table 3.1 Per-locus diversity measures and *F*-statistics. For *F*-statistics, numbers in bold are significant at P=0.05 after Bonferroni correction for multiple tests. N_A = number of alleles; H_E = expected heterozygosity.

Locus	Allelic Range	N_A	H_E	F_{IT}	F_{ST}
CO4.140	24	11	0.771	0.082	**0.087**
AHTh171	12	6	0.484	0.078	**0.037**
REN105L03	20	10	0.816	0.028	**0.023**
CPH3	26	10	0.720	**0.190**	**0.073**
AHT121	28	13	0.823	0.014	**0.013**
REN247M23	18	9	0.741	**0.140**	**0.051**
CPH9	12	7	0.700	**0.150**	**0.064**
CPH15	26	13	0.791	0.046	**0.083**
CO1.424	22	10	0.620	0.109	**0.104**
CXX109	22	8	0.600	-0.018	**0.029**
CXX250	14	8	0.743	0.080	**0.028**
CXX172	24	7	0.678	0.106	**0.133**
CXX173	34	8	0.229	0.020	**0.021**
CXX20	18	10	0.805	0.021	**0.064**
CXX377	26	10	0.609	**0.137**	**0.118**
Mean	21.7	9.33	0.678	0.079	**0.062**

Table 3.2 Measures of diversity and F_{IS} for ten sampling localities of red foxes in the eastern subarctic region of Canada. N_A = number of alleles; k = allelic richness; H_E = expected heterozygosity. Sampling locality codes are given in Table 2.1.

Locality	N_A	K	H_E	F_{IS}
MONT	7.47	4.32	0.637	-0.105
ABTE	6.67	4.71	0.667	-0.030
JAMA	6.27	4.81	0.678	-0.058
KUUK	4.93	4.30	0.650	-0.067
INUQ	4.87	4.57	0.661	-0.092
KUUQ	5.00	4.00	0.608	-0.049
LABC	6.07	4.52	0.648	0.0290
NOWR	5.80	4.31	0.635	-0.007
POHS	5.60	4.08	0.610	-0.057
CART	4.33	3.71	0.587	0.0090
Mean	5.70	4.33	0.638	-0.0430

Table 3.3 Microsatellite differentiation among ten localities of red foxes in the eastern subarctic region of Canada. F_{ST} values are below the diagonal and R_{ST} values are above the diagonal. Measures in bold are significant at P=0.05 after Bonferroni correction for multiple tests (adjusted P=0.00111). Locality abbreviations are as given in Table 2.1.

	MONT	ABTE	JABA	KUUK	INUK	KUUQ	LABC	NOWR	POHS	CART
MONT	-	0.011	**0.022**	**0.029**	0.046	**0.043**	**0.054**	**0.060**	**0.056**	**0.073**
ABTE	**0.014**	-	0.011	0.006	0.019	**0.027**	**0.032**	**0.052**	**0.033**	**0.071**
JABA	**0.069**	**0.036**	-	-0.018	0.039	0.027	0.021	0.023	0.005	0.035
KUUK	**0.089**	**0.056**	0.006	-	0.018	0.002	0.011	0.010	-0.004	0.034
INUK	**0.112**	**0.071**	0.003	-0.006	-	-0.002	-0.012	0.025	0.010	0.064
KUUQ	**0.111**	**0.074**	**0.024**	0.006	0	-	-0.010	-0.006	-0.003	**0.031**
LABC	**0.107**	**0.068**	**0.013**	0.006	-0.010	-0.004	-	0.005	-0.004	0.025
NOWR	**0.112**	**0.079**	0.011	0.007	-0.002	-0.005	-0.002	-	0.006	0.017
POHS	**0.122**	**0.083**	0.011	0.021	0.003	0.011	0	0.002	-	0.030
CART	**0.151**	**0.109**	**0.046**	**0.053**	**0.040**	**0.030**	**0.018**	**0.019**	0.024	-

Table 3.4 Analysis of Molecular Variance (AMOVA) of different sampling localities groupings of red foxes in the eastern subarctic region of Canada. F_{CT} gives the proportion of molecular variance attributable to differences among groups. K is the number of groups. Locality codes are as given in Table 2.1.

K	Grouping	F_{CT} (P)
3	(ABTE,MONT)(CART)(Other Localities)	0.082 (0.004)
3	(MONT)(ABTE)(Other Localities)	0.072 (0.022)
3	(ABTE,MONT)(JABA)(Other Localities)	0.077 (0.007)
4	(ABTE,MONT)(JABA)(CART)(Other Localities)	0.077 (0.001)
4	(ABTE,MONT)(JABA)(KUUK)(Other Localities)	0.074 (0.004)
5	(ABTE,MONT)(JABA)(KUUK)(CART)(Other Localities)	0.075 (0.001)
5	(ABTE,MONT)(JABA)(KUUK)(INUK)(Other Localities)	0.071 (0.004)
6	(MONT)(ABTE)(JABA)(KUUK)(CART)(Other Localities)	0.071 (0.008)
6	(ABTE,MONT)(JABA)(KUUK)(INUK)(CART)(Other Localities)	0.072 (0)
7	(ABTE,MONT)(JABA)(KUUK)(INUK)(KUUQ)(CART)(Others)	0.068 (0.004)

Chapter 4-Discussion

Maintenance and reservoir host species play important roles in transfer of virus from one place to other. If a host is identified it is very important to see the comparative population structure in order to interpret how far and from where the infections can be transferred, so as to identify causes of disease spread and provide information to help prevention programs. The red and arctic foxes have been positively identified as the preferred host to the arctic strain of the rabies virus. The aims of this study were to assess the phylogeography and distribution of the rabies virus host the red fox in the Eastern Subarctic area of Canada in comparison to the viral strain distribution. More specifically, I wanted to describe detailed 15-locus phylogeography of red foxes in ten sampling locations in the region and use it to help understand rabies virus spread and distribution. Population genetic structure of red foxes in Montreal, Abitibi-Témiscamingue, James Bay, Kuujjuarapik, Inukjuak, Kuujjuaq, Labrador City, North West River, Port Hope Simpson, and Cartwright were investigated in this study. The outcome of the phylogeographic assessment can then be correlated with the distribution of the rabies viral strain since it was assumed that migration or transport of viral strains was in fact host dependent.

The main finding in this study is that the 15-locus genotypes revealed substantial genetic heterogeneity across the region investigated. Primarily, interpretationof the results clearly demonstrates that there is a distinction between Montreal and Abitibi-Témiscamingue in the southwest compared with northern Quebec and Labrador in the northeast. James Bay area seems to be a mixture of genetic groups, as does Abitibi-Témiscamingue to a lesser extent. Despite positive and significant pairwise differentiation among many localities within the northern Quebec and Labrador population, only Cartwright seems to be substantially different. Thisphylogeographysuggests possible barriers of gene flow and isolation by distance. Below I

will review characteristics of the loci and population samples studied here, then discuss the phylogeographic structure of the coloured fox across the region, and implications of this structure for understanding rabies virus spread.

Characteristics of loci: null alleles, linkage disequilibrium, and diversity

Nine of the markers or loci used in this study are the same as markers used by Goldsmith et al. (2016) for identification of rabies virus in hosts in Alaska. With the addition of 6 more loci here, these microsatellite markers provide plentiful variability for investigating population structure across the Eastern Subarctic region of Canada. The methodology used is similar to the Côté et al. (2012) methodology, in which they also identified the migration routes and population structure by using software and statistics such as F_{ST} to study rabies virus in raccoons.

In this study, we observed a few microsatellite loci with null alleles in several populations. Null alleles occur due to mutation in the primer sites of targeted DNA sequences which prevents efficient annealing and results in failure of the amplification during the PCR procedure (Rico et al 2017). Also, they can occur in homozygote state in which case the samples do not produce any amplification at all (Rico et al 2017). Montreal and Abitibi-Témiscamingueshowed evidence of null alleles at locus of CPH9. In addition to Montreal and Abitibi-Témiscamingue, James Bayalso showed null alleles and stuttering at locus REN247M23. We also observed that the samples fromKuujjuarapikand Inukjuakhad null alleles at locus CXX250 and those from Labrador City and Port Hope Simpson had null alleles at locus CPH3. Because we did not observe many loci with null alleles across most samples, this issue was not further considered in investigation of population structure. Four loci did show overall departures from Hardy-Weinberg expectations (Table 3.1) including three of those with null alleles, but

again this was not consistent across populations, nor did any populations show overall departures from Hardy-Weinber expectations across loci. Goldsmith et al. (2016) observed null alleles with REN247M23 in arctic foxes but not red foxes.

The results generated withGenePop showedsignificant linkage disequilibrium (LD) detected betweencertain locus pairs in particular geographic locations such Montreal, Abitibi-Témiscamingue, North West River, and Cartwrightinvolving loci from the same or different multiplexes but instances of LD were not substantially consistent either across loci or across samples and were not a high proportion of all comparisons. Goldsmith et al. (2016) observed 5 comparisons of 36 to be inLD in their study, but none significant after correction for multiple comparisons.

Allelic range, which describes the difference in size between the largest and smallest alleles at a locus in the sample, was found to be highest for CXX173 marker and lowest for AHTh171 and CPH9 markers. These levels of heterozygosity are a little lower than those revealed in Côté et al. (2012) who reported a range of H_E=0.65 to H_E=0.91 in different samples across 10 loci. Given that 15 loci were used in my study with comparable levels of genetic diversity to other studies, this indicates a variable system within which to investigate patterns of genetic diversity and differentiation.

Population diversity

It was discovered that the fox population in Montreal carried the greatest number of alleles whereas the Cartwright carried the least compared to the average number of alleles found in all the populations of N_A=5.7. Number of alleles for a gene in a population is an indication of its heterogeneity and the interbreeding which would have caused numerous alleles to appear.

However, we cannot ignore the large sample size that was collected from Montreal, which would also explain the increase in the number of alleles carried by the Montreal samples. The allelic richness and expected heterozygosity were found to be highest in the sample of foxes from the James Bayand the Abitibi-Témiscamingue regions, consistent with the mixture of genetic clusters that occurs in these two regions. F-statistics were applied to calculate the expected level of heterozygosity compared with that of Hardy-Weinberg equilibrium. A negative value for inbreeding suggests that the fox population in the chosen regions were a result of interbreeding between different populations; however, the values observed here were not significant. The lowest diversity was observed in the location of Cartwright, which as discussed earlier, may be influenced by past fur farming activity in that location.

Population structure and phylogeography of the red fox in the Eastern Subarctic region of Canada

From the pairwise analysis, it was evident that more distant localities are more differentiated. To add spatial context, Geneland analysis was performed and correlated and uncorrelated frequency models were tested. According to Geneland manual, the correlated model is more realistic and more powerful but more sensitive to violation of model assumption such as isolation by distance, so both results are presented here. The uncorrelated model shows a clear result where the southern localities of Montreal and Abitibi-Témiscamingue are distinct from the northeastern localities (Figure 3.7), with an admixture zone containing James Bay and Inukjuak. The correlated model as expected revealed a larger number of populations (Figure 3.6). Montreal has own population, also most Abitibi-Témiscamingue individuals have their own, a third cluster contains some James Bay and coastal northern Quebec individuals and a fourthcontains other

James Bay and northern Quebec and Labrador individuals. James Bay appears to be admixture zone between two areas.

To farther understand population structure and phylogeography in northern Quebec and Labrador an additional STRUCTURE analysis was done (Figure 3.4) that included samples from Churchill Manitoba and the Island of Newfoundland. At K=2, two clusters were observed, one containing Montreal and part of Abitibi-Témiscamingue and the second containing James Bay, northern Quebec, Labrador localities and Churchill. At K=3, Churchill individuals from Montreal but some individuals from Churchill, Abitibi is a bit mixture between Churchill and Montreal, northern Quebec and Labrador localities are separated with some input from Churchill especially in the western localities and James Bay and Newfoundland are mixed between Churchill and the northern Labrador and Quebec masses.

Putting all the analyses performed together, the picture that emerges is of a northwestern cluster (Churchill), a related northeast group (north Quebec and north Labrador) and a southern group (Montreal) with a mixture between northwestern and northeaster clusters at James Bay and mixture between the northwest and southern at Abitibi-Témiscamingue. Newfoundland is also a mixture between the northereastern and the northwestern clusters. How widespread the northwestern genetic cluster is in other parts of Canada is unknown, but it clearly extends into Abitibi-Témiscamingue, the Island of Newfoundland, the northeast coast of Labrador and the eastern coast of Hudson Bay including James Bay. Similarly, the northeastern group has spread to the Island of Newfoundland and the eastern coast of Hudson Bay including James Bay but not further south into Montreal or Abitibi. Finally, many analyses done here also suggest the coastal location of Cartwright is also distinct.

The phylogeography observed from the microsatellite data suggests patterns of movement of the red fox in the region investigated here. Foxes seem to be able migrate along the coast of Hudson Bay and the northeast coast as well as into the interior from both directions. The coastal route may allow relatively easy movement of foxes as well as good sources of food such as fish. However, there are also barriers to movement or movement is limited by distance. Red foxes known to be sedentary with home range 16 km^2 in the tundra for example (Goldsmith et al 2016; Jones & Theberge 1982). Montreal maybe distinct due to urbanization of the red fox in the area or foxes remaining sedentary there due to resources or habitat associated with the St. Lawrence River system, or Montreal may be part of a larger southern cluster that has not been sampled here. Whether there are any geographical barriers to movement such as lack of resources across the landscape remains to be investigated in more detail.

Within Labrador only Cartwright was differentiated from other locations. It is worth noting that the sample of foxes from this locality was dominated by cross or silver foxes trapped on small islands in the bay. It is possible that farming activity conducted in the region in the 1910s, may have influenced the genetic structure here. Alternatively, the genetic distinctiveness of this locality may be due to its isolation on small islands within the bay.

Comparison between mitochondrial DNA analysis and current study

Aubry et al. (2009) used analysis of archaeological data and mitochondrial DNA (mtDNA) to interpret the red foxphylogeography in North America. Unlike the work being done here, they sequenced the mtDNA, specifically cytochrome b and control region genes, from fossil specimens, and used phylogenies to infer the possible migration of foxes between regions. They found using Spatial Analysis of Molecular Variance (SAMOVA) that there were three

groups of foxes throughout North America with some differentiation between the west and the east due to the different frequencies of an Eastern subclade and a Widespread subclade that diverged from a common ancestor more than 40,000 years ago. They attributed this to migration and differentiation of southern refugial foxes during the Wisconsin glaciation. The region of study investigated here was partially represented in their study, in that some of their samples were Quebec foxes, although here I include sampling further east and north and include the Island of Newfoundland as well. As in their study the northwestern locality of Churchill was differentiated from the eastern localities with microsatellite. The microsatellite data and greater intensity of sampling here show the existence of finer phylogeographic structure than was detectable using mtDNA, which often reflects older events. As mentioned by Hanke et al. (2016) however mtDNA and microsatellites should recover consistent population structures due to mating and dispersal patterns shown by foxes. Control region sequencing of some of the foxes in my study was performed in Nadin-Davis et al. (in preparation) and was broadly consistent with the study of Aubry et al. (2009) in that foxes in the Eastern Subarctic were mostly of the Eastern subclade of Aubry's study, with a few from the Widespread subclade and the Holarctic clade.

Implications of host population structure and phylogeography for rabies virus movement

The study of raccoon host-virus interaction has been a prominent part of the literature for some years, in particular with raccoons. Côté et al. (2012) claimed significant results which are closely related to my findings; they worked to identify rabies virus in raccoons and claimed that according to their findings raccoons are found near residential areas and are the basic cause of zoonotic infections when they interact with other animals or even humans. Quebec has been the

subject of this where the Richelieu River was investigated as a possible barrier to gene flow. Similarly, a sexual activity period in winter also cause spreading of this virus from parents to offspring. The identification of host is thus really important in order to help stop migration and eventually infections. In another study, Kyle et al. (2014) examined raccoon populations and found out that 85% of population was infected to a strain of raccoon rabies. Among populations, higher genetic diversity of major histocompatibility complex loci was linked to temporal exposure by rabies virus but not to susceptibility. They also reported differences in this diversity and microsatellite population structure which suggested that local adaptation of host populations has occurred.

Foxes as a host of rabies have also been studied. Previous studies such as Carmichael et al. (2007) and Noren et al. (2011) showed little evidence of population structure in arctic foxes based on microsatellites, and consistent with long distances movements they are capable of. The findings of Goldsmith et al. (2016)suggested that the arctic foxes are the main carrier of rabies virus. They worked to differentiate between the population of foxes which acts as host and reservoir. Similar to my procedure they also took nine microsatellites to determine the population structure between two species. Microsatellite analyses gave varying results. Bayesian clustering found two groups ofarctic foxes in the coastal tundra region, but for red foxes it identified tundra and boreal types. In their findings, they noted that arctic foxes showed 3-4 genetic clsuters, whereas red foxes showed 8-9 groups. They concluded that arctic foxes are the primary maintenance species for the rabies virus and the role of the redfox is less clear. Hanke et al. (2016) also compared host genetics patterns with virus type distribution of arctic foxes in Greenland using mitochondrial DNA sequences, and found evidence for three mtDNA clusters with different geographic locations. They concluded that there is a fine-scale spatial structure of

arctic foxes but no evidence of independent strains of rabies virus having evolved in them (Hanke et al. 2016).

In my study the observed phylogeographic structure suggested possible barriers to gene flow as well as isolation-by-distance within the northeastern group of foxes that could influence the rabies virus distribution.It is notable that rabies-positive foxes as indicated in Figure 3.3 A, B, and D, associate with the genetic cluster of their region. Furthermore, the first, third, and fourth foxes in Figure 3.4A and B are also rabies positive – the first is a fox from Nunavut, and the third and fourth were from Churchill. These foxes also associate with the blue Churchill or northwestern cluster. This pattern suggests that red foxes are probably infected by arctic foxes *in situ*. However, the existence of a northeastern cluster with some evidence of isolation-by-distance does suggest that, although it is not considered the main host, theredfox movements, while more limited than the arctic fox, could be important for moving the rabies virus from the north to the south along the coast of Labrador. This is consistent with the rabies outbreaks observed in recent years which tend to spread along the coast (Nadin-Davis et al., in preparation) and also suggests adifferent role for the red fox in regard to the rabies virus than was discussed by Goldsmith et al. (2016) in Alaska. Admixture in the James Bay area along with some input from the northwestern cluster in the northern Quebec and Labrador region indicate that there could also be some movement of the virus from further northwest. The differentiation with respect to Montreal however indicates that the coloured fox is not moving the virus further into the southern interior.

Other studies of relevance with regard to fox host movement patterns and the spread of rabies include those looking specifically at short distance movements. According to findings of Adkins and Stott (1998), for example, radio tracking of fox migration among locations in

suburban Toronto, Ontario, showed evidence that redfoxes migrate more at night and about 2-20 km movement is usual, and they move towards inhabited areas where they can easily spread rabies to animals and humans.

Distribution of rabies virus strains compared to host population structure

According to Nadin-Davis et al. (2006), in the Ontario region of Canada, four rabies variants called ONT.T1 to ONT.T4 were first identified. The Quebec region outbreak was due to the incursion of ARC.T5 variant, which is the arctic fox rabies variant that migrated to Quebec. More recent nomenclature and updated results show that most rabies variants circulating in the Eastern Subarctic region are closely-related and recently-evolved phylogenetically and are now designated subtypes 17 and 18 of the A3 serotype (Nadin-Davis et al. in preparation). Both subtypes are found throughout the region in consideration here, with subtype 18 being more prevalent on the coast of Labrador although also seen in Labrador City and further north in Quebec. An older subtype, subtype 2, is also found on the northeast coast of Hudson Bay in northern Quebec.

Given the relative homogeneity of the northeastern cluster of foxes and the lack of structure of virus strains in the region, it is difficult to do more than conjecture any further for more specific role for the red fox with regard to specific transmission into these areas. Within the broader region of northern Canada, subtypes 17 and 18, as well as older subtypes 2 and 8, have been observed in the period of 2010-2018. Subtype 17 in particular seems to dominate the Eastern Subarctic in these years, suggesting perhaps transmission of a virus from an arctic fox in the north to one or a few red foxes, which then carried it through the region. Interestingly, LABC

was noted for having a mixture of subtypes present perhaps suggesting an attraction to this region of a greater number of foxes of both species.

Conclusions

This study provides an improvement in our knowledge with the findings associated with spreading of rabies virus through foxes in different localities of Canada. Possible migration routes of coloured foxes were indicated by these results. That migration could possibly be due to lack or depletion of food and shelter supplies in certain areas. The phylogeographic analyses exhibited strong dominance of a particular genetic populationof foxes in Montreal perhaps suggesting genetic isolation of this population due to high level human inhabitation of the area. It was observed that Quebec and Labrador showed easterly increasing frequency of a northeastern genetic cluster. Due to the location of Abitibi-Témiscamingue near Montreal, it exhibited genetic mixture of different genetic clusters of foxes, while James Bay, located within Hudson between Abitibi-Témiscamingue and further north appeared to be mixed between the northeast cluster and a northwest genetic group associated with Churchill, Manitoba. The distinctiveness of the Cartwright locality may be due to past farming activity in the area. The observed phylogeographic structure suggested possible barriers to gene flow and isolation by distance between the south and north and that can influence the virus strains distribution such as the subtypes 17 and 18 of A3 serotype in the Eastern Subarctic regions. This study provides a stage for the analysis of surveillance data that might be applicable to other zoonotic infections and diseases and illustrates better the ecological association of red foxes with the environment, their dispersal patterns and genetic structure, in order to help reduce the chances of infections in wildlife.

Future directions

The current work can be expanded by including more detailed genetic profiles for the foxes in this study, such as could be obtained by genotype-by-sequencing or to use next generation sequencing approaches which include many methods, such as RAD sequencing, to provide panels of single nucleotide variants. Nonetheless, the current data are informative and will help inform theoretical models of rabies movement with different scenarios of climate change by members of the ArcticNet One Health group, with which this project was associated.

In other rabies work it has been suggested that a controlled environment is required for successful experimentation to avoid unnecessary migration during examination. As explained by Carmichael et al. (2007), isolation of a virally-infected population area by a river increased the effectiveness of the results. Similarly, isolation of a fox population is necessary could help further evaluate behavioral change. Moreover, each progeny of foxes must be vaccinated and monitored in an isolated space to determine if the rabies sustain. After successful trials and efforts, rabies can be reduced by increasing vaccination effort and increasing technological analysis for a large population to estimate affect. According to the research done by Charles et al. (1987), production of monoclonal antibodies by true cell lines of each variant of rabies virus can be effective for studying epidemiology.

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Appendix A. Sample identification (ID), concentration, and absorbance ratios at 260/280 and 260/230 for the DNA extracted from fox tissues in this study.

Sample ID	Concentration(ng/µL)	260/280	260/230
TH001	65.0	1.78	0.84
TH002	65.5	1.78	0.76
TH003	46.9	1.77	0.63
TH004	182.9	1.83	0.34
TH005	186.8	1.74	0.55
TH006	42.2	1.70	0.42
TH007	58.8	1.55	0.27
TH008	69.2	1.86	0.54
TH009	89.1	1.77	0.83
TH010	7.4	1.38	0.44
TH011	100.5	1.80	0.23
TH012	38.4	1.71	0.58
TH013	34.7	1.85	0.80
TH014	28.6	1.76	0.55
TH015	26.2	1.71	0.42
TH016	22.5	1.77	0.55
TH017	73.2	1.55	0.29
TH018	43.3	1.68	0.33
TH019	264.8	1.52	0.42
TH020	55.8	1.57	0.27
TH021	41.7	1.77	0.51
TH022	32.4	1.90	0.56
TH023	35.2	1.46	0.43
TH024	62.6	1.87	1.41
TH025	21.0	1.69	0.36
TH026	37.6	1.88	1.15
TH027	22.8	1.64	0.33
TH028	47.6	1.63	0.39
TH029	116.3	1.46	0.25
TH030	46.8	1.61	0.27
TH031	190.0	1.39	0.35
TH032	122.8	1.40	0.28
TH033	54.9	1.64	0.27
TH034	11.9	1.75	0.31
TH035	51.5	1.60	0.28
TH036	86.2	1.55	0.48
TH037	39.8	1.80	0.25
TH038	92.2	1.46	0.29
TH039	117.6	1.43	0.45
TH040	60.2	1.44	0.28

TH041	10.2	1.87	0.33
TH042	14.5	1.68	0.39
TH043	7.9	1.37	0.67
TH044	45.5	1.59	0.25
TH045	105.3	1.58	0.27
TH046	16.2	1.68	0.21
TH047	20.3	1.97	0.33
TH048	7.4	1.71	0.20
TH049	75.0	1.61	0.34
TH050	8.2	1.96	0.43
TH051	21.0	2.12	0.76
TH052	12.5	1.57	0.25
TH053	13.8	2.14	0.62
TH054	8.0	2.32	0.40
TH055	18.0	1.69	0.35
TH056	9.5	1.80	0.26
TH057	6.6	1.85	0.18
TH058	50.1	1.43	0.21
TH059	10.2	2.03	0.54
TH060	12.4	2.02	0.54
TH061	47.1	1.52	0.24
TH062	12.7	1.65	0.44
TH063	19.5	1.79	0.73
TH064	15.3	1.92	0.39
TH065	24.0	1.69	0.47
TH066	8.9	1.77	0.23
TH067	21.5	1.53	0.24
TH068	14.0	1.73	0.25
TH069	12.4	1.77	0.26
TH070	11.5	1.76	0.47
TH071	65.7	1.56	0.25
TH072	11.5	2.08	0.59
TH073	10.7	1.95	0.44
TH074	22.0	1.99	0.84
TH075	31.0	2.04	1.50
TH076	704	1.72	0.31
TH077	59.9	1.56	0.27
TH078	26.1	1.75	0.43
TH079	12.8	1.71	0.29
TH080	18.2	1.89	0.48
TH081	100.3	1.46	0.27
TH082	25.4	1.54	0.27
TH083	43.3	1.65	0.25
TH084	47.6	1.70	0.39
TH085	17.5	1.57	0.36

TH086	31.3	1.62	0.42
TH087	16.1	1.90	0.40
TH088	20.3	1.60	0.22
TH089	28.5	1.07	0.39
TH090	92.0	1.58	0.26
TH091	319.7	1.44	0.59
TH092	53.4	1.58	0.29
TH093	9.9	2.01	0.46
TH094	11.8	1.71	0.24
TH095	39.2	1.66	0.22
TH096	22.2	1.48	0.29
TH097	38.5	1.61	0.27
TH098	10.8	1.40	0.20
TH099	17.1	1.67	0.25
TH100	35.8	1.55	0.36
TH101	61.6	1.51	0.30
TH102	15.1	1.42	0.34
TH103	9.9	1.90	0.35
TH104	164.2	1.51	0.56
TH105	65.7	1.55	0.24
TH106	10.6	1.69	0.28
TH107	39.8	1.57	0.22
TH108	7.0	1.46	0.21
TH109	11.9	1.75	0.47
TH110	9.2	1.96	0.41
TH111	14.0	1.59	0.22
TH112	14.6	1.63	0.47
TH113	17.5	1.54	0.33
TH114	15.8	1.73	0.56
TH115	10.9	1.65	0.35
TH116	48.1	1.59	0.25
TH117	8.5	1.80	0.38
TH118	56.6	1.52	0.30
TH119	20.0	1.62	0.22
TH120	43.4	1.56	0.83
TH121	41.6	1.51	0.58
TH122	50.5	1.44	0.43
TH123	65.4	1.13	0.58
TH124	20.4	1.46	0.63
TH125	78.6	1.11	6.56
TH126	25.2	1.38	0.44
TH127	118.5	1.38	0.35
TH128	28.9	1.41	0.45
TH129	33.8	1.42	0.35
TH130	53.0	1.40	0.31

TH131	28.9	1.43	0.55
TH132	142.5	1.36	0.31
TH133	21.8	1.60	0.25
TH134	6.9	1.68	6.02
TH135	5.8	1.31	0.32
TH136	17.9	1.74	0.75
TH137	8.9	1.52	0.60
TH138	79.2	1.46	0.25
TH139	13.2	1.65	0.27
TH140	69.5	1.58	0.30
TH141	3.8	1.31	0.16
TH142	17.7	1.43	0.24
TH143	9.7	1.40	0.21
TH144	27.0	1.83	0.99
TH145	20.6	1.68	0.50
TH146	14.5	1.68	0.42
TH147	137.2	1.22	0.25
TH148	17.0	1.76	0.44
TH149	30.2	1.60	0.33
TH150	15.7	1.63	0.36
TH151	44.7	1.66	0.34
TH152	51.0	1.47	0.25
TH153	62.8	1.59	0.27
TH154	20.0	1.60	0.23
TH155	11.4	1.57	0.30
TH156	23.0	1.69	0.68
TH157	31.4	1.77	0.39
TH158	11.2	1.61	0.31
TH159	45.6	1.68	0.37
TH160	89.0	1.57	0.26
TH161	18.0	1.56	0.30
TH162	15.1	1.71	0.34
TH163	22.5	1.70	0.56
TH164	16.6	1.70	0.26
TH165	18.7	1.80	0.37
TH166	27.4	1.88	0.62
TH167	31.5	1.75	0.74
TH168	18.3	1.88	0.61
TH169	19.7	1.57	0.31
TH170	164.0	1.93	2.53
TH171	6.7	1.87	0.52
TH172	82.5	1.87	1.67
TH173	53.2	1.90	1.39
TH174	111.3	1.96	2.46
TH175	135.3	1.87	2.32

TH176	134.7	1.86	2.16
TH177	74.6	1.90	3.24
TH178	48.4	1.89	2.33
TH179	70.5	1.81	2.22
TH180	-2.2	1.69	0.31
TH181	68.1	1.89	1.30
TH182	76.1	1.94	2.38
TH183	123.4	1.91	2.74
TH184	175.9	1.93	2.53
TH185	137.5	1.91	2.77
TH186	104.7	1.92	2.61
TH187	-1.0	1.50	0.14
TH188	97.8	1.86	1.79
TH189	820	1.84	1.70
TH190	82.8	1.86	2.67
TH191	-1.4	1.47	0.32
TH192	94.5	1.80	1.89
TH193	28.5	1.85	1.84
TH194	13.2	1.49	0.74
TH195	17.7	1.79	1.58
TH196	18.8	1.86	1.32
TH197	61.2	1.76	1.48
TH198	56.5	1.85	1.45
TH199	44.7	1.79	1.39
TH200	37.3	1.75	1.12
TH201	24.0	1.68	0.94
TH202	50.8	1.86	1.93
TH203	12.3	1.62	1.49
TH204	90.3	1.88	2.16
TH205	85.3	1.88	1.63
TH206	116.7	1.86	1.86
TH207	190.0	1.87	1.92
TH208	70.7	1.81	1.60
TH209	73.0	1.77	1.46
TH210	41.7	1.80	1.30
TH211	17.4	1.75	1.51
TH212	6.8	1.58	0.67
TH213	74.3	1.71	1.08
TH214	34.0	1.56	0.63
TH215	15.6	1.64	1.19
TH216	52.2	1.79	0.93
TH217	172.2	1.89	2.01
TH218	108.2	1.84	1.65
TH219	128.3	1.81	1.40
TH220	7.4	1.78	0.55

TH221	94.7	1.95	1.42
TH222	82.1	1.74	0.87
TH223	87.2	1.84	1.66
TH224	40.0	1.76	1.00
TH225	96.1	1.74	1.05
TH226	18.9	1.62	0.34
TH227	25.2	1.43	0.38
TH228	9.7	1.33	0.26
TH229	112.0	1.91	2.12
TH230	15.7	1.33	0.32
TH231	37.9	1.68	0.65
TH232	98.2	1.88	2.01
TH233	28.9	1.90	1.26
TH234	63.9	1.75	1.00
TH235	17.1	1.65	0.53
TH236	18.1	1.74	0.52
TH237	30.9	1.66	1.03
TH238	25.5	1.86	1.15
TH239	42.7	1.83	1.31
TH240	170.5	1.88	1.85
TH241	120.9	1.88	1.73
TH242	70.9	1.78	1.06
TH243	102.2	1.56	0.69
TH244	82.6	1.86	1.91
TH245	53.1	1.89	1.65
TH246	53.6	1.62	0.80
TH247	66.0	1.72	0.98
TH248	70.7	1.84	1.70
TH249	33.6	1.70	1.00
TH250	122.4	1.83	1.65
TH251	59.2	1.79	1.26
TH252	114.0	1.77	1.28
TH253	78.5	1.76	0.89
TH254	146.4	1.48	0.55
TH255	135.4	1.85	1.38
TH256	49.2	1.72	0.77
TH257	24.4	1.91	1.26
TH258	68.4	1.92	1.73
TH259	22.1	1.58	0.57
TH260	116.6	1.81	1.31
TH261	45.9	1.80	1.35
TH262	33.9	1.69	0.73
TH263	7.9	2.03	0.36
TH264	148.8	1.86	1.99
TH265	92.9	1.65	0.97

TH266	227.6	1.88	2.07
TH267	90.1	1.29	0.38
TH268	55.7	1.72	0.91
TH269	54.6	1.83	1.63
TH270	87.3	1.78	1.34
TH271	31.8	1.69	1.13
TH272	37.8	1.51	0.59
TH273	70.4	1.75	1.20
TH274	15.3	1.50	0.42
TH275	32.0	1.68	0.82
TH276	113.1	1.91	2.19
TH277	50.4	1.80	1.08
TH278	135.2	1.83	1.39
TH279	47.7	1.87	1.83
TH280	69.9	1.79	1.29
TH281	125.4	1.82	1.48
TH282	61.2	1.73	1.33
TH283	33.0	1.79	1.27
TH284	14.3	1.65	0.53
TH285	30.2	1.81	1.16
TH286	5.9	1.14	0.19
TH287	162.7	1.31	0.42
TH288	193.3	1.57	0.62
TH289	8.1	1.57	0.57
TH290	64.6	1.83	1.27
TH291	126.2	1.88	1.95
TH292	62.7	1.77	1.14
TH293	75.6	1.83	1.28
TH294	34.7	1.69	0.89
TH295	17.8	1.83	0.66
TH296	83.8	1.81	1.69
TH297	44.8	1.68	1.11
TH298	57.7	1.80	1.57
TH299	18.0	1.54	0.63
TH300	23.9	1.55	0.64
TH301	160.6	1.90	1.90
TH302	203.2	1.37	0.44
TH303	84.08	1.83	0.99
TH304	59.9	1.79	0.95
TH305	29.3	1.63	0.79
TH306	22.5	1.48	0.47
TH307	156.3	1.85	1.85
TH308	14.2	1.52	0.51
TH309	57.6	1.61	0.72
TH310	36.9	1.66	0.89

TH311	23.5	1.65	0.70
TH312	136.6	1.82	1.69
TH313	86.1	1.80	1.14
TH314	101.9	1.81	1.43
TH315	212.2	1,42	0.53
TH316	148.1	1.69	1.01
TH317	195.6	1.84	1.72
TH318	14.4	1.49	0.57
TH319	29.7	1.75	0.76
TH320	23.6	1.55	0.51
Average	60.4	1.69	0.85