# ANALYSIS OF THE GENETIC STRUCTURE OF RACCOONS (*PROCYON LOTOR*) ACROSS EASTERN NORTH AMERICA: APPLICATIONS FOR WILDLIFE DISEASE MANAGEMENT

A Thesis Submitted to the Committee on Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Faculty of Arts and Science

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#### Abstract

Analysis of the Genetic Structure of Raccoons (*Procyon lotor*) Across Eastern North America: Applications for wildlife disease management

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Raccoon populations are infected with a raccoon-specific rabies virus strain across the eastern seaboard of the United States. Since rabies is fatal and can infect non-host mammalian species, control and eradication programs, especially in urban centres, are needed to protect human health. To provide information about raccoon dispersal for management purposes, genetic analyses at both a broad and fine scale were conducted. Mitochondrial control region DNA sequence data were analyzed for over 1000 raccoons across the eastern seaboard revealing 111 haplotypes that formed three distinct lineages. Despite the lack of geographic association of the lineages, considerable population structure was evident. At the broad scale geographic distance was found to be an important variable in explaining genetic distance, but a large amount of variation was left unexplained indicating important regional effects. At a fine scale major rivers were associated with population structure. Additional fine scale analysis was conducted using ten highly variable microsatellite loci isolated from raccoons; parentage assignment was used as a novel means of dispersal measurement and population genetic structure across the landscape was used to detect geographic barriers to movement. Parentage analysis of individuals in the Niagara (N = 296) and St. Lawrence (N = 593) regions revealed the majority of male and female raccoons did not frequently disperse large distances, where 80% of individuals dispersed less than 3 km. Spatial genetic analysis of these data indicated that females are more philopatric than males. Landscape analysis of genetic

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structure indicated the Niagara River (N = 744) is a barrier to gene flow, but the same was not found for the St. Lawrence (N = 802). The two regions have different habitat composition and the rivers have different characteristics, both having potential effects on raccoon dispersal across the rivers. The information obtained from these analyses implicates major rivers as an important factor in affecting genetic structure. Additionally, other ecological factors may contribute to the genetic structure and mobility of raccoons and implementation of rabies control programs should account for regional differences. **Keywords:** Disease management, landscape genetics, microsatellite, *Procyon lotor*,

phylogeography, raccoon, subspecies.

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# **Chapter 1**

#### Introduction

The use of genetic information in wildlife management has seen a dramatic increase over the past two decades as the ability to obtain large amounts of genetic data and analytical capabilities have improved (Hedrick 1999; Manel *et al.* 2003; Storfer *et al.* 2007). With these developments come opportunities to investigate management problems from a new perspective. For example, for wildlife disease management to be effective managers need a comprehensive understanding of the host's behaviour if they are to be successful in disease control and eradication. Understanding the dynamics of host movement and how individuals respond to the landscape is a critical parameter for designing effective management strategies (Sterner and Smith 2006). The use of genetics in estimating dispersal parameters has not been utilized for disease management and the raccoon (*Procyon lotor*) rabies epizootic affecting the eastern coast of North America provided an opportunity to use such information to better understand the movement and behaviour of this species.

Broad and fine scale genetic analyses have been conducted using samples obtained opportunistically during disease control programs from wildlife management agencies, including the Ontario Ministry of Natural Resources, and the United States Department of Agriculture – Wildlife Services. These genetic analyses of raccoon populations across eastern North America will provide important information to wildlife management agencies for decisions on implementing control actions to prevent further spread and eventual eradication of rabies in North America. As well, this work will demonstrate the

importance of a multidisciplinary approach, and the importance of using appropriate analytical methods to properly address biological questions.

The raccoon, one of the most successful mammals in North America with continuous populations across diverse habitats, presents an interesting challenge. The current subspecific delineations (Hall 1981) suggest there should be significant large scale population structure. However, the level of differentiation may have been obscured as raccoons have been transported in thousands across hundreds of kilometers (Nettles et al. 1979; Smith et al. 1984). The sequence variation in mitochondrial control region DNA (mtDNA) was investigated and reported in Chapters four and five to determine (a) if subspecific designations are supported by genetic data, (b) assess what effects translocations have had on raccoon populations, and (c) to examine the broad scale genetic structure of raccoon populations. In addition to using mtDNA sequences to determine whether separate lineages exist, haplotype distributions were also assessed to indicate the level of genetic differentiation across eastern North America and to provide information on large scale demographic events which may have occurred in the past and are occurring in the present. It is an ideal molecular marker for a number of reasons: it is considered selectively neutral, has an appropriate level of mutation for historical demographic process to be analyzed ( $\sim 2 \%/my$ ), is non-recombining, and is maternally inherited in vertebrates (Avise et al. 1987). Analyses of mtDNA haplotypes across the geographic range can allow the inference of demographic events by looking at their sequence and frequency differences; these include range expansion, restricted dispersal, high gene flow and long distance dispersal (Avise et al. 1987; Excoffier et al. 1992; Templeton 2004). In addition, as mtDNA is transmitted maternally; dispersal differences

between males and females will produce differences in mtDNA genetic structure in comparison to genetic structure determined by nuclear co-dominant markers.

To investigate raccoon dispersal a fine temporal and spatial approach is necessary; nuclear microsatellite markers provide this resolution. Analysis of microsatellite loci for parentage, spatial genetic structure and clustering is discussed in Chapters six and seven. The objective of the parentage analysis was to estimate juvenile dispersal directly by measuring the distance between mother-offspring pairs; the spatial genetic structure provides an alternative perspective on dispersal analysis. Assessing genetic population structure will identify clusters; genetic discontinuities among these can then be correlated with landscape features that may act as barriers to gene flow. The knowledge of both dispersal distances and how landscape features affect raccoon movement are critical measures for management to optimize prevention and eradication tactics. Microsatellites were employed because they are bi-parental markers, ideal for parentage analysis; they evolve rapidly at a rate of approximately  $10^{-3} - 10^{-4}$  events per locus per generation (Dallas 1992; Weber and Wong 1993), and thus are highly variable. For confident assessment of parentage and accurate population assignment, highly polymorphic markers were vital. There did not exist raccoon specific microsatellites; therefore I developed marker panels for the raccoon using methods adapted from Hamilton et al. (1999) and Refseth et al. (1997) as described in Chapter three and in Cullingham et al.(2006).

# **Chapter 2**

#### Literature Review

#### Raccoon ecology

The common raccoon (*Procyon lotor*) is a mesocarnivore belonging to the family Procyonidae (Goldman 1950). The procyonids appeared in the early Miocene but the centre of their radiation is unknown due to a scarce fossil record; it most likely occurred in the neotropics where there is their greatest diversity (Kurten and Anderson 1980; Zeveloff 2002). The ancestors of today's raccoon were well established across much of North America, primarily in the eastern and southern United States by the Pleistocene (Kurten and Anderson 1980); studies of the fossil record have uncovered at least three of these early species of raccoon, Procyon priscus in Illinois, Procyon simus in California, and Procyon nanus in Florida and after comparison with modern samples Kurten and Anderson (1980) subsumed them all within the same species, P. lotor. Procyon lotor likely evolved in the Pleistocene since the earliest fossils were uncovered from the Oligocene (Kaufman 1982). In the Americas there are currently 25 subspecies of *P. lotor* and five closely related species recognized; these designations were primarily based on morphological work by Goldman (1950) and later revised by Hall (1981). These classifications have since been investigated more thoroughly using morphometrics (Kennedy and Lindsay 1984; Helgen and Wilson 2003), allozymes (Dew and Kennedy 1980; Kennedy and Lindsay 1984; Hamilton and Kennedy 1987; White et al. 1998) and mitochondrial sequence analysis (Pons et al. 1999). Morphometric analysis has not strongly supported many of these subspecies as there is considerable individual variation creating clines in characteristics rather than distinctive groups; even Goldman (1950)

stated "Individual variation …is extensive in scope and may render difficult the determination of some specimens, especially if from unknown localities." Generally, the trend found by many studies have noted that smaller raccoons occur in the south (Kennedy and Lindsay 1984; Helgen and Wilson 2003), and much larger raccoons are found in the far west (Goldman 1950). Studies that have looked at geographical genetic variation found significant differences only between the northern west coast and eastern United States (Hamilton and Kennedy 1987), whereas the eastern United States exhibited less geographic patterning (Dew and Kennedy 1980; Pons *et al.* 1999). Both Dew and Kennedy (1980) and Pons *et al.* (1999) indicated the causes of this are likely the relocation of large numbers of raccoon for hunting purposes. These studies had low sample sizes and used protein electrophoresis (Hamilton and Kennedy 1987; Dew and Kennedy 1980); these markers display low variability in the Carnivora (Selander and Kaufman 1973), and there are now more appropriate and powerful markers such as microsatellites.

Presently the raccoon is widely distributed across North America reaching well into the central and southern parts of Canadian provinces (Zeveloff 2002) with very low densities found in higher elevations, i.e. the Appalachians and Rocky Mountains (Sanderson 1986; Gehrt 2003). The habitat originally occupied by raccoons includes bottomland forests, wooded streams, hardwood forests and forested areas associated with aquatic habitats (Sanderson 1986; Gehrt and Fritzell 1999; Chamberlain *et al.* 2002). Raccoons were generally distributed in low densities across their range, but a population explosion occurred in the 1940's (Sanderson 1986; Zeveloff 2002; Gehrt 2003). There are a number of potential interacting factors that could have contributed to this growth

including the extensive establishment of row crops (especially corn), increasing urban areas, and persecution of predators including coyotes and wolves (Zeveloff 2002). By the 1980s population growth and expansion has created densities 15-20 times higher than during the 1930s (Sanderson 1986), making the raccoon one of the most successful native species in North America.

Research conducted by Gehrt and Fritzell (1998a, 1998b, 1999) and Gehrt et al. (2002) showed that female raccoons are solitary and generally have large home range overlap with other females only when aquatic resources are scarce. Males have been found to reside in social groups that are spatially distinct from other male groups and solitary males, but are generally solitary themselves, with ranges that typically encompass more than one female home range. It has been proposed that females having home range overlap with other females are likely mother-daughter pairs (Gehrt and Fritzell 1998b), and this was shown to be true in a genetic study conducted by Ratnayeke et al. (2002) indicating strong female philopatry. Field-based studies investigating dispersal behaviour in raccoons indicate a range of dispersal distances observed from 0 - 20 km (Seidensticker et al. 1988; Gehrt and Fritzell 1998a), with males being more likely to disperse and moving greater distances than females. Studies of translocated raccoons reported distances traveled upon release of up to 20 km (Belant 1992; Mosillo et al. 1999) suggesting raccoons are capable of traveling considerable distances. Lynch (1967) reported movements of over 200 km for both a female and a male. Despite their dispersal capabilities, they maintain a relatively small home range of < 1 - 4 km<sup>2</sup> (Hoffman and Gottschang 1977; Seidensticker et al. 1988; Gehrt and Fritzell 1998a) but will adjust their home range size depending on their resources, where productive habitats will be able to

support smaller home ranges (Seidensticker *et al.* 1988; Gehrt and Fritzell 1999). While these investigations have contributed to our understanding of raccoon behaviour, they have been conducted in limited study areas; as raccoons occupy diverse habitats with different environments they may have different behavioural responses in different portions of their range.

#### Raccoon rabies and rabies management

Rabies is an almost always fatal infectious disease of mammals and is caused by a Lyssavirus of the family Rhabdoviridae. Exposure to the virus is typically caused by a bite as viral particles are shed from the salivary glands (Niezgoda *et al.* 2002). The route of the virus from site of infection is through the peripheral nervous system, to the motor neurons in the spinal cord where replication occurs, and following that, rapid infection of the brain, where neuronal dysfunction will begin to occur. Once the brain is infected virus will spread along nerves to salivary glands, skin, cornea and other internal organs (Jackson 2002). Behavioural changes do result as the main site of infection is brain tissue, changes which include increased alertness, loss of natural timidity, aberrant sexual behaviour and aggressiveness (Johnson 1971); these behavioural changes result in what is considered the furious phase. The behaviour during the furious phase, together with the viral shedding in the salivary glands allows for efficient infection (Jackson 2002). Aside from this method of infection, infection can also occur before the clinical phase (as virus can be shed at this point) and natural behaviours will involve exposure to the virus bearing saliva including, copulation, predation, resource defense, carrying of infants, food sharing, play and grooming (Jackson 2002). These opportunities may be just as, or more important, for transmission than during the furious phase.

Prevention of zoonotic disease is often important for wildlife managers when it can have damaging impacts on endangered mammalian populations, livestock, companion animals and human health (Cleavland *et al.* 2002; MacDonald and Laurenson 2006). Although rabies has specific reservoir hosts in North America including the raccoon, arctic fox (*Alopex lagopus*), red fox (*Vulpes vulpes*), gray fox (*Urocyon cinereoargenteus*), skunk (*Mephits mephitis, Spilogale putoris*) and a number of insectivorous bats (incl. *Myotis* spp., *Eptsesicus* spp., *Lasionycterus* spp.) (Nadin-Davis *et al.* 2001; Smith 2002; Krebs *et al.* 2003); there are cases of cross-species transmission, or spill-over. Most often spill-over will not lead to further infections within that species, but rabies does mutate rapidly and recently a strain of bat virus infected and persisted in skunks (Engeman *et al.* 2003; Slate *et al.* 2005).

Currently the raccoon rabies situation is more critical than the others due to the hosts' success in occupying a broad range of habitats and their especially dense populations in urban environments (Rosatte 2000; Smith and Engeman 2002; Prange *et al.* 2003), which lead to higher rates of intraspecific infection as well as increased contacts with livestock (Rupprecht and Smith 1994), companion animals and humans (Gordon *et al.* 2004). The cost of post-exposure treatment ranges from \$1000-\$4000 (USD) in biologicals per human case (Kreindel *et al.* 1998; Chang *et al.* 2002); this could cause a serious burden on health care. In New York State, approximately \$13.9 million (USD) has been spent on rabies control from 1993-1998 (Chang *et al.* 2002) and in Massachusetts human exposure treatment cost an estimated \$2.4-6.4 million (USD) in the first year of detected raccoon rabies cases in the state (Kreindel *et al.* 1998). In addition to human health, there is a serious danger to other wildlife; infection from this strain has been reported in other

animals such as the skunk (*Mephitis mephitis*), woodchuck (*Marmota monax*), beaver (*Castor canadensis*), and eastern cottontail (*Sylvilagus floridanus*) (Childs *et al.* 1997). Finally, there is the danger to the raccoon population, and its effects on the fur trade; the raccoon represents the largest revenue source of any furbearer in North America (Sanderson 1986; Gehrt 2003; Kamler and Gipson 2004). Since raccoon populations in North America exhibit high densities, it is estimated that 85% of infected raccoon populations will be decimated by the raccoon rabies epizootic (Kirby 1995). This may seem to be surprising, but studies have shown that no significant immunity has developed in raccoon populations after an initial rabies epizootic (Childs *et al.* 2000); this will maintain high mortality rates when another outbreak occurs.

The first recorded case of raccoon rabies was in Florida in 1947 (Rupprecht and Smith 1994). The spread of rabies from Florida into neighbouring states occurred at an approximate rate of 40 km/yr, and by 1977 cases were recorded for Georgia, Alabama and South Carolina (Rupprecht and Smith 1994). At around this time a new focal point emerged in Virginia well ahead of the front. Shipments of raccoons from Florida to Virginia for restocking hunting reserves documented at least one case of a rabid raccoon among more than 3500 raccoons shipped from 1971-1977 (Nettles 1979; Rupprecht and Smith 1994; Kirby 1995). From this new focal point rabies spread both north into West Virginia and Maryland at significant levels by 1981, and south, where it converged with the initial front in North Carolina in 1994 (Childs *et al.* 2000). The front has since continued northward and the first documented case in Ontario occurred in July 1999 near Brockville, less than 20 km from the U.S. border (Rosatte *et al.* 2001).

In the 1960's the World Health Organization (WHO) saw the importance of implementing rabies control in reservoir populations and formed the WHO Expert Committee on Rabies (WHO 1966) to call for research into developing vaccines and methods of delivery. This research led to the distribution of vaccine of baits in the wild in Switzerland in 1977 to control rabies in red foxes (Winkler and Jenkins 1991). Interestingly, by this time the Canadian Wildlife Rabies Control Program to eliminate fox rabies was already 10 years old; it began in Ontario in 1967 after the death of a four year old girl from rabies contracted from a stray cat (Johnston and Tinline 2002). Since that time this program has been successful in nearly eliminating the arctic fox strain in Ontario and a number of protocols developed have been adapted to control raccoon rabies (Rosatte et al. 1992, 1997, 2001). The Ontario Ministry of Natural Resources (OMNR), Wildlife Services-United States Department of Agriculture (WS-USDA), and the New Brunswick Department of Agriculture (NBDOA) and various wildlife organizations are all working to eliminate raccoon rabies (Slate et al. 2005). In Ontario numerous control strategies have been implemented including population reduction, point infection control (PIC), trap-vaccinate-release (TVR), and oral vaccination with baits (OVR). These control measures have held rabies prevalence at significantly lower levels than in neighbouring US states (Rosatte et al. 2001). Even though there has been some success in establishing vaccinated zones between infected and non-infected regions, there have also been instances of barriers being breached: in Ontario along the St. Lawrence River, in Cape Cod, Massachusetts, in Ohio, and most recently in Quebec.

At present this rabies epizootic is one of the largest documented in wildlife history (Childs *et al.* 2001). In part, because of this, numerous studies have attempted to

understand spatial use and population association of raccoons (Childs et al. 1997; Pedlar et al. 1997; Wilson et al. 1997; Riley et al. 1998) and model development that will predict the spread of the rabies epizootic (Coyne *et al.* 1989; Broadfoot *et al.* 2001; Childs et al. 2001; Smith et al. 2002). These have met with limited success. The model by Smith et al (2002) found that local spread of rabies was slower when associated with rivers, but had no significant correlation with human population density. This is puzzling because Rupprecht and Smith (1994) demonstrated the spread of rabies was channeled along the eastern seaboard where U.S. human populations are most dense. Childs et al. (2001) looked at the relationship between magnitude of epizootics in the northern United States, human population density and health care spending, finding a direct positive relationship indicating the more spending and more people, the better the surveillance. In addition, they found differences in spatial and temporal dynamics not accounted for by this relationship and felt that habitat composition could have an important role in affecting the spread of raccoon rabies. Although there have been numerous studies dedicated to understanding the rabies/raccoon dynamic, there does not currently exist an ability to predict emerging disease threats for implementing effective control programs for raccoon rabies (Rupprecht and Smith 1994; Wilson et al. 1997; Broadfoot et al. 2001; Smith et al. 2002).

Understanding the spread of rabies is important and being able to test management scenarios prior to rabies outbreaks is critical in ensuring their success (Sterner and Smith 2006), this might be achieved by building models that represent the study system. Anderson *et al.* (1981) and Smith and Harris (1991) modeled the dynamics of fox rabies in Europe to determine the proportion of the population that must be targeted to achieve

immunity and Coyne et al. (1989) compared different strategies, including culling and vaccination, to find the best method or combination of methods in controlling rabies. These were relatively simple models and may not mimic a natural system accurately which is critical to effective rabies management (Johnston and Tinline 2002). To this end a model has been developed in efforts to more accurately represent populations and to better determine how management strategies can affect rabies dynamics, the Ontario Raccoon Rabies Model (ORRM – Tinline et al. in prep). This is an individual based, spatially explicit model adapted from a model developed for fox rabies (Voigt et al. 1985). It mimics raccoon population dynamics based on parameter estimates that have been obtained from the literature and field studies. The problem with this approach is that critical parameters are sometimes imprecisely estimated due to small sample sizes, with a wide range of possible values. Dispersal distance is obviously important as it will dictate the distances and speeds at which rabies can spread, but rabid raccoons may not behave in the same manner as non-infected ones (Jackson 2002) therefore it is important to know dispersal distances for both healthy raccoons, for prevention management, and rabid raccoons to effectively control rabies (Sterner and Smith 2006). Similar to dispersal, understanding the landscape features that act as barriers or corridors to raccoon movement is also critical. Information on landscape barriers can be used in establishing vaccination zones which, in turn, will reduce costs for control programs. Data on dispersal corridors will indicate areas needing more attention to better prevent breaches of vaccination zones.

#### Dispersal

Understanding dispersal patterns is important as it is likely the most critical parameter in species persistence and evolution, and plays an obvious and critical role in disease spread. To model and predict a population's behaviour the dispersal distance and frequency have to be measured accurately. It is often assumed that the frequency of dispersal distances is fixed for a species; distances are obviously constrained by morphology and physiology but there is considerable variability. As well, it is difficult to predict under what hypothesis of dispersal individuals of a species act; it could be resource competition (Greenwood 1980), mate competition (Dobson 1982) or inbreeding avoidance (Wolff 1993; Pusey and Wolff 1996). In the attempts to generalize animal behaviour it is clear as Dobson (1982) stated that, "No single hypothesis sufficiently explains the dispersal pattern of all mammal species. Therefore the factors influencing dispersal are likely to either vary between species, or vary in their importance in different species." and furthermore to vary within a species depending on an individual's circumstances. Therefore the "why" and "how far" an individual disperses are both plastic responses constrained by capabilities and the context of the landscape.

Dispersal, despite being one of the most important parameters in understanding and managing populations, is also one of the most difficult parameters to measure. Traditionally dispersal has been measured by direct methods such as capture-markrecapture (CMR); this involves trapping and tagging individuals for identification, and then re-trapping an area and contrasting the locations individuals were originally captured with their new capture locations. Some of the issues with this method include: the cost, as it requires intense effort to capture the maximum number of individuals to ensure it is

possible to describe the dispersal function accurately; the difficulty in capturing individuals and the inability to detect long distance dispersal is limited by the extent of the trapping area (Koenig *et al.* 1996).

More recent methods of measuring dispersal use molecular data and are considered indirect methods as they are not directly measuring individuals' movements. Initially dispersal estimates were obtained using summary statistics such as F<sub>ST</sub> (Wright 1951), and the utility of these measures was debated as there was considerable disparity between these and field-based estimates. The problem with this approach is the temporal scale because F<sub>ST</sub> reflects a historical perspective rather than a contemporary estimate (Bohonak 1999; Sork et al. 1999). As well, F<sub>ST</sub> as a dispersal measure is based on an island model with assumptions not often met by natural populations; therefore its use in calculating migration rate is less than optimal (Bossart and Prowell 1998; Whitlock and McCauly 1999). However, with the development of assignment tests, which use allele frequency data to assign individuals to their source population (Paetkau *et al.* 1995; Rannala and Mountain 1997; Cornuet et al. 1999; Pritchard et al. 2000; Guillot et al. 2005; Francois et al. 2006), has come a better measure of dispersal. Although this approach can detect individuals in a population that actually belong to a different population (Berry et al. 2004; Paetkau et al. 2004) and thus have dispersed, there is no actual distance measure to say where they physically started from in their original population. We are therefore still missing a precise distance measure, which is especially critical for effective disease management.

Parentage analysis using molecular techniques has been developed for the purpose of understanding mating systems and has resulted in a number of outcomes that challenge

hypotheses that were proposed based on observational studies (see, Hughes 1998;

DeWoody 2005). The analysis of parentage in wildlife systems can also provide a more accurate measure of dispersal than either the traditional direct method of trapping or the indirect genetic assignment tests. The premise is to determine the parent(s) of juveniles and measure the distances between where the parent(s) and the juvenile are found. This methodology was first used in plant systems (Sork *et al.* 1998; Sork *et al.* 1999) where sampling of potential parents is easier as they are not mobile; the technique has proven successful (Nason *et al.* 1996; Trapnell *et al.* 2005; Pospíšková and Šálková 2006). Waser *et al.* (2006) utilized parentage in comparison to CMR in Kangaroo rats, *Dipodomys spectabilis* and found a significant difference, where dispersal distance was considerably underestimated by CMR and a number of dispersal events went undetected as juveniles had dispersed before capture occurred. Telfer *et al.* (2003) also found the same pattern in water voles *Arvicola terrestris* when parentage was compared to CMR.

#### Landscape genetics

The emerging field of landscape genetics focuses on identifying the genetic structure of populations across the landscape and examines the causes of the restricted gene flow. From these analyses a better understanding emerges of how a species interacts with and is influenced by its landscape. The first article describing landscape genetics as a field was published in 2003 by Manel *et al.*, yet the concept and importance of landscape genetics, the effects of landscape on the genetic structure of a species, has long been recognized (see Haldane 1940; Wright 1942; Fisher and Ford 1947; Manicacci *et al.* 1992; Koenig 1999; Sork *et al.* 1999; Barbujani 2000). The ability to explore this relationship has unfortunately been hindered because of inadequate analytical methods and a lack of

effective communication between geneticists and ecologists. To look at this relationship one needs to be able to determine the population structure, display this structure and analyze the significance of landscape as a factor in causing the structure, while incorporating the biology of the species.

Initial studies aimed at this type of approach attempted to make inferences based on the results of  $F_{ST}$  statistic (Wright 1951) analysis (Lougheed *et al.* 1999; Hale *et al.* 2001; Saenz-Romero *et al.* 2001; Matocq *et al.* 2000; Toda *et al.* 2003). F statistics or their derivatives evaluate average allelic differences between groups and because of this, a large amount of variation is lost (Paetkau *et al.* 1995). Most studies now use highly polymorphic microsatellite loci (Sunnucks 2000; Balloux and Lugon-Moulin 2002) where each allele can contribute new information if analyzed separately instead of being part of an average. An additional disadvantage of using F statistics for landscape genetics is the need to designate *a priori* individuals into populations. If individuals are assigned to populations before the analysis, the inferences being made about population structure could be incorrect (Epperson and Li 1996). For example, if individuals were assigned to populations based on the assumption that rivers caused restricted gene flow (when in fact roads were the cause), there may not be any indication of significant genetic structure when it does exist.

The next step in looking at the relationship between genetic structure and geography was through the isolation by distance (IBD) model. Wright (1942) and both recognized that most, if not all populations deviate from the ideal panmictic unit, and proposed the relationship of geographic and genetic distance, where the further apart populations were, the stronger the genetic differences. Investigators have recognized the importance of this

simple model and have further developed it to incorporate more landscape data in the distance measure (Keyghobadi *et al.* 1999; Michels *et al.* 2001); rather than only compare genetic distance to Euclidean distance, they developed distance matrices that account for landscape barriers. This method has been used for numerous studies, but the relationship of a species to its landscape may be more complex than the simple IBD and the ability to investigate these multifaceted relationships is necessary.

Understanding how genetic variation is partitioned in space can provide additional insight into the role landscape plays in creating and maintaining structure. This paradigm is common and many investigators have adopted and modified spatial statistic methods applied to genetic data. One commonly used method of looking at spatial variation is that of spatial autocorrelation (SA: Moran 1950; Geary 1954; Legendre and Fortin 1989); it was initially used by ecologists to look at the environment and how samples taken in close proximity are more similar than those further apart (Legendre and Fortin 1989), very similar to the underlying theory of IBD. A number of studies have utilized this method for human (Piazza et al. 1981) and plant populations (Smouse and Peakall 1999; Diniz and Telles 2002); the major disadvantage in using this method is that it only describes the pattern and this does not indicate how the genetic structure relates back to the landscape. Another method often associated with spatial statistics is the geostatistical method of kriging (Legendre and Fortin 1989). Kriging goes one step further than SA in that it actually uses the spatial autocorrelation in the data to create an entire surface for the variable, and this has been adapted to map genetic variation over a region (Le Corre et al. 1998). There is now a method to map the variation, but without an analytical tool to associate this variation with the landscape, again only descriptions can be made.

To get closer to a landscape genetic study, researchers have tried to utilize statistical clustering methods such as Principal Components Analysis (PCA) (Piertney *et al.* 1998; Lugon-Moulin and Hausser 2002; Jacquemyn *et al.* 2004), Canonical Correspondence Analysis (CCA) (Angers *et al.* 1999), and Multidimensional Scaling (MS) (Davison and Clarke 2000). The approach here is to graph the partitioned variation and compare it to landscape features, but again like the previous methods it is limited to descriptions.

Assignment tests have been developed in an attempt to better define population structure. These tests can detect structure using individual multi-locus information without the need to define populations *a priori*, and so analyses are more powerful. Using Bayesian (Rannala and Mountain 1997; Pritchard 2000; Guillot *et al.* 2005; Francois *et al.* 2006) and maximum likelihood (Paetkau *et al.* 1995; Piry *et al.* 2004) statistics, population structure can be derived and these defined clusters can be used to look for associations with the landscape to understand the causes of genotypic discontinuities. The most recent Bayesian methods (Guillot *et al.* 2005; Francois *et al.* 2006) have shown to be the most sensitive and can detect cryptic population genetic structure (Coulon *et al.* 2006). This can be advantageous when looking for recent changes in population dynamics as species respond to anthropogenic changes, but are not necessary when looking for genetic discontinuities that have developed as a result of long standing physical barriers to movement.

More powerful methods to detect population structure have been developed which have the ability to examine this structure and its interaction with the landscape. The choice of the most appropriate method is now a daunting task for genetic researchers without experience in spatial analyses (Excoffier and Heckel 2006); this makes necessary

a multi-disciplinary approach to population management questions; investigations should be customized according to the questions to be addressed and the biology of the species.

# Chapter 3

# Isolation, Characterization and Multiplex Genotyping of Raccoon Tetranucleotide Microsatellite Loci

#### Abstract

Twelve raccoon-specific polymorphic tetranucleotide microsatellite loci were isolated and characterized. Three multiplex panels comprising the 12 loci were developed and 80 individuals from southeastern and western Ontario were genotyped; allele sizes were assigned without difficulty. One locus isolated was identified as an X-linked marker. The number of alleles per locus ranged from 6-25 with the average heterozygosity ranging from 0.674-0.925. These loci will be useful in characterizing raccoon population structure across North America and the data useful in further understanding the spread of raccoon rabies.

#### Introduction

Raccoons (*Procyon lotor*) are medium-sized carnivores of the family Procyonidae. They are found across much of North and Central America with exceptions occurring at elevations over 2000 m and in northern Canada where winters are harsh (Sanderson 1987; Gehrt 2003). Raccoons are considered one of the most successful mammals in the Americas due to their adaptive abilities (Zeveloff 2002), and have been an important species to European immigrants, both as a symbol in culture as well as to the furbearing industry (Sanderson 1987; Zeveloff 2002; Gehrt 2003).

Raccoon management has typically been concerned with maintaining sustainable harvests for hunting and trapping, but there are growing reasons for careful management. Raccoons carry a strain of rabies that is particularly virulent to them, and has spread

rapidly across the eastern seaboard of North America (Rupprecht and Smith 1994; Childs *et al.* 2001). Understanding raccoon population dynamics, such as dispersal distances and dispersal rates, contributes to the information managers need to plan effective rabies control programs.

#### Materials and Methods

DNA profiling of raccoon populations would provide these estimates but presently there are no raccoon specific microsatellite primer pairs. To this end, I isolated whole genomic DNA from a male and female raccoon using standard phenol-chloroform procedures (Sambrook et al 1989). This DNA was used to construct an enriched genomic library following a procedure adapted from Hamilton et al. (1999) and Refseth et al. (1997). Briefly whole genomic DNA was digested with Hae III, and SNX linkers were ligated to the digested fragments. Enrichment was carried out using  $GATA_8$  biotin labeled probe and streptavidin coated beads (Dynal Biotech). Four hundred ng of DNA were first hybridized to 100  $\mu$ mol of probe in a 50  $\mu$ L volume, and the complex was added to 50 µL of washed beads and the two were mixed for 15 min at room temperature. The unbound DNA was washed away using three washes each of 100  $\mu$ L of 2X and 1X SSC. Single stranded DNA, once released from the probe, was amplified to double stranded form. The resulting product was ligated into vector and transformed into cells using pCR<sup>®</sup>2.1-TOPO<sup>®</sup> vector, and TOP10 chemically competent cells following the manufacturers instructions (Invitrogen). Colonies positive for insert were amplified using M13 forward and reverse primers, and the resulting product was sequenced using the DYEnamic<sup>TM</sup> ET terminator cycle sequencing kit, and run on a MegaBASE 1000 (GE Healthcare).

Fifty-six sequences were selected for primer design. Primer design was performed by eye and primer pairs assessed for secondary structure using Fast PCR<sup>©</sup> (http://www.biocenter.helsinki.fi/bi/bare-1 html/oligos.htm). Unlabelled primers were used to determine optimal amplification conditions. PCR reactions were carried out using 1X PCR buffer, 1.25 mM MgCl<sub>2</sub>, 0.1 µM each dNTP, 0.3 µM forward and reverse primer, 1 unit Taq DNA polymerase (Invitrogen), and 5 ng DNA in a 10 µL reaction. The amplification conditions were as follows, 95° C for 5 min, 30 cycles of 95° C for 30 sec, T<sub>a</sub> for 1 min, 72° C for 1 min, and a final extension at 65° C for 45 minutes. All reactions were carried out on a PTC-225 thermocycler (MJ Research). To avoid the cost of purchasing fluorescent primers for all loci, variation was tested using five samples each from Florida and Ontario and running the product on a 4% agarose gel stained with Sybr®Green (Molecular Probes). This allowed sufficient allele separation to ensure loci were polymorphic before purchasing fluorescently labeled primers. From the 56 primer sets 12 were selected for multiplex design. Multiplex reaction conditions were the same as above except the MgCl<sub>2</sub> concentration for PCR1 was 1.0 mM and changes to primer concentrations as listed in Table 3.1.

#### **Results and Discussion**

Eighty samples obtained from southeastern and southwestern Ontario from the Ministry of Natural Resources were genotyped using the three multiplex reactions to evaluate their performance and to look at their overall heterozygosities, and alleles per locus. All loci were easily and consistently scored without ambiguity. Locus PLO3-117 was identified as an X-linked marker, with all males being hemizygous. The expected

PCR	Locus	GenBank accession	Primer Sequences	Repeat motif	Ta	Primer	Dye
		number		•	u	μΜ	2
PCR1	PLO3-117F	DQ388431	TCACTTGACTGGACCTGAGC	GAAA <sub>(20)</sub>	60	0.12	HEX
	PLO3-117R	DQ388431	CAACTTCATCAGATAAGTGATTTGC	(==)	60	0.12	
	PLO-M15F	DQ388432	ACTGAGACCACTGAGC	AGAT <sub>(13)</sub>	60	0.2	HEX
	PLO-M15R	DQ388432	CAGATTTCAGTCGATCGATG		60	0.2	
	PLO2-117F	DQ388433	ATATGCCGGGTCTAGGACAACACTG	$GATA_{(13)}GT_{(16)}$	60	0.4	6FAM
	PLO2-117R	DQ388434	CTAGTAATATCATGAGAAGCG		60	0.4	
PCR2	PLO-M3F	DQ388435	CTCCCATCTTCCTCTTTTCG	ATCT <sub>(15)</sub>	56	0.1	NED
	PLO-M3R	DQ388435	GTTGACAATTGCAGGACCAC		56	0.1	
	PLO3-71F	DQ388436	GCTTCCTTTAATTTTAACTAATGG	$ATCT_{(14)}$	56	0.35	
	PLO3-71R	DQ388436	CAATCCTGTATCAGGTTTCC		56	0.35	HEX
	PLO-M20F	DQ388437	GATTCTTATGTCTCTTGGGA	TCTA <sub>(17)</sub>	56	0.15	
	PLO-M20R	DQ388437	AAGTGCTTCAAGAGAAAGTGC		56	0.15	NED
	PLO2-14F	DQ388438	AAGAGCGTAATAAAAGCTTAC	GAAA <sub>(21)</sub>	56	0.35	6FAM
	PLO2-14R	DQ388438	CAAATAACAAGTTTCAATTTGG		56	0.35	
	PLO-M2F	DQ388439	GGAAAACCACAGAGAGACGG	TCTA <sub>(7)</sub> TCTG <sub>(6)</sub> TCTA <sub>(8)</sub>	56	0.3	
	PLO-M2R	DQ388439	CTTGGCACAGAGCAGAATCC		56	0.3	HEX
PCR3	PLO-M17F	DQ388440	CTGCTGAGTAAGGAGTAAGG	GTTT <sub>(3)</sub> TATC <sub>(12)</sub>	56	0.4	HEX
	PLO-M17R	DQ388440	TCCCCTGTACATATTCAGGC		56	0.4	
	PLO3-86F	DQ388441	GATTGATAGATTAATTGGTCTTAACTTCC	$CTTT_{(20)}$	56	0.27	
	PLO3-86R	DQ388442	CTGGATTATAAATCTGGCAAGAGCC		56	0.27	6FAM
	PLO2-123F	DQ388443	GTCTACTCAGTGCATAGCATTGTGC	GATA <sub>(~15)</sub>	56	0.15	
	PLO2-123R	DQ388444	TTCCTGCAATGTCCTCTAACTG		56	0.15	HEX
	PLO3-CF	DQ388445	AGTGGAATTGCCCTAACAATTTCATGCC	ATCT <sub>(24)</sub>	56	0.4	6FAM
	PLO3-CR	DQ388446	CCAGATTATCAGCTTTCAGATTTCG		56	0.4	

Table 3.1. Characterization of loci isolated from *Procyon lotor*, T<sub>a</sub> is the reaction specific annealing temperature. GenBank accession numbers refer to the clones the primers were designed from (all primer sequences were confirmed unique through a BLAST search on NCBI).

Locus	Size Range	Number of Alleles	H <sub>O</sub>	$H_{\rm E}$
*PLO3-117	263-295	8	0.674	0.782
PLO-M15	157-203	13	0.725	0.806
PLO2-117	284-354	25	0.875	0.914
PLO-M3	266-286	6	0.675	0.758
PLO3-71	157-201	12	0.763	0.864
PLO-M20	179-219	11	0.850	0.854
PLO2-14	228-316	17	0.825	0.847
PLO-M2	281-329	12	0.900	0.887
PLO-M17	208-228	6	0.688	0.793
PLO3-86	301-460	23	0.925	0.934
PLO2-123	579-619	11	0.813	0.848
PLO-3C	700-740	19	0.881	0.923
	1 10 0	1 1 01 10		

Table 3.2. Microsatellite diversity indices. Size range and number of alleles observed across 80 samples.  $H_0$  is the observed heterozygosity,  $H_E$  is Nei's expected heterozygosity.

\* Values calculated for females only (N=46)

and observed heterozygosities for females alone are not significantly different (Table 3.2).

For the remaining loci, the number of alleles per locus ranged from 6 - 25, and overall heterozygosities ranged from 0.675 - 0.925 (Table 3.2). Linkage disequilibrium tests were conducted using GenePop v. 3.4, and no significant values were found after Bonferroni correction. All loci were found to be in Hardy-Weinberg equilibrium using CERVUS (Marshal *et al.* 1998). These multiplex panels will be used to study raccoon population dynamics across the eastern seaboard of North America to assist in rabies disease management.

### **Chapter 4**

### Examination of Subspecific Designations of Raccoons (*Procyon lotor*) using Mitochondrial DNA Control Sequences

#### Abstract

To assess the currently recognized subspecific designations of the raccoon (Procyon *lotor*), a 467bp fragment of the mitochondrial control region from 313 individuals was compared across the ranges of the four subspecies: Eastern raccoon (P. l. lotor), Florida raccoon (P. l. elucus), Alabama raccoon (P. l. varius), and Upper Mississippi Valley raccoon (P. l. hirtus). Seventy-seven haplotypes were found, 59 of these were restricted to one of the four subspecies and the remaining 18 were shared with at least one other subspecies. Phylogenetic analysis of the sequences revealed two closely related lineages with 2.2% sequence divergence. Haplotypes from both lineages were found across much of the range but there were considerable haplotype frequency differences between the Florida raccoon (95%-lineage I) and the Eastern raccoon (73%-lineage II). These differences resulted in significant analysis of molecular variance for both a two-group comparison ((1) Florida, (2) Eastern, Alabama, Upper Mississippi) and a three-group comparison ((1) Florida, (2) Eastern, (3) Alabama, Upper Mississippi). Although there were significant differences among these defined groups, there was no evidence of reciprocal monophyly and no one lineage was restricted to one subspecies range. The subspecific designation of the Florida and Eastern raccoon subspecies were supported by the mitochondrial DNA sequence data; however growth of raccoon populations occurring concurrently with anthropogenic landscape changes and human mediated translocations have led to a mixing of the lineages particularly in the Mississippi and Alabama ranges.

### Introduction

The definition of subspecies and the criteria necessary to delineate separate subspecies (as opposed to distinct populations) has been an issue of continuous debate for biologists (e.g. Cronin 2006; Avise and Ball 1990; Mayr 1970; Moritz 1994). The utility of defining subspecies has been thoroughly questioned (Ryder 1996; Cronin 1997; Zink 2004) but their use for management is relevant (Cronin 2006; Geist *et al.* 2000) provided they are defined following appropriate criteria. Criteria that are generally agreed upon include evidence of historical separation (Moritz 1994) and the importance of concordance of independent characters, where these characters can be morphological or genetic (Avise and Ball 1990; Avise 2000).

The common raccoon, *Procyon lotor*, is endemic to the Americas and evolved from other procyonids in Central America where the majority of procyonids are found (Baskin 1982). The fossil record indicates procyonids were present during the Miocene, and potentially earlier; however this cannot be confirmed by the fossil record because the habitats of forests and woodlands that were likely used by prehistoric procyonids were not conducive to fossilization (Gehrt 2003; Kurten and Anderson 1980). The earliest representative fossils of the raccoon were found in Kansas and Texas and date back to the mid-Blancan of the Pliocene (2.5 mybp). The fossil record is scarce until the late Irvingtonian of the Pleistocene where the first appearance of the modern raccoon was in Florida; by the Wisconsian (0.2 mybp) the raccoon was well distributed across the United States especially in the east and in the south (Kurten and Anderson 1980). Prior to the 1940's, raccoons were rarely found in southern Canada, the Rocky Mountains or the western deserts (Gehrt 2003; Sanderson 1997), but since then have experienced

significant population growth and range expansion that corresponds well with anthropogenic modifications to the landscape and climatic changes (Zeveloff 2002). Raccoons can now be found across southern Canada, including portions of northern Ontario (Larivière 2004), in deserts and at low elevations in the Rocky Mountains (Gehrt 2003).

The most thorough description of raccoon (genus Procyon) species and subspecies was carried out by Goldman (1950), where he described 5 species and 25 subspecies based on morphology. These designations have been more recently reviewed by Hall (1981), where minor changes were made to some geographic delineation of subspecies. Subspecific designations primarily in eastern North America have been investigated using genetic information from allozymes to complement morphometric information (Dew and Kennedy 1980; Hamilton and Kennedy 1987; Kennedy and Lindsay 1984; White et al. 1998). Minimal differences in allozyme allele frequencies among the designated groups, with the exception of western United States (US) vs. eastern US have been found (Hamilton and Kennedy 1987). Differences among populations were noted in the morphology; however they were not reflective of the subspecies and characteristics showed a gradation along latitude (Kennedy and Lindsay 1984), suggesting the subspecific designations should be reconsidered. The validity of the West Indies island species (P. maynardi, P. minor and P. glovraini) has been investigated using mitochondrial (mt) DNA sequence information which indicates they are not separate species. Rather, the origin of these apparent species was most likely raccoons from North America and they are not indigenous as was originally thought (Helgen and Wilson 2003, Pons et al. 1999).
The validity and value of the raccoon subspecies designations has been questioned by Lazell (1989) and Whitaker and Hamilton (1998), and even Goldman (1950) indicated the difficulty in assigning individuals to a subspecies without knowing the origin due to the extreme variability in features. In light of this subspecific designations were re-evaluated eastern North America, specifically, the Eastern raccoon (*P. l. lotor*), Florida raccoon (*P. l. elucus*), Alabama raccoon (*P. l. varius*) and Upper Mississippi Valley raccoon (*P. l. hirtus*) (Figure 4.1) through mtDNA sequence analysis. If these designations are taxonomically valid, evidence of separate genetic lineages would be expected. If the genetic data do not agree with the subspecific designations, understanding how the genetic variation is partitioned is of considerable value. The raccoons in these regions are of substantial management importance due to the presence or threat of raccoon rabies, the largest documented wildlife epizootic in North America (Rupprecht and Smith 1994; Kirby 1995). Knowing where gene flow occurs can be used to focus raccoon management efforts in preventing further spread of raccoon rabies.

## Materials and Methods

#### Sample collection

Samples were obtained across eastern North America covering the ranges of four raccoon subspecies as displayed in Figure 4.1. Numerous agencies were involved in sample collection. They are listed in Table 4.1 with sampling state/provinces, number of samples, tissue type and location resolution. Hair and pelt samples were stored dry until DNA extraction and brain samples were stored in 1X lysis buffer which consists of 2 M urea, 0.1 M NaCl, 0.25% n-lauryl sarcosine, 5 mM 1,2, cyclohexanediaminetetraacetic acid and 0.05 M Tris HCl pH 8.



Figure 4.1. Map of eastern North America indicating approximate subspecific ranges (from Hall 1981) together with locations of raccoon samples. Symbols represent subspecific designations: ● Eastern raccoon, ▲ Alabama raccoon, ■ Upper Mississippi raccoon, ○ Florida raccoon.

Agency	Ν	State/Province	Tissue type	Location data
Canadian Food	6	New Brunswick, Ontario	Brain	County:
Inspection Agency				Resolution 100 km
Center for Disease	92	Florida, Alabama, Georgia,	Brain	State:
Control		Virginia		Resolution >100 km
Fur Harvesters	102	Florida, Iowa, Illinois, Kansas,	Pelt	Trap line:
Association		Louisiana, Maryland,		Resolution 10 km
		Michigan, Minnesota,		
		Missouri, Mississippi, New		
		Brunswick, Nebraska, Nova		
		Scotia, New York, Ohio,		
		Ontario, Quebec, Utah,		
		Wisconsin, Wyoming		
North American Fur	36	Arkansas, Georgia, Iowa,	Pelt	Trap line:
Auctions		Illinois, Indiana, Kansas,		Resolution 10 km
		Missouri, Mississippi		
New York	11	New York	Brain	Town:
Department of				Resolution 10 km
Health				
Ontario Ministry of	15	Ontario	Hair	UTM:
Natural Resources				Resolution 5 m
United States	46	Alabama, Florida, Kansas,	Hair	Lat/long :
Department of		Maine, North Carolina, New		Resolution 5 m
Agriculture –		York, Tennessee, Vermont		
Wildlife Services				

Table 4.1. Sample summary for subspecies analysis. Collecting agencies samples were obtained from, including numbers of individuals (N), state/province collected from, tissue type and location resolution.

### DNA extraction and Quantification

Samples were digested using 1X lysis buffer and 600 U/mL proteinase K. Extraction was carried out using an automated 96-well plate magnetic bead procedure; all liquid handling being carried out by an Evolution P3 (Perkin Elmer). Briefly, 50  $\mu$ L of sample lysate was mixed with 180  $\mu$ L Blood Lysis Buffer (Promega Corporation, Madison, WI) and 20  $\mu$ L of MagneSil<sup>®</sup> ONE (Promega) magnetic beads for 7 min. With DNA bound to the beads a magnet was applied to allow for waste to be discarded. The DNA/bead complex was then washed with 100  $\mu$ L of Promega wash buffer to remove lipids and proteins. After thorough mixing the magnet was once again applied and the waste discarded. Two more washes were carried out each using 140  $\mu$ L 70% ethanol to remove salts. The plate was then heated to 65°C for 2 min. to remove excess ethanol. DNA was eluted from the Magnesil<sup>®</sup> ONE beads by mixing with 100  $\mu$ L of 70°C 0.1 Tris-Ethylenediaminetetraacetic acid. The DNA was quantified using PicoGreen® (Invitrogen) which binds to double-stranded DNA and the level of emitted fluorescence is directly proportional to the quantity of DNA.

## PCR amplification

To ensure the most variable region of the mtDNA control region was assessed a 1400 bp fragment was amplified using the forward primer L15997 (Ward *et al.* 1991) and the reverse primer H00651 (Kocher *et al.* 1989). Polymerase chain reactions (PCR) were performed using 1ng of DNA, standard buffer conditions, 2 mM MgCl<sub>2</sub>, 160 uM of each dNTP, 0.3 uM of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen) and 30 cycles following steps: 94° C for 30 s, 60° C for 30 s, and 72° C for 30 s, preceded by 5 min of initial denaturing, and followed by 2 min of final extension. PCR products were

purified for sequencing using ExoSAP-IT (USB) following the manufacturers'

instructions. Sequencing using both forward and reverse primers was carried out using the DYEnamic<sup>TM</sup> ET terminator cycle sequencing kit, and the resulting fragments were analyzed on a MegaBACE 1000 (GE Healthcare). Fragments were visually inspected, corrected and aligned manually in BioEdit (Hall 1999) and from these sequences internal primers were designed. For the 5' segment, the forward primer L15997 and the reverse primer PLO-CR1R (CAGTTATGTGTGATCATGGG) were used to produce a 450 bp fragment; for the central portion, PLO-CR2F (ACATAACTGTGGTGTCATGC) and PLO-CR2R (AGGTATTTGTGCGTTCTGG) resulted in a 500 bp fragment; for the terminal end the reverse primer H00651 and the forward primer PLO-CR3F (CGTAACTCCAAGAACGCACAAA) were used which produced a 350 bp fragment. Each fragment was sequenced in two individuals to determine the most variable region; the 3' end of the initial fragment and the 5' end of the central portion were found to be the most variable. A new reverse primer was designed within the central fragment (PLO-CRL1 – CGCTTAAACTTATGTCCTGTAACC) to be used with the forward primer L15997 resulting in a fragment that was approximately 700 bp; sequencing this fragment resulted in 467 bp of clean sequence. Three hundred thirteen raccoon DNA samples were sequenced and haplotypes found in only one sample were sequenced in both directions for confirmation.

## Sequence analysis

Estimates of haplotype (H) and nucleotide ( $\pi$ ) variability were calculated for each subspecies group, where H is the probability that two randomly chosen individuals will not have the same haplotype, and  $\pi$  is the average number of nucleotide differences per

site between any two sequences (Nei and Kumar 2000). To test for in situ population growth, both Fu's *Fs* neutrality test (Fu 1997) and Tajima's *D* procedure (Tajima 1989) were applied. Fu's *Fs* is calculated by determining if there is an excess of recent mutations (excess of rare alleles) and negative values indicate significance (*Fs* should be considered significant if P-value < 0.02) (Fu 1997). Negative values of *D* also signify an excess of rare variants; in contrast to *Fs*, *D* looks at the number of segregating sites in relation to the average number of nucleotide differences between DNA sequences (Tajima 1989).

# Phylogenetic analysis

For phylogenetic analysis both distance and likelihood-based methods were employed as each carries different assumptions and congruence of the trees indicates strength in the data (Nei and Kumar 2000). The transition/transversion ratio and the  $\alpha$  parameter of the gamma distribution of rate heterogeneity were estimated using TREE-PUZZLE (v 5.0 – Schmidt *et al.* 2002). Maximum likelihood (ML) trees were drawn using 1000 quartet puzzling steps in TREE-PUZZLE. MEGA3 (Kumar *et al.* 2004) was used to calculate minimum evolution (ME) trees using the Kimura 2-parameter model and the calculated  $\alpha$ parameter of the gamma distribution, and significance of the trees was tested using 1000 bootstrap replicates. Sequence divergences between clades identified by the phylogenetic analyses were calculated in MEGA3 using the same model as above. Mismatch distributions were calculated in ARLEQUIN (Schneider *et al.* 2000) with 10 000 permutations for each subspecies. The pattern of mismatch distribution can indicate population growth (Rogers and Harpending 1992), and the patterns can also show if more than one lineage is present (Avise 2000).

#### Differentiation

Pair-wise genetic differences were calculated between subspecies to test for support of subspecific designations using  $\Phi_{ST}$  (Excoffier *et al.* 1992) an analogue of Wright's (1951)  $F_{ST}$ . Genetic differences were then used to formulate hypotheses of alternative groups and these were then tested for significance using an analysis of molecular variance (AMOVA – Excoffier *et al.* 1992). All above calculations implemented in ARLEQUIN (Schneider *et al.* 2000) were tested for significance using non-parametric permutation with 10 000 replicates.

In addition to the *a priori* subspecies groups,  $F_{ST}$  values were calculated between state/provinces where sample size was sufficient (N  $\ge$  10) to look at general connectivity across the range studied.

## Results

### Sequence analysis

A 467 bp fragment of the mitochondrial control region was used for all analyses. Three hundred thirteen samples were sequenced and 77 haplotypes were identified with 43 variable sites: 39 transitions, two transversions, one with both a transition and a transversion and one indel (GenBank accession numbers are listed in Table 4.2). Of those 77 haplotypes, 18 were shared among more than one subspecies range, with the remaining 59 confined to one of the four subspecies ranges (Table 4.2). Both haplotype and nucleotide diversity varied across the subspecies; haplotype diversity ranged from 0.841 - 0.969, and nucleotide diversity ranged from 0.007-0.014 (Table 4.3). The Alabama raccoon had the highest levels for both diversity measures, whereas the

Haplotype	State/Province	Lineage	GenBank
PLO4	Florida -4	Ι	EF030343
PLO5	Florida -3	Ι	EF030344
PLO13	Arkansas-1, Georgia -1, Louisiana -3, Minnesota -2, Missouri-1,	Ι	EF030345
	Mississippi -1, North Carolina -1, Ohio -1, Utah -1, Wisconsin -1,		
DI O14	Wyoming -1	т	EE030346
PLO14	Minnesota -2, Missouri -2, Wisconsin -4	I	EF030347
PL015	Minnesota-1, Wisconsin -1	I T	EF030348
PLOI6	Mississippi -1, North Carolina -2, wyoming-1	I T	EF030349
PLO1/	Arkansas -1, Louisiana -1, Wisconsin -2	I T	EF030350
PLO21	Iowa -1 Kansas -1 New York -2 Ontario -2 Ouebec -1 Vermont -	1	EF030351
PLO22	1, Wisconsin-2	Ι	LI 050551
PLO23	Ontario -4	Ι	EF030352
PLO24	Florida -1, Georgia -1	Ι	EF030353
PLO25	Florida -13	Ι	EF030354
PLO26	Florida -3	Ι	EF030355
PLO27	Alabama -2, Florida -3	Ι	EF030356
PLO28	Ontario -2	Ι	EF030357
PLO29	New York -1, Ontario -2, Vermont -1	Ι	EF030358
PLO32	Kansas -1, Minnesota-2	Ι	EF030359
PLO41	Missouri -1, Mississippi -1	Ι	EF030360
PLO42	Missouri -2	Ι	EF030361
PLO44	Mississippi -2	Ι	EF030362
PLO46	Maryland -1	Ι	EF030364
PLO47	Maryland -1	Ι	EF030365
PLO48	Alabama -2, Florida -13, Georgia -1, Missouri-3	Ι	EF030366
PLO49	Mississippi -2	Ι	EF030367
PLO51	Georgia -1, Utah -1	Ι	EF030368
PLO53	Florida-5	Ι	EF030369
PLO57	Minnesota -1	Ι	EF030370
PLO62	Alabama -3, Florida-2	Ι	EF030371
PLO63	Florida -4	Ι	EF030372
PLO68	Illinois -1	Ι	EF030373
PLO74	Georgia -1	Ι	EF030374
PLO75	Missouri -1, Nebraska -2	Ι	EF030375
PLO76	Arkansas -1	Ι	EF030376
PLO77	Florida -3	Ι	EF030377
PLO78	Florida -7	Ι	EF030378
PLO79	Florida -5	Ι	EF030379
PLO80	Florida -1	Ι	EF030380
PLO84	Georgia -1	Ι	EF030381
PLO85	Alabama -1, Florida -1	Ι	EF030382
PLO86	Georgia -1	Ι	EF030383
PLO88	Florida -2	Ι	EF030384
PLO89	Florida -2	Ι	EF030385
PLO90	Florida -4	Ι	EF030386
PLO91	Alabama -1	Ι	EF030387

Table 4.2. Haplotypes for subspecies analysis. List of haplotypes, their frequencies in each subspecies, their lineage (as determined from phylogenetic analysis) and GenBank accession numbers.

Haplotype	State/Province	Lineage	GenBank
PLO93	Florida -1	Ι	EF030388
PLO97	Alabama -1, Florida -1	Ι	EF030389
PLO99	Florida -2	Ι	EF030390
PLO104	Alabama -3	Ι	EF030391
PLO1	New York -5, Ontario -24	II	EF030392
PLO2	Alabama -5, Arkansas -1, Florida -2, Iowa -4, Illinois -2, Indiana -1, Kansas -5, Louisiana -2, Michigan -1, Minnesota -2, Missouri -1, New York -6, Ontario -8, Pennsylvania -1, Tennessee -1, Virginia -2, Wisconsin -2, Wyoming -10	II	EF030393
PLO3	New York -2	II	EF030394
PLO7	Iowa -2, Ontario -2, Pennsylvania-1	II	EF030395
PLO8	Vermont -1	II	EF030396
PLO10	Prince Edward Island -1	II	EF030397
PLO11	Nova Scotia -1	II	EF030398
PLO20	Florida -2	II	EF030399
PLO33	Indiana -1	II	EF030400
PLO34	Indiana -1	II	EF030401
PLO36	Iowa -1, New York -5, Ontario -1	II	EF030402
PLO38	Louisiana -2	II	EF030403
PLO40	Michigan -2	II	EF030404
PLO50	New York -2	II	EF030405
PLO52	New York -1	II	EF030406
PLO64	Utah -1	II	EF030407
PLO65	Georgia -1, Iowa -1	II	EF030408
PLO66	Kansas -1	II	EF030409
PLO67	Alabama -2	II	EF030410
PLO70	Alabama -2	II	EF030411
PLO72	Alabama -1	II	EF030412
PLO73	Ohio -1	II	EF030413
PLO82	Alabama -1	II	EF030414
PLO83	Alabama -1, Georgia -1	II	EF030415
PLO102	Alabama -1, Georgia -1	II	EF030416
PLO107	Alabama -1	II	EF030417
PLO108	Alabama -1	II	EF030418
PLO109	Alabama -1	II	EF030419
Lineage I	Eastern 0.28, Florida 0.95, Alabama 0.59, Mississippi 0.55		
Lineage II	Eastern 0.72, Florida 0.05, Alabama 0.41, Mississippi 0.45		

Table 4.3. Haplotype diversity measures for defined subspecies. Haplotype and nucleotide diversity, Tajima's *D* and Fu's *Fs* values calculated in ARLEQUIN with 10 000 permutation significance test for each subspecies and the entire data set. Haplotype and nucleotide diversity are presented with their confidence intervals. Tajima's *D* and Fu's *Fs* are presented with their p-values (significance assigned at p < 0.02 for Fu's *Fs*)

Subspecies	Haplotype	Nucleotide	Tajima's D	Fu's Fs
	Diversity	Diversity		
Eastern	0.861±0.024	0.010±0.006	0.190(0.653)	-5.05(0.078)
Florida	0.933±0.013	$0.007 \pm 0.004$	-1.043(0.145)	-8.43(0.005)
Alabama	0.969±0.011	0.136±0.007	0.262(0.669)	-15.9(<0.001)
Mississippi	0.841±0.037	0.011±0.006	0.792(0.828)	-3.51(0.142)
All	$0.945 {\pm} 0.008$	0.013±0.007	-0.224(0.482)	-24.7(0.0001)

Mississippi raccoon had the lowest value for haplotype diversity and the Florida raccoon had the lowest nucleotide diversity. Haplotype diversity was observed to be high, while nucleotide diversity was low. This can be indicative of rapid population growth, which results in an accumulation of new haplotypes without large sequence differences (Avise 2000). Overall *Fs* was highly significant with a value of -24.7 (p < 0.001); the statistic was negative for all of the subspecies and ranged from -3.51 to -15.9, but only Alabama and Florida had significant values (Table 4.3). Tajima's *D* was not significant for either the global test (-0.224, p = 0.482) or for any of the subspecies and ranged from -1.04-0.792 (Table 4.3), the null hypothesis of neutral evolution of the mtDNA control region sequences could not be rejected, suggesting the evolution of this region in these populations is not under strong selection.

#### *Phylogenetic analysis*

There was phylogenetic structure among the 77 haplotypes for both tree building methods. The ME tree displayed two lineages (50% bootstrap) and was in agreement with the ML tree, which for the same lineages indicated 91% bootstrap. The ML tree had a further lineage split from lineage I, but this was not supported by the ME tree and had lower bootstrap support. The un-rooted ML tree is displayed in Figure 4.2. The sequence divergence between the two lineages is  $2.2 \pm 0.6\%$ , while the within lineage divergence is  $1.1 \pm 0.2\%$  (Lineage I) and  $0.7 \pm 0.2\%$  (Lineage II). The overall mismatch distribution also supports the existence of two lineages as it is bimodal (Figure 4.3), which indicates two haplotype lineages. The haplotype distribution for the Florida raccoon indicates there is one primary lineage. The Eastern raccoon has two very distinct peaks indicating the



\_\_\_\_0.01\_\_\_\_

Figure 4.2. Unrooted maximum likelihood consensus tree from TREE-PUZZLE. Branches with bootstrap support are shown. Due to the number of haplotypes and the lack of resolution within lineages, haplotype names are not displayed. For a list of haplotypes and their respective lineages refer to Table 4.2.



Figure 4.3. Mismatch distributions of mutational differences between sequences calculated for subspecific ranges and entire dataset in ARLEQUIN (Excoiffier *et al.* 1992)

presence of both lineages with a higher frequency of more similar haplotypes, and from Table 4.2 lineage II is considerably more common than lineage I. For the Mississippi raccoon there is a greater frequency of dissimilar haplotypes as it has equal numbers of both lineage I and II. The Alabama raccoon, like the Mississippi raccoon, has equal numbers of both lineages I and II, but has greater diversity so its mismatch distribution is most similar to the pattern observed for the overall distribution.

## Differentiation

All pairwise genetic differences between subspecies regions were significant (Table 4.4) following sequential Bonferroni correction (Rice 1989). The difference between Florida and the other subspecies was 2-4 times greater than all other pairwise comparisons. Based on the  $\Phi_{ST}$  values, two AMOVAs were calculated, one with two groups: (1) Florida and (2) Alabama, Eastern and Upper Mississippi valley raccoons, and one with three groups: (1) Florida, (2) Eastern and (3) Alabama and Upper Mississippi valley. The two group comparison explained more variation than the three group comparison, with the global  $F_{ST}$  being 0.394 compared with 0.309.

Pairwise  $F_{ST}$  values were calculated for nine province/states using conventional Fstatistics since there is lineage mixing across the range and the sequence differences are not reflective of geographic separation. Results are listed in Table 4.5. Following Bonferonni correction for significance testing, no province/states sharing borders had significant  $F_{ST}$  values: Florida, Ontario and Wyoming were the most differentiated.

between the subspecies (lower diagonal), 1 values in the upper diagonal (10 000 permatations)						
Subspecies	Eastern	Florida	Alabama	Mississippi		
Eastern	-	< 0.001	< 0.001	< 0.001		
Florida	0.488	-	< 0.001	< 0.001		
Alabama	0.110	0.268	-	0.005		
Mississippi	0.127	0.429	0.052	-		

Table 4.4. Genetic differentiation among subspecies. Pairwise  $\Phi_{ST}$  values calculated in ARLEQUIN between the subspecies (lower diagonal), P-values in the upper diagonal (10 000 permutations)

Table 4.5. Pairwise genetic differences between sampling areas. Pairwise  $F_{ST}$  values calculated in Arlequin v3.1 (Excoffier *et al.* 2005) using conventional F statistics and 10 000 permutations for significance testing, above diagonal are P-values and below diagonal are  $F_{ST}$  values. Sample sizes for each province/state are indicated in brackets.  $F_{ST}$  values in bold are significant after Bonferroni correction (P = 0.001)

State/	Alabama	Florida	Georgia	Minnesota	Missouri	New	Ontario	Wisconsin	Wyoming
Province						York			
AL (29)		< 0.001	0.196	0.056	0.050	0.004	< 0.001	0.004	< 0.001
FL (84)	0.039		0.118	0.002	0.002	< 0.001	< 0.001	< 0.001	< 0.001
GA (10)	0.013	0.021		0.263	0.363	0.010	< 0.001	0.039	< 0.001
MN (10)	0.034	0.072	0.025		0.330	0.032	0.001	0.567	0.006
MO (11)	0.035	0.035	0.010	0.019		0.005	< 0.001	0.181	< 0.001
NY (24)	0.052	0.094	0.075	0.069	0.096		0.006	0.012	< 0.001
ON (45)	0.164	0.181	0.189	0.197	0.213	0.079		< 0.001	< 0.001
WI (12)	0.063	0.093	0.062	-0.014	0.033	0.086	0.214		< 0.001
WY (12)	0.211	0.278	0.351	0.262	0.337	0.217	0.358	0.308	

# Discussion

The results indicate the presence of two distinct, although shallow, mt lineages in raccoon populations across eastern North America. Lineages I and II were present in all subspecies ranges; however the frequencies differ markedly between the Florida and Eastern raccoon. The Florida raccoon had 95% lineage I and 5% lineage II, the Eastern raccoon had 28% lineage I and 72% lineage II, and the Alabama and Mississippi raccoon had almost equal representation of both lineages. The association of the two lineages across the majority of the study region suggests the historical processes that created the lineages is no longer effective and the historical signal is being erased by subsequent gene flow, both natural and human mediated.

# Phylogeography

It is not surprising the Florida raccoon is representative of primarily one lineage, resulting in strong genetic differences from the other subspecies; this pattern has been noted in a number of studies in a variety of plants and mammals (Avise 1989, 2000; Bulgin *et al.* 2003; Ellison *et al.* 2004; Hayes and Harrison 1992). Florida has been studied extensively due to its physiogeographic history; between approximately 200 000 and 70 000 years ago there were three to four time periods when sea levels were high enough to submerge portions of Florida separating it from the mainland (Cronin *et al.* 1987). This would have prevented gene flow from occurring between Florida and the mainland allowing for sequence differences to accumulate. The divergence between lineages I and II is  $2.2\pm0.6\%$ , and with an approximate estimate of 10-12% divergence per million years for the control region (Aquadro *et al.* 1982; Arborgast *et al.* 2001; She *et al.* 1990); the time of separation is approximately 130,000 to 280 000 years ago, which

is within the time frame of the Florida separation. Although Florida does represent a separate lineage for *Procyon*, the physiographic features that historically prevented gene flow are no longer effective as is evidenced by the occurrence of lineage I across eastern North America.

During the Pleistocene, lineage II was present in the habitable regions of the Eastern raccoon range since we see its dominance and few lineage I haplotypes at low frequencies. The presence of lineage I in the eastern range could be the result of natural dispersal, or human-mediated translocations. It has been well documented that thousands of raccoons were shipped from Florida to the Virginias for purposes of hunting (Nettles *et al.* 1979; Smith *et al.* 1984) which could account for the presence of lineage I. If this were the only source of lineage I one would expect the majority of lineage I haplotypes to be found in the areas receiving translocations. Although lineage I is found in North Carolina, 75% of lineage I haplotypes found in the eastern range occur in the northern states/provinces, including Maine, New Brunswick, New York, Ontario, Quebec and Vermont. This could indicate natural dispersal of the translocated raccoons; however this could also be the result of raccoons traveling considerable distances through hitch-hiking on transports and railway cars (Rosatte *et al.* submitted; Smith *et al.* 2002).

The Alabama and Upper Mississippi raccoons have equal representation of both lineages; however the Alabama raccoon has the greatest diversity, even though it is represented by the lowest sample size. This is surprising, as it would be expected that with increasing sample size there would be increased diversity (Kalinowski 2004). This implies the Alabama raccoon is the first region of admixture of the two lineages. The Upper Mississippi raccoon, unlike the Alabama raccoon, has the lowest haplotype

diversity. The raccoon was well established to the south and east of the United States by the end of the Pleistocene (Kurten and Anderson 1980), and prior to colonial times the raccoon was primarily found in the deciduous forests of the East and Gulf coasts and Great Lakes regions (Zeveloff 2002). This suggests the populations found in the Mississippi range are more recent. Further, the Mississippi raccoon has half the unique haplotypes than any of the other subspecies regions. The founding animals likely came from both the Alabama and Eastern raccoon since there are a number of shared haplotypes, and the most frequent haplotype in the Mississippi range is the second most frequent in the Eastern range.

#### Genetic differentiation

All pairwise genetic comparisons were large and significant involving the Florida subspecies; this is a result of the dominant presence of lineage I in Florida. The Eastern range also demonstrated strong genetic differences as indicated by the significant three group AMOVA, due to the high frequency of lineage II. Using the global  $F_{ST}$  value as irrefutable support for subspecies is not commonly used as there can often be significant values that are not large enough to indicate gene flow barriers. Palma *et al.* (2005) used a global  $F_{ST}$  of 0.256 to support evidence for subspecific structure in mice, whereas Drew *et al.* (2003) considered an  $F_{ST}$  of 0.518 between fisher populations to correspond to the subspecific designations but not indicate full support; rather they felt it indicated significant population subdivision, and likely reflected a pattern of isolation by distance. This is similar to Tomasik and Cook (2005) who found a Global  $F_{ST}$  of 0.206 to indicate significant population structure in wolverines, but felt this may not be reflected in other genetic markers due to male mediated gene flow.

Province/state  $F_{ST}$  comparisons do support the *a priori* subspecific groupings since most of the significant  $F_{ST}$  values are between regions from different subspecies, although this could be due to the large distances separating the areas included in the comparison, as well as effects of sample size, where small samples may not reflect accurate haplotype frequencies. Additionally many of the  $F_{ST}$  values are large, but not significant as a conservative test was used. For instance, the Ontario and New York comparison was not significant but the  $F_{ST}$  value is higher than any other comparison between adjacent regions; interestingly their borders are separated by two major rivers (Niagara and St. Lawrence) as well as Lake Ontario. These rivers are likely barriers to raccoon movement and are reducing the level of gene flow between these regions as rivers have been implicated as a barrier to raccoon movement (Grenfell 2002; Smith *et al.* 2002). This does indicate genetic population structure exists at a more regional scale and there are local effects of gene flow.

# Subspecific designation

The mitochondrial haplotype data do not correspond to the four subspecific designations. There appears to be two lineages represented by the Florida and Eastern raccoons. The Mississippi and Alabama ranges may have resulted from a mixing of the two lineages due to anthropogenic impacts on the landscape and raccoon population dynamics. The mixing of lineages due to anthropogenic impacts has also been noted in other species where the mixing has made it difficult to determine the pure lineages (Wayne *et al.* 1992; Buiteveld and Koelewijn 2006; Kyle *et al.* 2006). To fully investigate this issue for raccoons the use of additional data using nuclear markers such as microsatellites might provide insight into the strength of these genetic differences.

## Management implications

The raccoon populations of the eastern seaboard of North America have been infected with rabies since the late 1940's. The first documented case was in Florida and thereafter, sporadic cases were reported; by 1953 cases were found in Florida, Georgia and Alabama and it remained restricted in this area until 1977 (Rupprecht 1992). A new focus of rabies cases emerged in the Virginias and it is likely due to infected animals being translocated from the southeast for hunting purposes (Nettles et al. 1979; Smith et al. 1984). It is interesting that rabies remained in the southeast for approximately 30 years and once the new focus occurred in 1977 in less than 20 years almost the entire eastern seaboard of the United States was infected. Disease modeling also found a significant difference in the spatial and temporal behaviour of rabies infections between the southern and northern regions (Childs *et al.* 2001). The genetic data presented here adds additional evidence to suggest differences in dynamics among the ranges of the four subspecies. There is evidence of considerable movement to the north of the Florida range, but little movement occurring into the Florida range, similar to what we have seen with the movement of raccoon rabies. For managers to be successful in eradicating rabies the cause of this dispersal to the north needs to be addressed. There is evidence of substantial movement of raccoons by transport trailers and railway containers over large distances (Nettles et al. 1979; Smith et al. 2002; Rosatte et al. submitted). As a first step in management new legislation should be implemented requiring inspection before departure to ensure no hitch-hiking. Further assessment of the contribution of natural dispersal and impacts of landscape on dispersal is also required. This will involve future investigation with higher resolution genetic markers, such as microsatellites. Rabies-

affected regions found with substantial natural dispersal will require enhanced rabies surveillance and vaccination control.

The range of the Mississippi raccoon is currently free of raccoon rabies, but both the Eastern and Alabama ranges are epizootic. The presence of shared haplotypes between the northern ranges of the Eastern and Mississippi raccoons indicate dispersal between the ranges; whether this is natural dispersal or human mediated still needs to be addressed. The Mississippi and Alabama raccoon share a considerable number of haplotypes and have the lowest F<sub>ST</sub>. The evidence of substantial movement between epizootic and non-epizootic regions indicates the importance of rabies management for the Mississippi raccoon range. It would be unfortunate for a rabies outbreak to occur in the Mississippi range as there has been years of containment effort and considerable funding invested in raccoon rabies control and eradication (Bogel et al. 1992). Management plans for the Mississippi raccoon should include at a minimum, increased surveillance with plans in place for implementing vaccination programs upon the occurrence of rabid cases. The success of a vaccination program depends upon the effectiveness with which wildlife managers can respond to positive cases (Rosatte et al. 2001).

# **Chapter 5**

# Beyond Phylogeography: Multiple Processes Affecting Intraspecific Variation in a Continuously Distributed Species

#### Abstract

Typical phylogenetic analyses utilize the information in the sequence differences and frequencies of mitochondrial DNA haplotypes to infer historical demographic processes. However, when intraspecific variation reveals multiple lineages that no longer correspond to geographic isolation, alternative methods of investigation need to be employed. Analysis of sequence variation in the mitochondrial control region of 1106 raccoons (*Procyon lotor*) across eastern North America revealed three distinct sequence lineages. Based on their frequency differences across the study area their likely origins are glacial refugia during the Pleistocene in Florida (lineage I), the southern east coast (lineage II), and the south central states (lineage III). The isolating barrier between lineage II and III most likely is the Mississippi River. Following the Pleistocene glaciations, range expansions occurred to the north as determined from a significant correlation of haplotype and nucleotide diversities with latitude. Since then geographic and anthropogenic changes together with the raccoon's ability to utilize a variety of habitats appear to have led to lineage mixing across the eastern portion of the continent. Despite this mixing, spatial analysis of haplotypes revealed significant genetic structure and Mantel tests indicated a significant effect of geographic distance on genetic distance. However, a large amount of variation in genetic differences was still unexplained suggesting additional effects on genetic structure that are specific to regional landscapes. Further analysis at a regional level using Mantel and partial Mantel tests indicated major rivers, the Niagara and St. Lawrence, acted as significant barriers to gene flow. By

incorporating multiple analyses and a variety of scales an understanding of raccoon population history and current demographics has been accomplished.

# Introduction

Phylogeography, the study of the processes that dictate the structure and diversity of intraspecific lineages across geographic ranges, has grown considerably since its formal distinction as a field by Avise et al. (1987). Using this approach the historical processes that have shaped the genetic structure of numerous species have been defined, and these include, fragmentation due to biogeographic barriers (Tibbets and Dowling 1996; Burbrink et al. 2000; Brant and Ortí 2003; Runck and Cook 2005; Howes et al. 2006), range contraction and expansion due to glacial cycles (Tibbets and Dowling 1996; Bernatchez and Wilson 1998; Runck and Cook 2005; Howes et al. 2006; Kelly et al. 2006; Magoulas et al. 2006), colonization from multiple refugia (Wilson and Hebert 1998; Austin et al. 2002), and recent contact of historically separated lineages (Burbrink et al. 2000; Donovan et al. 2000; Omland et al. 2000). In addition, phylogenetics has been used to refine current subspecific designations for a number of species for conservation and management (Lansman et al. 1983; Lehman and Wayne 1991; Ellsworth *et al.* 1994; Ellison *et al.* 2004). Due to the diversity of applications and utility of this method there has been considerable research into determining the best methodological approach(es) to studying phylogeography.

The methods applied to phylogenetics are wide ranging, including tree building algorithms, nested clade analysis (NCA), population expansion statistics, population differentiation statistics and population clustering algorithms. Each of these methods provides insight into a particular process at a particular time frame. For instance, studies

of population structure across a large extent have traditionally relied on the use of distance, maximum likelihood and parsimony tree building methods (Avise 2000; Nei and Kumar 2002). Since these methods were developed for interspecific studies, they are informative only if there is a strong phylogenetic signal. For intraspecific studies they are often inappropriate; intraspecific data have internal nodes, where tree building assumes ancestral haplotypes are no longer present, and sequences are frequently not divergent enough to produce trees with statistical confidence (Crandall et al. 1994; Smouse 1998; Nei and Kumar 2002). There have been significant developments in phylogenetic analysis and methods more amendable to intraspecific data are being utilized. Nested clade analysis (Templeton et al. 1987, 1992, 1995; Templeton and Sing 1993) tests for geographic associations of closely related haplotypes, and this allows for hypothesis testing, including range expansion, isolation by distance, restricted gene flow and population bottlenecks. This hypothesis testing is based on a minimum spanning network of haplotypes, where significant looping of the network will prevent effective hypothesis testing. Crandall and Templeton (1993) developed rules to resolve looping in the network including haplotype frequency being associated with haplotype age, and the use of geography to resolve mutational connections.

To use these rules some assumptions regarding the data are made. Often studies do not have sufficient sample sizes to be confident in the frequencies (Excoffier and Smouse 1994), therefore they many not accurately reflect age, and using the geography to resolve mutations in the network is a circular argument since the same network is used to infer processes across that geography (Smouse 1998). Additional statistical measures to infer population histories include mismatch distribution analysis (Rogers and Harpending

1992), which infers population expansion, and neutrality tests (Tajima 1989; Fu 1997) that approximate the time of event with the expectation that population growth and decline will result in particular patterns of nucleotide site differences between individuals. These statistics are also used to infer population growth, so concordance among these three statistics will better support the conclusions (Ahrens *et al.* 2005). Haplotype and nucleotide diversity measures can indicate past population processes, for example if there is high haplotype diversity but low nucleotide diversity there has likely been rapid population growth which has resulted in haplotype recovery without the accumulation of large differences (Avise 2000).

Demographics can also be analyzed with the use of F-statistics (Wright 1951) to determine the level of population structure. This is especially useful when phylogenetic signals are weak, since it indicates which populations are potentially exchanging effective migrants. Many studies have taken this one step further to test for hypotheses of structure based on potential gene flow barriers (both current and historic) using an analysis of molecular variance (AMOVA- Excoffier *et al.* 1992). The problem with this type of approach is the *a priori* assumptions that are required for hypothesis testing and if the species history is unknown, hypothesis testing is difficult.

The use of the above methods together could not only define the relationships but also identify the processes that created the relationships. From this Bernatchez (2001) and Althoff and Pelmyr (2002) have effectively argued that these methods utilized together allow for a comprehensive historic and contemporary population analysis. This multistep approach has been utilized effectively in a number of studies (Hoffmann and Blouin 2004; Seagraves and Pelmyr 2004; Ahrens *et al.* 2005; Laffin *et al.* 2005). Although the

above methods have been effectively applied and provided insights for a considerable number of species, the next step forward is to utilize spatial analysis methods to make use of the geographic information more effectively. Kidd and Ritchie (2006) have recently made an effective argument to focus on the "geography" of phylogeography as the focus has primarily been on the "phylo" component. Utilizing geographic information more completely will particularly benefit studies where intraspecific phylogenetic signals are not strong enough or concordant with geography to make historical demographic inferences.

The raccoon (Procyon lotor) poses a challenge for phylogenetic analysis. Procyon lotor is found across most of North America from southern Canada to Mexico with exceptions at higher elevations, primarily the Rocky Mountains (Sanderson 1987; Gehrt 2003). Recent expansions have occurred in central Canada (Larivière 2004) and it is considered one of the most successful mammals of North America (Sanderson 1987; Gehrt 2003). It has often been found at unusually high densities in urban settings (Rosatte 2000; Smith and Engeman 2002). *Procyon lotor* is considered a relatively young species in the order Carnivora (Kurten and Anderson 1980; Bininda-Emonds et al. 1999) and the fossil record is scarce with the earliest records dated from the mid-Pleistocene (Kurten and Anderson 1980). Procyon lotor has not been studied extensively from a genetic perspective as it has not been a species of concern. Recently, however, populations along the eastern seaboard of North America have been seriously affected by a rabies virus strain specific to raccoons (Rupprecht and Smith 1994; Kirby 1995; Childs et al. 2000; Rosatte et al. 2001; Jackson and Wunner 2002). Understanding their history and current demographic situation could provide insight as to the best approach to

population management to control and eventually eradicate raccoon rabies from the eastern seaboard. Additionally *P. lotor* provides an opportunity to utilize all of the phylogenetic methods described above to study a species that is found across North America, which has a number of interesting biogeographic features including mountain ranges and major rivers, that have been important in forming the population structure in other species (Wilson and Hebert 1997; Bernatchez and Wilson 1998; Donovan *et al.* 2000; Burbrink *et al.* 2000; Weisrock and Janzen 2000; Austin *et al.* 2002; Brant and Ortí 2003; Ellison *et al.* 2004; Runck and Cook 2005; Howes *et al.* 2006; Kelly *et al.* 2006).

*Procyon lotor* populations have shown little genetic variation across the eastern seaboard (Dew and Kennedy 1980; Kennedy and Lindsay 1984; Hamilton and Kennedy 1987; White *et al.* 1998). Here I propose that species that have high densities, are mobile over large distances and are habitat generalists are not amenable to traditional phylogenetics. I will demonstrate that not all methods of analysis are appropriate as the choice of method will depend on the nature of the data. I will show how the use of demographic and spatial analyses can still lead to additional understanding of the species current population processes across central and eastern North America.

#### Materials and Methods

#### Sampling

Samples were obtained across the eastern and portions of central North America and were collected opportunistically through wildlife management agencies and fur dealers (Table 5.1). Samples collected by the Ontario Ministry of Natural Resources consisted of pulled hairs during their Trap-Vaccinate-Release program (TVR), and brain tissue, which

Table 5.1. Source and number of samples used for phylogeographic analysis for each province/state. Abbreviated forms of agencies are as follows: CDC, Centers for Disease Control; CFIA, Canadian Food Inspection Agency; FHA, Fur Harvesters Association; OMNR, Ontario Ministry of Natural Resources; NAFA, North American Fur Association; NYDOH, New York Department of Health; USDA-WS, United States Department of Agriculture – Wildlife Services.

	Sample sizes and sources						
PROV/STATE	CDC	CFIA	FHA	OMNR	NAFA	NYDOH	USDA-WS
AL	10						19
AR					4		
CA			1				
DE	1						
FL	75		6				3
GA	5				5		
IA			6		3		
ID					1		
IL			3		3		
IN			4		6		
KS			2		1		6
KY					2		
LA			8				
MA	2						
MD	3		14				
ME							15
MI			20		1		
MN			10				
МО			8		3		
MS			3		5		
NB		27	12				
NC			1				11
NE			1				
NH	2						
NJ			1				
NS			12				
NY			12			101	93
OH			28				
OK					1		
ON		57	155	89	117		
PA			10		1		3
PE			10				
QC			24				
SC	1						
TN					1		16
UT			3				
VA	8		10		1		
VT							13
WA					1		
WI			11		1		
WV	2		2				
WY			12				

were collected during their Point-Infection-Control (PIC) program (Rosatte *et al.* 1992, 1997, 2001). Samples collected by the United States Department of Agriculture, Wildlife Services also consisted of hairs collected during TVR, and ear punches collected from survey animals, which were typically road kills. Samples obtained from the Canadian Food Inspection Agency, the Centers for Disease Control and the New York Department of Health consisted of brain tissue preserved from surveys and positive rabies cases. Skin punches from pelt samples were obtained from both the North American Fur Harvesters Association and the Fur Harvester Auction. Pelt and hair samples were stored dry at room temperature until extraction; brain tissue and ear punches were stored at -20°C in 1X lysis buffer (2 M urea, 0.1 M NaCl, 0.25% n-lauryl sarcosine, 5 mM 1,2, cyclohexanediaminetetraacetic acid and 0.05 M Tris HCl pH 8).

Location data for the samples had different levels of resolution. Sample locations for all Ontario Ministry of Natural Resources and United States Department of Agriculture, Wildlife Services were obtained in the field using Geographic Positioning System instruments (5 m resolution). Samples from the New York Department of Health were referenced based on the city or town nearest to capture location. Samples obtained from the Centers for Disease Control were referenced to their state of origin and were assigned the centroids of the state. Samples from Canadian Food Inspection Agency were assigned to the county in which they were found and were geo-referenced at the county centroid. All samples from fur houses were referenced back to the trapper and the trap line to which that trapper was assigned.

#### DNA extraction and PCR amplification

Samples were dissolved using 1 X lysis buffer and digested with 600 U/mL proteinase K. Extraction of genomic DNA was carried out using an automated 96-well plate magnetic bead procedure as outlined in Chapter 4; all liquid handling was carried out by an automated Evolution P3 pipettor (Perkin Elmer). Quantity of DNA was determined using PicoGreen® (Invitrogen) where the level of emitted fluorescence is directly proportional to the quantity of DNA.

A 700 bp nucleotide segment of the mitochondrial control region was amplified using the forward primer L15997 (Ward *et al.* 1991) and the reverse primer PLOCR-L1 (described in Chapter 4). Polymerase chain reactions (PCR) were performed using 1ng of template DNA, standard buffer conditions, 2 mM MgCl<sub>2</sub>, 100 uM of each dNTP, 0.3 uM of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). The cycling protocol was initiated with 5 min. at 94°C for full denaturing followed by 30 cycles of 94° C for 30 s, 60° C for 30 s, and 72° C for 30 s, and finishing with a 2 min final extension. ExoSAP-IT (USB) was used to prepare PCR products for sequencing following the manufacturers' instructions. Sequencing using the reverse primers was carried out using the DYEnamic<sup>TM</sup> ET terminator cycle sequencing kit, and the resulting fragments were analyzed on a MegaBACE 1000 (GE Healthcare). Fragments were visually inspected, corrected and aligned manually in BioEdit (Hall 1999). For haplotypes found in only one sample, samples were sequenced with the forward primer to confirm the sequence.

# Phylogenetic Trees

Sequences were first analyzed in Modeltest v3.7 (Posada and Crandall 1998) to determine the best model of nucleotide evolution. Modeltest chooses among 56 different

models and Akaike's information criterion (AIC) was used to select the best model that fit the data (Posada and Buckley 2004). Using the parameters estimated in Modeltest, TREE-PUZZLE v5.0 (Schmidt *et al.* 2002) was used to calculate a maximum likelihood (ML) tree with 10 000 quartet puzzling steps. MEGA3 (Kumar *et al.* 2004) was implemented to calculate both neighbour-joining (NJ) and maximum parsimony (MP) trees again using the model estimates from Modeltest where appropriate.

# Nested Clade Analysis

A minimum spanning network of all haplotypes was calculated using TCS (Clement *et al.* 2000), which utilizes the 95% statistical parsimony method of Templeton *et al.* (1992). This network was then used to create one-step clades beginning at the tips and working inward to the more ancestral haplotypes. The clades were tested for significant associations of haplotypes with geography to infer population processes that have created the current population structure. Significance testing is performed by random permutations of the data against a null hypothesis of a random geographic distribution using GeoDis (Posada *et al.* 2000). Looping between haplotypes is resolved based on the criteria of Crandall and Templeton (1993) as summarized in Pfenninger and Posada (2002).

### Spatial Group Assignment

Samples were assigned to spatial groups with the goal of minimizing assumptions about population structure while maximizing the sample size per unit. Spatial groups were assigned in ArcView3.3 (ESRI) following these guidelines: the optimal goal was  $\geq$ 20 individuals per group, but samples were not grouped, if they were separated by more than 120 km. If units could be created with N $\geq$ 20 with less distance separating them they

were broken down into smaller geographic groups. Where possible, landscape features that potentially restricted raccoon movement, i.e. elevation and large rivers, were taken into account. Polygons were drawn around sample points to simplify visual presentation. *Demographics* 

For each spatial group, estimates of haplotype (H), and nucleotide ( $\pi$ ) diversity were calculated. H is the probability two randomly chosen individuals will not share the same haplotype and  $\pi$  is the average number of nucleotide differences per site between two sequences (Nei and Kumar 2002). To test for *in situ* population growth, Fu's *Fs* neutrality test (Fu 1997) was applied. This test is calculated by determining if there is an excess of recent mutations, negative values of Fu's *Fs* indicates population growth and should be considered significant if P-value  $\leq 0.02$  (Fu 1997). All calculations were performed in ARLEQUIN v3.1 (Excoffier *et al.* 2005) with 10 000 iterations. *Spatial Analysis* 

Significant population structure was detected without the use of *a priori* assumptions by using the program, Spatial Analysis of Molecular Variance (SAMOVA 1.0– Dupanloup *et al.* 2002). This program uses a simulated annealing approach to find geographically continuous groups that are maximally differentiated where the level of differentiation is measured using AMOVA (Excoffier *et al.* 1992). The program iteratively seeks for a user-defined number of groups (*K*) that maximizes  $F_{CT}$ , the proportion of total genetic variance resulting from differences among groups of spatial groups, and minimizes  $F_{SC}$ , the proportion of total genetic variance shared between spatial groups within groups. Simulations were run with 10 000 iterations each with 200 random initial starting conditions. To select for the optimal group configuration I chose

*K* as the minimum value when  $F_{SC}$  displayed a large decline before reaching a plateau. Since smaller sample sizes do not properly reflect haplotype frequencies and SAMOVA uses this information to form the groups, I performed a second SAMOVA using only spatial groups with sample sizes  $\geq 20$ .

To understand the effect of geographic distance and rivers on the genetic structure of raccoons, Mantel and partial Mantel tests were conducted using *zt* (Bonnet and Van de Peer 2002). Pairwise genetic distances were calculated using  $\Phi_{ST}$ , an analogue of  $F_{ST}$  (Wright 1951), in Arlequin. Pairwise distances between spatial groups were calculated based on the centroids of the polygons in the statistical package R v2.3.1 (2006). Mantel tests assessing the relationship between geographic and genetic distance were performed for the full set of 40 spatial groups and the subset of spatial groups with N≥20. Partial Mantel tests were performed using rivers as an explanatory variable for the N ≥ 20 spatial groups using the following major rivers: Chattahoochee, Connecticut, Niagara, Potomac, St. Lawrence, Savannah, and the Mississippi system. For the Ontario and New York data, both Mantel and partial Mantels were used to test the effects of geographic distance and major rivers, specifically, the Niagara and St. Lawrence, both collectively and individually.

# Results

#### Sequences

From the 700 bp fragment, 467 bp of clean sequence from the mt control region was analyzed in 1106 samples from across the range of *P. lotor*, of these, 111 haplotypes were identified. Forty-nine variable sites were found among the 111 haplotypes, comprising 43 transitions, two transversions, two indels, one transition/transversion and one

deletion/transition. Of these 111 haplotypes, 77 have been previously identified (GenBank Accession numbers: EF030343-EF030419) and the 34 new haplotypes have been assigned GenBank accession numbers (EF517136-EF517169).

# Phylogenetic Analysis

The twelve best models of nucleotide evolution found in Modeltest based on AIC selection criteria were all within less than six AIC units of the minimum, indicating model selection uncertainty (Burnham and Anderson 2002). All of these models included proportion of invariable sites (I) and the gamma distribution shape parameter (G) and differed in their nucleotide substitution; large differences (>21 AIC units) did occur once the parameter G was removed. This indicates that the best model needs to include I and G and the nucleotide substitution model used will not make a large difference. Using this information I chose the HKY model of substitution as it was found to be optimal and the estimated values of I and G from Modeltest (0.802 and 0.460, respectively) were used to build both the ML and NJ trees. The NJ tree displayed poor resolution with very little bootstrap support for the majority of the branches. Using a maximum likelihood algorithm however, revealed phylogenetic structure with bootstrap support (Figure 5.1). Three lineages of sequences were supported, two of which were previously identified in Chapter 4 as lineage I and II; accordingly, the third group is designated here as lineage III. Using the parameters from Modeltest, mean net sequence distances among lineages I and II were 1.30% as well as between II and III; lineages I and III differed by 0.90% (Figure 5.1). The frequencies of each lineage in each state/province are displayed visually in Figure 5.2.



Figure 5.1. Maximum likelihood consensus tree indicating three ancestral lineages in *Procyon lotor* obtained from the analysis of 467bp of mt control region sequence. Due to the lack of within lineage structure only the bootstrap values for the lineages are displayed. As well, due to the large number of haplotypes their designations are not displayed.


Figure 5.2. Distribution of *Procyon lotor* mitochondrial lineages (Lineage I -  $\bullet$ , Lineage II -  $\circ$ , Lineage III -  $\bullet$ ) in eastern and central North America. Pie diagrams are shown for each state and province. Sample sizes are indicated for each.

## Nested Clade Analysis

The haplotype network produced by TCS had a considerable amount of looping. Following the rules to resolve these ambiguities as summarized in Pfenninger and Posada (2002), the first and second rules utilize the haplotype frequencies where older haplotypes will be more frequent and have more mutational connections, but without knowing if they have been sufficiently sampled, their estimated frequencies may be inaccurate and more importantly there are a large number of low frequency haplotypes involved in the looping. The third rule indicating new haplotypes are not likely to be found in a distant population is also inapplicable for resolving the ambiguities as a number of low frequency haplotypes are found in distant populations. Due to the inability to resolve the ambiguities found in the network I was unable to proceed with NCA to test hypothesis of range expansion, founder events and long distance colonization.

## Spatial Group Assignment

A total of 40 spatial groups were formed (Figure 5.4), their sample sizes ranging from 8-83 (Table 5.2), 18 of these had an N  $\ge$  20. These spatial groups were used for all spatial analyses.

## **Demographics**

Nucleotide diversity within spatial groups ranged from 0.000 - 0.014, and haplotype diversity ranged from 0.000 - 1.00 (Table 5.2), where the lowest values for both were found on Navy Island, ON (Group 36, N = 23), and the highest values were found in Alabama (Group 3, N = 27) and Georgia (Group 2, N = 10). Nucleotide and haplotype diversity were significantly, negatively correlated with latitude with r values of -0.326 (p

ŜG	Ν	π	Н	Fs		
1	83	0.007±0.004	0.932±0.013	$-3.40 \times 10^{20} (< 0.001)$		
2	10	0.014±0.008	1.00±0.045	-18.5 (<0.001)		
3	27	0.014±0.007	0.943±0.025	-24.8 (<0.001)		
4	8	0.012±.007	0.929±0.084	-8.48 (<0.001)		
5	21	0.013±0.007	0.933±0.031	-21.1 (<0.001)		
6	8	0.006±0.004	0.643±0.184	-4.83 (0.006)		
7	19	0.009±0.005	0.848±0.059	-15.0 (<0.001)		
8	32	0.010±0.005	0.804±0.069	-20.3 (<0.001)		
9	10	0.001±0.001	0.200±0.154	-2.50 (0.016)		
10	12	0.001±0.001	0.303±0.147	-2.72 (0.010)		
11	46	0.009±0.005	0.456±0.065	-0.370 (0.448)		
12	8	0.004±0.003	0.250±0.180	-0.872 (0.103)		
13	17	0.010±0.006	0.559±0.083	0.490 (0.686)		
14	18	0.013±0.007	0.922±0.039	-15.4 (<0.001)		
15	19	0.011±0.006	0.766±0.059	-4.17 (0.023)		
16	38	$0.008 \pm 0.005$	$0.809 \pm 0.040$	-13.2 (<0.001)		
17	16	$0.008 \pm 0.005$	0.575±0.112	-1.61 (0.168)		
18	30	0.010±0.006	$0.860 \pm 0.044$	-18.0 (<0.001)		
19	15	0.009±0.005	0.771±0.072	-5.05 (0.006)		
20	10	$0.009 \pm 0.006$	0.911±0.077	-9.27 (<0.001)		
21	12	$0.010 \pm 0.006$	$0.864 \pm 0.072$	-7.57 (0.005)		
22	10	0.012±0.007	0.756±0.129	-5.11 (0.006)		
23	11	0.012±0.007	0.909±0.066	-7.11 (0.002)		
24	10	0.009±0.006	0.911±0.062	-8.64 (<0.001)		
25	33	0.013±0.007	$0.748 \pm 0.440$	-4.47 (0.034)		
26	51	$0.006 \pm 0.004$	$0.457 \pm 0.080$	-3.79 (0.078)		
27	60	$0.000 \pm 0.000$	0.977±0.052	-6.68 (<0.001)		
28	30	0.012±0.007	0.572±0.052	-1.77 (0.242)		
29	20	$0.013 \pm 0.007$	0.621±0.063	-1.55 (0.268)		
30	36	0.012±0.006	0.767±0.041	-3.03 (0.131)		
31	27	0.010±0.006	0.801±0.051	-7.81 (0.001)		
32	19	0.011±0.006	0.714±0.074	-1.41 (0.278)		
33	51	$0.007 \pm 0.004$	0.516±0.069	-1.01 (0.407)		
34	9	$0.006 \pm 0.004$	0.639±0.126	-2.44 (0.074)		
35	14	$0.007 \pm 0.004$	0.494±0.151	-3.82 (0.010)		
36	23	0.000	0.000	NA		
37	19	0.008±0.005	0.813±0.067	-10.9 (<0.001)		
38	19	0.011±0.006	0.854±0.043	-8.23 (<0.001)		
39	22	0.006±0.003	0.762±0.077	-7.93 (<0.001)		
40	41	$0.004 \pm 0.003$	0.700±0.045	-10.1 (<0.001)		

Table 5.2. Diversity measures for each spatial group. Sample size (N), nucleotide diversity ( $\pi$ ), haplotype diversity (H), Fu's (1987) *Fs* for the 40 spatial groups (SG) as calculated in Arlequin v3.1. Confidence intervals for nucleotide and haplotype diversity are included, and P values for *Fs* are included in brackets (P values  $\leq 0.02$  are considered significant and are bolded).

= 0.040) and -0.539 (p  $\leq$  0.001) (respectively). Of the 40 spatial groups, 17 were found to have significantly negative *Fs* values after Bonferonni correction (p  $\leq$  0.001) indicating positive population growth. Interestingly, none of the spatial groups found north of 44.5° N latitude had *Fs* values that were significant and only two spatial groups south of 40.0° N latitude were not significant.

## Spatial Analysis

SAMOVA was carried out using all spatial groups and only spatial groups with N  $\geq$ 20 with similar results. For the 40 spatial group comparison,  $\Phi_{SC}$  exhibited the greatest change at 14 groups (Table 5.3, Figures 6a and 7). There was not an associated decrease for  $\Phi_{CT}$ , as discussed later in the Results and Discussion. This combination explained 33.4% of the variation among groups, while 64.9% was due to within-spatial-group variance. Using only spatial groups with N > 20, the 18 spatial group comparison resulted in the greatest change in the  $\Phi_{SC}$  value at seven groups (Table 5.3, Figure 5.3b) (again without an associated decrease for  $\Phi_{\rm CT}$ ), with results consistent with the grouping using 40 spatial groups. The variation explained by these groups is 35.5% and similar to the 40 spatial group analysis; there is a large amount of variance within spatial groups (61.4%). Both AMOVAs resulted in large significant global  $\Phi_{ST}$  values (40 spatial group -0.351 (p < 0.001), 18 spatial group -0.386 (p < 0.001)). There were two differences from the 40 spatial group comparison; spatial groups 25 and 28 grouped with 29 and 30, where before they formed two separate groups, and spatial group 18 grouped with spatial groups 8, 16, 31, 33, 39 and 40, where as before it grouped with 19, 22, and 23, none of which were included in the  $N \ge 20$  analysis.

Table 5.3. Output for SAMOVA analysis. The values of within-group variation ( $F_{SC}$ ), between-group variation ( $F_{ST}$ ), and among-group variation ( $F_{CT}$ ) for the 40 and N≥20 spatial group analyses calculated in SAMOVA for all number of groups tested (K). Both  $\Phi$  and conventional F statistics are presented. For the value of K where the within-group value is minimized and among-group is maximized is bolded for each data set.

Number of Groups (K)	<b>Φ</b> F-statistics			<b>Conventional F-statistics</b>		
40 spatial groups	F <sub>SC</sub>	F <sub>ST</sub>	<b>F</b> <sub>CT</sub>	F <sub>SC</sub>	F <sub>ST</sub>	<b>F</b> <sub>CT</sub>
2	0.255	0.516	0.351	0.274	0.255	-0.026
3	0.250	0.512	0.350	0.270	0.269	-0.002
4	0.184	0.466	0.345	0.253	0.294	0.055
5	0.170	0.458	0.347	0.255	0.289	0.046
6	0.153	0.446	0.346	0.236	0.304	0.088
7	0.160	0.449	0.344	0.240	0.302	0.081
8	0.152	0.444	0.344	0.218	0.318	0.129
9	0.154	0.443	0.342	0.220	0.316	0.123
10	0.114	0.417	0.342	0.170	0.324	0.186
11	0.115	0.417	0.342	0.172	0.323	0.183
12	0.117	0.416	0.339	0.173	0.323	0.181
13	0.113	0.408	0.333	0.170	0.319	0.179
14	0.025	0.351	0.334	0.094	0.288	0.214
15	0.026	0.351	0.334	0.101	0.288	0.208
16	0.017	0.346	0.335	0.092	0.285	0.212
17	0.002	0.336	0.334	0.064	0.278	0.229
18	0.002	0.336	0.334	0.062	0.278	0.231
19	-0.005	0.331	0.335	0.053	0.275	0.234
N≥20 spatial groups						
2	0.245	0.550	0.405	0.286	0.264	-0.029
3	0.232	0.524	0.381	0.288	0.262	-0.036
4	0.188	0.495	0.378	0.255	0.316	0.083
5	0.182	0.477	0.361	0.256	0.309	0.072
6	0.130	0.436	0.352	0.191	0.327	0.168
7	0.058	0.386	0.348	0.127	0.306	0.205
8	0.047	0.379	0.348	0.128	0.301	0.198
9	0.042	0.375	0.348	0.112	0.299	0.211
10	0.025	0.365	0.349	0.093	0.293	0.221
11	0.019	0.362	0.350	0.077	0.291	0.231
12	0.016	0.357	0.346	0.173	0.322	0.181





Figure 5.3. Analysis of molecular variance values for (a) 40 group and (b)  $N \ge 20$  group SAMOVA analyses. Both  $\Phi$  and F statistics for the groupings are shown.



Figure 5.4. Forty spatial groups for *Procyon lotor* sampled across central and eastern North America formed to minimize assumptions about population structure while maximizing sample size; colors correspond to group membership for the K=14 SAMOVA analysis for the 40 spatial groups.

SAMOVA uses  $\Phi$  F statistics because they use both haplotype frequency and sequence distances in their estimation making them more powerful for sequence data (Excoffier *et al.* 1992); however for *P. lotor* the lineages do not reflect geographic separation and this information does not reflect population structure. To correct for this I tested the group structure for all the SAMOVA outputs using only conventional F-statistics in Arlequin and the results are presented in Table 5.3. Removing the impact of sequence differences reduces the noise in the F<sub>CT</sub> values; as the number of groups increased, F<sub>CT</sub> steadily increased then reached a plateau as expected, rather than what was seen when using the sequence information (Figure 5.3). The number of K groups for each of the SAMOVA analyses remained the same as when using the  $\Phi$  F-statistics.

Mantel tests showed that genetic distance co-varied with geographic separation for both the full (40 spatial groups) dataset and spatial group subset with N  $\ge$  20 (r=0.290, p=0.003 and r=0.400, p=0.030, respectively). When testing for the effect of rivers with a partial Mantel test using the N  $\ge$  20 spatial groups, rivers did not explain any of the variation (r = 0.066, p = 0.241). Similarly, river effects for Ontario and New York were not significant using either Mantel (r = 0.160, p = 0.138) or partial Mantel testing (r = 0.150, p = 0.107). By contrast, partial Mantel tests when looking at the effects of Niagara and St. Lawrence Rivers individually were significant (r = 0.460, p = 0.010 and r = 0.730, p = 0.025, respectively) while the Mantel tests of geographic distance were not (r = 0.140, p = 0.361 and r = -0.250, p = 0.308).

## Discussion

*Procyon lotor* presents a species where the use of some traditional phylogeographic methods was inadequate, but the incorporation of phylogenetic, ecological and spatial

data provided an improved understanding of historic and current population demographics of raccoons. The following is a discussion of the origin of these clades, how their distribution has been affected by historical events, both natural and anthropogenic and how the current frequencies of haplotypes can be used to infer processes of genetic exchange between regions.

The presence of three evolutionarily distinct lineages as evident from the ML tree clearly indicates historical geographic separation which has allowed for the loss of intermediate haplotypes (Avise et al. 1987). P. lotor was affected by glaciations during the Pleistocene and would have survived in different refugia as other species did in North America (Ellsworth et al. 1994; Riddle 1996; Wooding and Ward 1997; Bernatchez and Wilson 1998; Burbrink et al. 2000; Donovan et al. 2000; Austin et al. 2002; Brant and Orti 2003; Lessa et al. 2003; Runck and Cook 2005; Guralnick 2006; Howes et al. 2006; Kelly et al. 2006). Although the geographic distributions of the lineages are no longer clearly defined, there exist sufficient differences in lineage frequencies to make inference as to the areas of potential refugia. Lineage I is found most frequently in Florida, where only 5% of the haplotypes found belong to other lineages. Cullingham et al. (in preparation) suggested the sea-level changes (Cronin 1987) and the widening of the Apalachicola River during the Pleistocene resulted in isolation of Florida allowing the formation of this separate genetic lineage. Lineage II is widespread along the east coast of North America and the formation of this and lineage III could have resulted from one of two potential geographic barriers that have been implicated for other species; the Appalachian Mountains or the Mississippi River (Burbrink et al. 2000; Brant and Orti 2003; Runck and Cook 2005; Howes et al. 2006). Although raccoons are able swimmers

(Sanderson 1987; Rosatte 2000; Gehrt 2003), the Mississippi River is the most likely isolating barrier as it was considerably wider then as a result of the glacial melting during the Pleistocene (Pielou 1991). Additionally, one would expect high frequencies of lineage III to the west of the isolating barrier and low frequencies of lineage II; this is what we do see with the Mississippi River and not the Appalachian Mountains (Figure 5.2).

The landscape changed following the Pleistocene and more evergreen forest developed in northern North America where glaciers had been present (Pielou 1991). As these changes occurred it is not unreasonable to hypothesize P. lotor colonized new areas expanding from their refugium. Tests of range expansions are often conducted using NCA (Templeton et al. 1987, 1992, 1995; Templeton and Sing 1993; Gomez-Zurita et al. 2000; Brant and Orti 2003; Fuerst and Austin 2004; Etter et al. 2005); however, due to the geographic inconsistencies of the lineages, I was unable to resolve the ambiguities in the TCS network using Crandall and Templeton's (1993) criteria. Rather, I utilized the information contained in the haplotype and nucleotide diversity data to make inference of population expansion after glacial retreat. Similar to Bernatchez and Wilson (1998) who found that populations of various fish species located north of 46° N latitude showed lowered levels of diversity as a result of colonization after glacial retreat; P. lotor spatial groups show a significant, negative correlation of both haplotype and nucleotide diversities with latitude. In addition, Fs values were found significant only south of 42° N, which fits closely to 40° N, the extent of the Laurentide ice sheet in North America (Hewitt 1993). These data are consistent with the hypothesis of southern refugial

populations expanding post-Pleistocene and that *P. lotor* continues to expand its range northward (Lariviere 2004).

As *P. lotor* expanded into new areas, the landscape saw further changes with the narrowing of major rivers, including the Mississippi and Apalachicola, as glacial melt water dissipated (Pielou 1991). Procyon lotor being a capable swimmer, and the narrowing of these rivers would diminish their effectiveness in isolating lineages as it had previously. Movement across these barriers by P. lotor over time would create a category II phylogeographic pattern of secondary admixture (Avise et al. 1987). For many species, secondary contact is restricted to the areas adjacent to the isolating barrier (Cronin et al. 1991; Taberlet et al. 1992; Wooding and Ward 1997), but this would be dependent on the time since the barrier has been crossed and on dispersal capability (Hewitt 1993) of the species. In fact, for a number of highly vagile species we see considerable geographic spread of common haplotypes (Wayne et al. 1990; Cronin et al. 1991; Arctander et al. 1996; Wooding and Ward 1997, Kyle et al. 2006) similar to the distribution of *P. lotor* lineages. However, *P. lotor* does not have a large average dispersal distance (Hoffman and Gottschang 1977; Fritzell 1978; Seidensticker et al. 1988; Prange et al. 2004); although anthropogenic effects may have further contributed to admixture of genetic lineages.

A considerable amount of landscape changes have occurred since the 1800's when Europeans began colonizing the east coast of North America (Williams 1992), but changes in the past 100 years have had the most significant impacts on *P. lotor* populations. The first effect has been on population size; in the 1930's raccoon population densities were considerably lower (Sanderson 1987; Gehrt 2003) due to loss

of traditional denning sites from deforestation, but by the 1940's population levels rose sharply (Sanderson 1987; Gehrt 2003). This growth was concomitant with a number of events that may have allowed for an increase in numbers. The extirpation of wolves represented the loss of a significant predator (Zeveloff 2002), enabling the raccoon to make use of open productive habitats like agricultural fields in the Great Plains. As well, forest re-growth due to the abandonment of agriculture (Williams 1990, 1992) caused an increase in traditional habitat, and an increase in urbanization (Riebsame 1990) also provided more resources for raccoons allowing for an increase in numbers. The second and conceivably a more significant impact on population structure was the improvement of human transport. This has allowed for the long distance translocations of thousands of animals from the south into the mid- and north-east (Nettles *et al.* 1979; Smith *et al.* 1984), as well as hitchhikers on railway cars and transports to traverse hundreds of kilometres (Smith *et al.* 2002; Real *et al.* 2005; Rosatte *et al.* submitted).

The extensive mixing of lineages across the range of *P. lotor* may suggest little population structure, but I found using SAMOVA at a large extent and Mantel tests at large and small extents that there is considerable structure. Since the sequence differences do not represent geographic distributions, the spatial population genetic structure was analyzed using conventional F statistics. This measure of differentiation is based on the variance in the haplotype frequencies, where frequencies can be strongly influenced by sample sizes (Kalinowski 2004). This could result in aberrant structure in the spatial analysis. To reduce the sample size from being the cause of anomalous structuring, I performed analysis on only those spatial groups with over twenty

individuals in addition to the initial analysis. I found consistent results between the two analyses indicating that significant structure exists.

It is difficult to determine what factors are affecting each region to create genetic structure when looking at a species across a large extent where there are numerous ecotypes and geographic features. Geographic distance explains a significant portion of the variance in  $F_{ST}$  values between the spatial groups. This implies natural dispersal over time and the translocation of animals across large distances has not been frequent enough to have had a homogenizing effect on mitochondrial genetic structure (Hedrick 2000). However we do see with the spatial groups some geographic inconsistencies from the SAMOVA (e.g. groups 6, 18 and 31, Figure 5.4); this could still be the result of sample size effects, but it could possibly reflect actual population structure that has resulted from long distance movements of raccoons (either natural or human-mediated).

The amount of variation left unexplained in  $F_{ST}$  values by geographic distance is considerable, but because the region is so large it is difficult to test what factors are creating the genetic structure with the present sample distribution. Physiographic effects will vary across the landscape (Cullinan and Thomas 1992; Hobbs 2003) and will then contribute to genetic differences only at a more local scale and therefore not create a strong enough signal to affect the entire study area. To look at the importance of local effects, Mantel and partial Mantel tests were used to analyze Ontario and New York since there were sufficient sample sizes in this region and two major rivers that could be potential barriers to movement. In these regions, the rivers had significant effects on the genetic structure of raccoon populations, but only when examined at each of the regions around the rivers separately. This demonstrates how the scale of the analysis can have

significant impacts on the result and the genetic structure is dependent on local physiogeographic effects.

Here I have demonstrated that using a comprehensive data set one can elucidate a diverse amount of information about population processes, from large scale historic events to current local physiographic effects. There is no single best method for studying the phylogeographic history of species; rather, the approach should be considered based on the scale of the analysis, what information needs to be obtained as well as the ecological information for the species in question.

# **Chapter 6**

# Direct and Indirect Genetic Methods of Estimating Dispersal: Applications for Raccoon Rabies Management

## Abstract

Predictive disease models can be useful tools in assessing management actions to efficiently control wildlife disease, but without accurate estimates of ecological parameters, simulated outcomes will not reflect biological reality. One of the critical parameters in modeling disease spread is dispersal of the vector population. Dispersal will determine the dynamics of disease spread; unfortunately dispersal of wildlife is one of the most difficult parameters to measure. To obtain an accurate measure of dispersal methods of genetic parentage assignment and analysis of spatial genetic structure were applied to two raccoon populations in Ontario, Niagara (N = 296) and St. Lawrence (N =593), that are at the front of the North American raccoon rabies epizootic. Using all available genetic and ecological data, a distribution of dispersal distances for raccoons were calculated. The majority of raccoons both male and female move < 3 km, although there was an observed effect of sex on dispersal, where females show stronger philopatry than males. The maximum dispersal distances of a few animals exceeded 20 km. Analysis of spatial genetic structure provided a similar assessment indicating stronger genetic structure for juveniles vs. adults, and females vs. males. Without the inclusion of life stage data, the results of the spatial genetic analysis could be misinterpreted due to the timing of juvenile dispersal and sample collection. This dispersal information could now be applied to predictive disease modeling to test management actions in preventing future rabies outbreaks and disease control and eradication.

## Introduction

Models in animal and plant epidemiology are valuable as they give us the tools to forecast future disease outbreaks (Dwyer and Elkinton 1995; Russell et al. 2005), investigate impacts of disease on naïve populations (Deal et al. 2000; Garner et al. 2006), determine causes of disease spread (Guerra et al. 2003; Russell et al. 2005), and explore the effectiveness of different management scenarios on disease eradication (Barlow 1993; White et al. 1995; Moore 1999; Broadfoot et al. 2001; Jin and Wang 2005; Sterner and Smith 2006). However, the accuracy of the output on which we rely depends on how well the model mimics the natural system (Bacon 1985; Hilborn and Mangel 1997; Rushton et al. 2000; Burnham and Anderson 2002). The models utilized in epidemiology range from simple SIR (susceptible, infected, recovered) to complex individual, spatially explicit, stochastic systems; and as our computational abilities improve, model complexity continues to grow (Efron and Tibshirani 1993; Mollison et al. 1994; Hilborn and Mangel 1997; Elliot et al. 2000; Grenfell et al. 2002). Independent of model complexity, there appear to be key parameters that will strongly influence model accuracy. For example, the transmission rate, often represented as  $R_0$ , the basic reproductive number (Mollison 1985; Mollison *et al.* 1994; Stemer and Smith 2006) or  $\beta$ , the disease transmission co-efficient (Barlow 1993), are critical in determining rate of spread, but these parameters are difficult to measure (Macdonald and Voigt 1985; White et al. 1995; Tompkins et al. 2002; Sterner and Smith 2006). The difficulty is primarily due to the influence of a number of other parameters including density, contact rate, pathogenicity and host dispersal (Anderson and May 1978, 1991; Voigt et al. 1985; Coyne et al. 1989; Arneburg et al. 1998; Hess et al. 2002; Tompkins et al. 2002;

Macdonald and Laurenson 2006) of which some are difficult to measure, i.e. contact rate and host dispersal. Host dispersal is a particularly important factor in disease transmission, as it is a critical parameter in determining rates of disease spread.

Knowing the hosts' dispersal patterns, including frequency and distance, will allow a model to assess how quickly a disease front can move (Barlow 1993; Deal *et al.* 2000; Wang and Mulone 2003; Jin and Wang 2005), whether it will move at all (Castillo-Chavez and Yakubu 2001; Wang and Mulone 2003; Jin and Wang 2005), what potential there is for long distance movements to create new out-breaks ahead of a disease front (White *et al.* 1995; Filipe and Maule 2004), what threat dispersal poses to the "reintroduction" of disease once an epizootic has passed (Macdonald and Voigt 1985), and the effectiveness of disease control strategies (Barlow 1996). Additionally the timing of dispersal will determine when disease fronts move, which can have a considerable impact on control strategies (Lomnikci 1978; Bacon 1985).

Dispersal is an important parameter not only in disease behaviour but in almost all ecological interactions (Bohonak 1999; Caro 1999; Clobert *et al.* 2001; Proctor *et al.* 2004; Pospíšková and Šálková 2006; Sork and Smouse 2006), but unfortunately there is a paucity of dispersal data due to the difficulties in their measurement. Direct field methods based on mark-recapture tend to result in inadequate estimates due to the limitations on size of study area (Koenig *et al.* 1996; Bohonak 1999). Indirect methods using genetic data have more recently been employed, but they do not provide actual distances; rather, they give an average rate of movement over many generations between genetically differentiated populations (Hedrick 2000), which often represents historical processes rather than contemporary gene exchange (Sork *et al.* 1999). In addition, they

are based on a number of assumptions, many of which are not met in natural populations including, equal population sizes, equal migration rates between populations and negligible levels of mutation (Bossart and Prowell 1998; Whitlock and McCauley 1999; Rousset 2002). An alternative to the above methods of dispersal estimation involves parentage assignment using unique individual genotypes. The intent is to identify parentoffspring pairs and measure the distance between them after dispersal has occurred.

Parentage assignment offers advantages over indirect genetic approaches as it can quantify both the distances offspring disperse as well as the frequencies of those distances; furthermore by measuring dispersal directly one can capture movement that may not result in successful gene flow (Sork et al. 1998). This is advantageous when applying the information to a disease situation because a dispersal event can have an impact on disease spread regardless of breeding success. Parentage assignment as a method of measuring dispersal was first used in plant systems (Sork et al. 1998, 1999; Trapnell and Hamrick 2005; Pospíšková and Šálková 2006) where both pollen and seed dispersal could be measured giving insight into the scale of population processes. This method has seen limited use for mammalian species (Telfer et al. 2003; Proctor et al. 2004; Waser et al. 2006); the time lag to its application in mammalian systems is related to the ease in measuring spatial locations; whereas plants are stationary, mammals have the ability to move. The incorporation of global positioning systems (GPS) and geographical information systems (GIS) in ecological analyses has improved our abilities to accurately measure parent-offspring distances.

Raccoon rabies is the largest documented epizootic with the infected region covering the east coast of North America from Florida into southern Quebec. Since raccoons

(*Procvon lotor*) are abundant in urban areas with dense human populations (Prange *et al.*) 2003; Smith and Engeman 2002; Riley et al. 1998; Rosatte et al. 1992), there is an increased risk of human-rabies contact (Rupprecht 1992) causing a considerable impact on human health spending in the form of post-exposure treatment (Kreindel *et al.* 1998; Chang et al. 2002). As a result, numerous models have been developed to study the spread of rabies, but most have been constructed to fit a particular outbreak that has already occurred (Moore 1999; Smith et al. 2002; Guerra et al. 2003; Russell et al. 2004). What is needed is a predictive model adaptable to different ecological conditions where management scenarios can be tested for disease eradication (Voigt et al. 1985; Hess et al. 2002). Juvenile dispersal in raccoons has not been well characterized; most field studies have been conducted measuring raccoon movement but they involved measuring home range movements and foraging distances (Hoffman and Gottschang 1977; Fritzell 1978; Seidensticker et al. 1988; Prange et al. 2004), and movement distances of translocated raccoons (Rosatte and MacInnes 1989; Belant 1992; Mosillo et al. 1999). The one study that has focused on juvenile dispersal obtained distances for only 20 radio-collared individuals of which only the males (N = 8) dispersed (Gehrt and Fritzell 1998a).

To provide accurate dispersal estimates for disease modeling I profiled raccoon samples collected by the Ontario Ministry of Natural Resources (OMNR) during their control programs in 2003, Trap-Vaccinate-Release (TVR) and Point-Infection-Control (PIC) (Rosatte *et al.* 1992, 1997, 2001). Both direct and indirect estimation methods were used through parentage analysis and spatial genetic analysis, respectively. It is difficult in natural systems to sample individuals sufficiently to enable successful parentage analysis. Comparing a highly accurate dispersal distribution determined from parentage analysis to results provided by spatial genetic analysis will validate the applicability of more recently developed indirect methods (Hardy and Vekemans 2002; Vekemans and Hardy 2004) which are more feasible methods of dispersal analysis in natural populations than parentage.

## Materials and Methods

#### Sample collection

Samples were obtained from two study areas, Niagara (NIA) and St. Lawrence (STL). Raccoon samples were collected by OMNR staff during their routine TVR program from July – October 2003 across an approximate 530 km<sup>2</sup> area in NIA. Samples consisted of pulled hairs and were stored dry in envelopes until extraction. In STL, samples were collected during both TVR and point infection control (PIC) programs from April – July 2003 over an area of 670 km<sup>2</sup>, TVR samples again were pulled hairs, samples from the PIC consisted of brain tissue stored in 1X lysis buffer (2 M urea, 0.1 M NaCl, 0.25% nlauryl sarcosine, 5 mM 1,2, cyclohexanediaminetetraacetic acid and 0.05 M Tris HCl pH 8). Information for all collected samples included: date, sex, age class, Universe Transverse Mercator (UTM) locations, ear tag information, previous capture status, and vaccination status.

#### Genotyping

Dry hairs were immersed in 1X lysis buffer and follicles treated with 25  $\mu$ L proteinaseK (600 U/mL) left overnight at 37°C. Brain tissue stored in 1X lysis buffer was digested using 25  $\mu$ L proteinaseK incubated for 2 hrs at 65° C and following, an additional 25  $\mu$ L left overnight at 37° C. DNA was extracted using an automated magnetic bead procedure from 50  $\mu$ L of tissue lysate and quantified following procedures

outlined in Cullingham *et al.* (2006). Ten highly polymorphic microsatellite loci developed specifically for the raccoon were used to profile samples using three multiplex reactions described in Cullingham *et al.* (2006). Results from the multiplex reactions were obtained using the MegaBACE 1000 (GE Healthcare), a 96 capillary electrophoresis system, and the raw data was processed in Genetic Profiler 2.0 software (GE Healthcare) to assign locus-specific genotypes.

### Parentage Analyses

Parentage assessment was achieved using two programs; CERVUS (Marshal *et al.* 1998) and NEWPAT (Wilmer *et al.* 1999). CERVUS uses a maximum likelihood approach to generate log-likelihood scores for assigning paternity at a specified level of statistical confidence. The simulation incorporates user-defined parameters including the frequency of errors in the data and confidence levels (80% - relaxed and 95% - strict). To maximize the number of mother-offspring dyads identified, all adult females were considered potential mothers while all females and males (both juvenile and adult) were potential offspring; analysis for each region conducted separately. Allele frequencies were calculated in CERVUS using all samples in the data set; mothers were assigned to potential offspring using an observed genotyping error rate of 0.001. Mother-offspring pairs that were assigned at the 95% confidence level (CL) and were compatible at all loci were the only ones considered. All pairs that were assigned at the 95% CL that were mismatched at one locus were retained for comparison of dispersal distribution to the zero mismatch pairs.

NEWPAT uses Queller and Goodnight's (1989) relatedness coefficient to determine potential fathers and utilizes a randomization approach to determine statistical

confidence. Since NEWPAT was designed to determine paternity given motheroffspring pairs, but has the option of entering only offspring without maternal information, all potential offspring (males, females, adults, juveniles) were entered and compared to all adult females to assign maternity rather than paternity. For these analyses mismatches were not accepted, a low acceptance of probability of null matches (p = 0.01) was used and all individuals had complete profiles. The intent was to use the most stringent conditions in finding mother-offspring pairs to ensure an accurate dispersal distribution; therefore, the assignments generated by NEWPAT were used only to corroborate the dyads identified in CERVUS. If the search for mothers of adult females resulted in duplicates, i.e. mother A-offspring B, and vice-versa, only one was retained. The choice of which pair to retain was unimportant as they would represent the same dispersal distance.

## Dispersal Distances

Trapping information was obtained from the OMNR for the years 2001-2003 for NIA and 1999-2003 for STL to obtain all location history of the individuals assigned to mother-offspring dyads. UTM locations for mothers and offspring were plotted in ArcView 3.3 (ESRI) and pairwise distances between all locations were calculated in metres using the Animal Movement extension (Hooge and Eichenlaub 2000). Three distributions of distances were calculated: zero mismatch, one mismatch and individual distances; the zero and one mismatch distances were calculated as follows: provided that the mother and offspring had either single locations or were trapped multiple times at the same location in the year of birth, the direct distance of juvenile to mother was used. In instances where an individual was recorded at multiple locations, the distance between

average locations were taken, unless distances indicated pre and post juvenile dispersal. To calculate individual distances, where individuals had multiple locations over time, a maximum-distance moved by that individual was calculated. This distribution was compared to the zero and one mismatch dispersal distances. Due to the non-normality of dispersal data, a non-parametric analysis of variance, PERNOVA (Anderson 2001), was used to test for differences in the dispersal distributions and to look for sex and age effects on the distributions in each region. PERNOVA allows for the comparison of multiple effects similar to ANOVA and statistical testing is performed through permutation. Since PERNOVA requires a balanced design, groups exceeding the smallest sample size were randomized and sub-sampled. Post-hoc analysis of where differences lie was also performed analogous to a t-test (Anderson 2001).

#### Spatial Genetic Analysis

Indirect dispersal estimates were inferred by analyzing the spatial genetic structure of each region using Spatial Pattern Analysis of Genetic Diversity (SPAGeDi v1.2 –Hardy and Vekemans 2002). SPAGeDi estimates pairwise genetic relatedness between individuals and associates those estimates with spatial distances. These values can be tested for isolation by distance through permutation of individual locations among all individuals resulting in what is effectively a Mantel test of isolation by distance (IBD; Hardy and Vekemans 2002). For permutation of locations, SPAGeDi provides one- and two-tailed tests at each distance class allowing the user to determine at what distance individuals are more related than would be expected by chance (H<sub>1</sub>: observed > expected), and are less related than would be expected by chance (H<sub>2</sub>: observed < expected). These output values were used to infer dispersal estimates rather than the

analysis of the correlogram as they are commonly misinterpreted (Vekemans and Hardy 2004). As well, since Bonferroni corrections are applied, the test is conservative (Rice 1989). For both NIA and STL ten distance classes were selected allowing the program to designate the distance intervals to provide approximately equal numbers of individual pairs. Additionally, analysis using distance intervals of 1 km up to 5 km were used to define more precisely the distance at which individuals are, on average, more similar than two random individuals. Ritland's (1996) kinship co-efficient was used to calculate relatedness. This estimator is correlation based and does not make assumptions about the degree of relatedness in the population (Ritland 1996). It also shows low sampling variance and high power when highly polymorphic markers are used (Lynch and Ritland 1999; Vekemans and Hardy 2004). Isolation by distance was performed by testing individual locations permuted among all individuals; significance was tested using 3000 permutations (1000 permutations allows for significance testing at  $\alpha = 5\%$ ; Hardy and Vekemans 2002). To test for female philopatry, females and males were analyzed separately for both regions, with the expectation that females would be more related than males. In addition, since there were equal numbers of juveniles and adults for NIA, samples were separated into sex and age class to test for effects on genetic structure due to potential pre-dispersal sampling, where juvenile genetic relatedness would be expected to be higher than adult.

## Results

#### Parentage Analyses

Complete profiles were obtained for 296 individuals in the NIA region consisting of 70 male juveniles, 79 female juveniles, 67 male adults, and 80 female adults, and 593

individuals trapped in the STL with 17 male juveniles, 11 female juveniles, 301 male adults and 264 female adults; because the trapping in the STL was focused in the spring (only 24% of samples were captured in June and July), fewer juveniles in comparison to adults were collected since juveniles do not emerge until June/July (Sanderson 1987; Rosatte 2000; Gehrt 2003). Using the multi-locus genotypes, CERVUS calculated the total exclusionary power of finding one parent not knowing the second to be 0.9997. Using allele frequencies calculated from the entire data set, CERVUS identified 18 mother-offspring pairs at the 95% CL with zero mismatches for NIA; of these 18 pairs, 15 involved juveniles (11 females, four males) and three involved adults (two females, one male). An additional 32 mother-offspring pairs were identified at the 95% CL with one mismatch consisting of 15 juveniles (seven females, eight males) and 17 adults (seven females and ten males). For the STL data set CERVUS identified 101 motheroffspring pairs with zero mismatches, 21 of the pairs were with juveniles (seven female, 14 male) and 80 of the pairs were with adults (34 females, 46 males), of the 46 males, four were representative of mother-son pairs as determined from the trapping history, two are likely father-daughter and the remaining 40 could not be determined. One hundred three mother-offspring pairs with one mismatch were identified at the 95% CL, all involving adults (49 females, 54 males).

## Dispersal Distances

The dispersal distributions for zero mismatch dyads, single mismatch dyads, and individual movements are presented for NIA and STL in Figure 6.1. For STL, zero mismatch, one mismatch and individual distances were found to be significantly different from each other (p = 0.001, n = 55) using a one-way PERNOVA. Post-hoc analysis



Figure 6.1. Dispersal distributions of raccoons in Ontario as calculated in ArcView3.3.  $\bullet$  - are the dispersal distances calculated between mother-offspring pairs determined using CERVUS with zero mismatches,  $\bullet$  - are the distribution calculated for individual movements over time, and  $\blacksquare$  - are the dispersal values calculated for mother-offspring pairs with one mismatch between genotypes for both Niagara (8a) and St. Lawrence (8b) raccoons.

indicated the one mismatch distribution was significantly different from zero mismatch (p = 0.001) and individual distance distributions (p = 0.001). The effect of sex was significant only for a one-way PERNOVA for the zero mismatch distribution (p = 0.044, n = 41). Sex/age effects could be tested only for the zero mismatch distribution due to small sample sizes of juveniles and no significant effects were found (sex: p = 0.933, age: p = 0.064, sex/age: p = 0.724, n = 7). Since all juveniles may not have dispersed prior to trapping, comparisons were performed using only adult mother-offspring pairs for zero and one mismatches to ensure distances were dispersal events and did not represent home-range movements. For individual distances only measured distances across years were included (n = 20). Again the distributions were significantly different from each other (p = 0.001, n = 20) with the post-hoc analysis indicating one mismatch was significantly different from both zero mismatch (p = 0.007) and individual movements (p= 0.001). Since the zero mismatch and individual distributions were not significantly different the data were pooled to test for effects of sex but they were not significant (p =0.148, n = 48). For NIA the distributions were significantly different (p = 0.018, n = 18), where individual distances were significantly different from zero mismatch (p=0.040) and one mismatch (p=0.002). Due to the small sample sizes, tests for effects of sex were conducted for only zero mismatch and individual distances where no significant effects were found, and for one mismatch there were no significant effects of sex or age from the two-way PERNOVA.

## Spatial Genetic Analysis

The kinship co-efficient for individuals in both STL and NIA showed a negative linear relationship with the logarithm of spatial distance (NIA: b = -0.008, p < 0.001;

STL: b = -0.004, p < 0.001) indicating an effect of isolation by distance. Average kinship values greater than expected were found for both NIA and STL at the first distance class (NIA: 0.00 - 4.41 km, p < 0.001; STL: 0.00 - 4.46 km, p < 0.001) suggesting the majority of raccoon dispersal is less than 5 km. For NIA, average kinship values less than expected were found at distance classes 7-10 (17.7 - 21.3 km, p < 0.001, 21.3 - 25.5)km, p < 0.001, 25.5 – 29.5 km, p = 0.002, 29.5 – 48.2 km, p < 0.001), similarly, STL distance classes nine and ten (16.7 - 22.8 km, p = 0.001, 22.9 - 36.6 km, p < 0.001) had lower than average kinship values suggesting infrequent dispersal of raccoons at these distances. Analyses of females and males separately resulted in similar findings for both regions where females have a higher average relatedness values than males (Figure 6.2a) and for males none of the upper distance classes had significantly lower average kinship values than expected. Looking at spatial genetic structure for 1 km intervals up to 5 km resulted in greater than average values of kinship up to 4 km for NIA females (> 1 km, p < 0.001, 2 km, p < 0.001, 3 km, p = 0.008, 4 km, p = 0.016), up to 1 km for NIA males (p < 0.001) and up to 2 km for both males and females in the STL (p < 0.001 for all comparisons) (Figure 6.2b).

Separating the NIA data into four categories (adult female – AF, adult male – AM, juvenile female – JF, and juvenile male – JM) indicates the average relatedness of juveniles was higher than that of the adults (Figure 6.3) with juvenile females showing the strongest genetic structure and adult males showing no genetic structure.

## Discussion

Models of disease dynamics for use in disease management must contain the appropriate parameters with realistic values (Bacon 1985). We know dispersal is a





Figure 6.2. Average genetic relatedness values calculated using Ritlands (1996) estimate for males and females for Niagara and St. Lawrence raccoons. (9a) Relatedness values calculated across the entire study area for ten distance classes (as determined by the program SPAGeDi to ensure equal sample sizes). (9b) Relatedness values calculated for five distance classes at 1km intervals.



Figure 6.3. Average genetic relatedness values calculated using Ritlands (1996) estimate for separate age and sex classes in the Niagara region for ten distance classes across the entire study area.

critical behaviour as it determines the rate and timing at which individuals will interact, representing potential for disease spread. With this in mind both direct and indirect genetic methods (parentage and spatial genetic structure, respectively) were used to characterize raccoon dispersal. The dispersal distributions calculated from the parentage analyses suggested the majority of raccoons do not move far (> 80%, < 3 km), but have the capability to move greater than 20 km. Spatial genetic analysis of the data support these findings as I found only at short distances (< 1 km) do average genetic kinship values exceed what would be expected at random. There does appear to be an effect of sex on dispersal distributions supported by the spatial genetic analysis, but the parentage analysis suggests a many females and males remain philopatric (Switzer 1997; Sutherland *et al.* 2000).

#### Parentage analysis

Parentage analyses using both CERVUS and NEWPAT resulted in similar findings for both NIA and STL with only a few additional dyads identified in NEWPAT. These methods were used as they calculate the probability of paternity using different estimators, but can both implement exclusion as an additional criterion. By only using pairs that fit with exclusion criteria, this may have increased the likelihood of type II errors, but this decreases the sample size, and minimized the risk of type I errors. The use of single mismatch dyads that are most likely at the 95% CL in CERVUS are often considered reasonable as they could be attributed to mutation, genotyping or data entry error (Marshal *et al.* 1998; Wilmer *et al.* 1999; Slate *et al.* 2000; Oddou-Muratorio *et al.* 2003; DeWoody *et al.* 2006; Vandeputte 2006), but the dispersal distributions for single mismatch dyads in the STL region does not support this. The dispersal distributions for STL mismatch dyads were significantly different from both zero mismatch and individual movements, with considerably larger distances found for one-mismatch. Additionally male and female distributions were not significantly different, where data from field observations suggested that females are more philopatric and do not disperse frequently at larger distances (Seidensticker *et al.* 1988; Gehrt and Fritzell 1998a). This indicates mutation/null alleles are not at a level to bias genetic parentage assessment (Dakin and Avise 2004; Pospíšková and Šálková 2006) and these data have error levels <0.001. Interestingly the same was not found for NIA, where zero mismatch and one mismatch were not significantly different. Although this could be attributed to smaller sample sizes, the NIA data were obtained entirely from hair samples, whereas only 32% of the STL data were obtained from hairs. Non-invasively collected samples could be prone to higher levels of genotyping error (Goossens *et al.* 1998; DeYoung and Honeycutt 2005; Waits and Paetkau 2005; Broquet *et al.* 2007).

## Dispersal

The literature suggests that juvenile raccoons do not disperse during the autumn or winter but prior to their first breeding season, especially to the north where the climate is harsh (Shirer and Fitch 1970; Sanderson 1987; Gehrt and Fritzell 1998a; Whitaker and Hamilton 1998). These data suggest that juvenile dispersal occurs in the first year as there were no effects of age on the STL distribution. However, the spatial genetic structure for NIA of both juvenile males and females, in comparison to adult males and females, show higher relatedness values indicating a portion of individuals have not yet dispersed which is most apparent in the male comparisons where the adults show no genetic structure and juveniles show higher average relatedness than adult females.

I found a significant effect of sex on the STL zero mismatch dispersal distribution and the spatial genetic structure of females was stronger for both STL and NIA than male structure supporting female philopatry. As well, at distances beyond approximately 23 km (STL) and 27 km (NIA) females were on average less related than expected indicating very infrequent dispersal of females beyond those distances. In contrast the adult males in both regions were not found to be less related on average than expected (maximum distances: NIA- 45 km, STL- 36 km) suggesting more frequent long distance male dispersal. The NIA dispersal data do not suggest an effect of sex on dispersal; however, the data set is considerably smaller. Although the data do support female philopatry, they do not suggest all females are philopatric or that all males disperse, contrary to Seidensticker *et al.* (1988) and Gehrt and Fritzell (1998a), who found females were entirely philopatric and all males dispersed.

Previous studies measuring raccoon dispersal have been limited in numbers of individuals, which have made the estimation of a frequency distribution of distances imprecise. This parameter is critical for accurate disease modeling as distances moved play an important role in disease spread where the most frequent distances moved will determine the rate the disease front moves (White *et al.* 1995; Filipe and Maule 2004) and many long distance movements could lead to an increased risk of reintroduction of rabies once it has passed through an area (Macdonald and Voigt 1985). While the data suggest the majority of raccoon dispersal is at short distances, there have been instances where raccoons travelled distances over 100 km in short periods of time (Lynch 1967; Belant 1992; Mosillo *et al.* 1999). Most of these were translocated animals, but the ability to travel far could play an important role in disease spread. Long-distance

movements of raccoons have already caused rabies outbreaks ahead of the disease front. However, these were human mediated (Nettles *et al.* 1979; Smith *et al.* 2002) and are therefore difficult to incorporate into a predictive model. This implicates juvenile dispersal as one of the primary measurable movement parameters for predicting disease spread (White *et al.*1995).

## Conclusion

Juvenile dispersal determined by genetic parentage assignment has been compared to mark-recapture data and been found to be a better measure since pre-capture dispersal of juveniles is often missed (Telfer *et al.* 2003; Waser *et al.* 2006). However, I have found that although I was able to successfully generate a reasonable dispersal distribution, this would not have been obtained without access to capture data. Additionally, interpretation of spatial genetic structure will provide a general assessment of dispersal behaviour, conditional on having ecological data (Double *et al.* 2005; Latch and Rhodes 2006). Without having information on age class, I would not have been able to separate out effects for the spatial genetic structure, making the interpretation of the dispersal behaviour different for NIA and STL. This study effectively demonstrates the importance of a multidisciplinary approach to answering ecological questions.

While obtaining a dispersal distribution for raccoons has been successful, it is based on movements of healthy individuals. Rabies is a disease, which is characterized by infection of the nervous system and the brain causing neuronal dysfunction (Jackson 2002) resulting in behavioural changes, including increased alertness, loss of natural timidity, aberrant sexual behaviour and aggressiveness (Johnson 1971). These behavioural changes could result in dispersal movements greater than those expected

which could result in an underestimation of disease spread in a predictive model (White *et al.* 1995; Sterner and Smith 2006).

# Chapter 7

# Analysis of Raccoon Genetic Structure in Two Regions Reveals Effects of Rivers and Resource Distribution

## Abstract

Understanding how the landscape affects the genetic structure of species is important in the conservation of threatened and endangered species, but the methods employed can also be of use in species affected by disease. Identifying features of the landscape that create genetic discontinuities suggests dispersal barriers, which might be exploited for establishing vaccination zones. Raccoons in two regions, Niagara (N=744) and St. Lawrence (N=802) at the front of a rabies epizootic, were genotyped at ten highly polymorphic microsatellite loci to quantify the effects of the Niagara and St. Lawrence rivers as barriers to gene flow. Genetic clusters were identified in each region using STRUCTURE and additional individual analysis was performed using SPAGeDi to quantify the spatial genetic structure. In the Niagara region, two clusters were identified by STRUCTURE corresponding to either side of the Niagara River. Spatial genetic analysis of individuals indicated relatedness values greater than expected at distances up to 45 km and less than expected beyond 60 km. For St. Lawrence, STRUCTURE was unable to identify any spatially congruent clusters, and spatial genetic analysis indicated relatedness beyond 7 km was no different than expected and at no distance were values less than expected (up to a maximum of 115 km). The differences in the spatial genetic structure between the study sites may indicate differences in behaviour in response to resource differences. Management efforts targeted at raccoon rabies will have to make adjustments for regional differences as dispersal behaviours are likely to vary dependent on habitat.
## Introduction

Dispersal of individuals in wildlife populations is an important process allowing connectivity among populations and preventing populations from becoming genetically isolated. Individuals of a species may have the ability to disperse long distances, but whether they do so is influenced by the cost or benefit associated with that movement. Studies have shown that for some species habitat fragmentation can have a significant impact (Kyle and Strobeck 2001; Driscoll and Hardy 2005; Mcrae et al. 2005; Antolin et al. 2006; Broquet et al. 2006; Keyghobadi et al. 2006; Riley et al. 2006). Fragmentation could be caused by either natural barriers to movement, like mountain ridges (Lougheed et al. 1999; Rueness et al. 2003; Funk et al. 2005) or rivers (Lugon-Moulin and Hausser 2002; Ernest et al. 2003), or anthropogenic changes such as major highways (Coulon et al. 2006; Riley et al. 2006) or general urbanization (Kyle and Strobeck 2001; Driscoll and Hardy 2005; Mcrae et al. 2005). All of these situations can have significant impacts on demography and population genetic structure, and it is in the understanding of the processes creating this structure that the field of landscape genetics has evolved (Manel et al. 2003; Holdregger and Wagner 2006; Storfer et al. 2007). The appropriate choice of a landscape genetic method has become a challenge due to the array of techniques available (Storfer et al. 2007) but investigators should tailor the method to the species being investigated; for species that are strongly affected by habitat types because of dispersal limitations and/or specializations, a complex least-cost modeling approach (Michels et al. 2001; Coulon et al. 2004; Spear et al. 2004) may be the most appropriate method to define all potential factors affecting dispersal and ultimately population

structure. However if the species is a generalist and has the ability to disperse through a varied habitat matrix, it is more appropriate to look at large-scale effects.

*Procyon lotor* (common raccoon) is a medium-sized habitat generalist occurring across most of North America except in the higher elevations of the Rocky Mountains and in northern Canada (Sanderson 1987; Gehrt 2003). Even though raccoons generally have small home ranges of less than 4 km<sup>2</sup> (Hoffman and Gottschang 1977; Seidensticker et al. 1988; Rosatte 2000) they do have the ability to traverse great distances; studies of translocated raccoons found they moved upwards of 20 km after relocation in attempts to return to their original capture sites (Belant 1992; Mosillo et al. 1999), and movements over 200 km have been documented (Lynch 1967). Unlike most species, raccoon populations have not been negatively impacted by anthropogenic changes; populations were at low densities until the 1940's when numbers significantly increased as raccoons opportunistically took advantage of agricultural production and urbanization (Zeveloff 2002). The raccoon is considered one of the most successful mammals in North America (Whitaker and Hamilton 1998) occurring at extremely high densities in urban settings (Prange et al. 2003; Smith and Engeman 2002; Riley et al. 1998; Rosatte et al. 1992) allowing for significant human interaction. This interaction is an important factor in our need to understand their dispersal abilities. Raccoon populations along the eastern seaboard of North America have been infected with a raccoon-specific rabies virus (Rupprecht 1992; Kirby 1995; Rosatte et al. 2001). Rabies is a fatal disease for the host and is able to infect across species, including wildlife, domestic livestock, companion animals and humans (Rupprecht and Smith 1994; Kirby 1995; Jackson and Wunner 2002). Treatment for the disease is mandatory if exposure is suspected due to the lethal

nature of the disease; however the treatment for the disease is costly (Kreindel *et al.* 1998; Chang *et al.* 2002; Slate *et al.* 2005). Due to these costs wildlife control agencies in Canada and the United States have made efforts to prevent further spread and eradicate this disease (Rosatte *et al.* 1992, 1997, 2001; Slate *et al.* 2005). Actions to control rabies outbreaks include creating buffer zones of vaccinated animals between infected and un-infected populations. The size and locations of these buffer zones are vital to successful disease control (Barlow 1993; White *et al.* 1995; Moore 1999; Broadfoot *et al.* 2001; Stemer and Smith 2006). Understanding the dispersal capabilities of raccoons could contribute to making control measures more effective.

Studying the landscape genetic structure of raccoon populations could provide answers for disease management; it also provides a unique opportunity to apply landscape genetic methodology, a relatively new research direction, to a species that is continuously and densely distributed to demonstrate the possible effectiveness or limitations of this approach. Most landscape genetic studies have focused on the analysis of already fragmented populations where the probability of detecting genetic structure is increased (Driscoll and Hardy 2005; Mcrae *et al.* 2005; Antolin *et al.* 2006; Broquet *et al.* 2006). To assess the impact of a natural barrier on raccoon dispersal, two regions were focused on, the Niagara and St. Lawrence (Figure 7.1), both of which are transected by their respective rivers and are at the front of the rabies epizootic. To quantify the effect of the river, population genetic structure of raccoons was investigated by analyzing highly polymorphic microsatellite markers (Cullingham *et al.* 2006) using the Bayesian clustering algorithm, STRUCTURE, developed by Pritchard *et al.* (2000) and further modified by Falush *et al.* (2003), and the spatial genetic structure using the correlation

between pairwise individual relatedness and distance measures (Hardy and Vekemans 2002; Vekemans and Hardy 2004). The objective of this analysis was to determine the genetic population structure in a continuously distributed species, to see if the genetic structure correlates to the major rivers, and if the rivers do pose a barrier to movement, quantify the number of migrants.

#### Materials and Methods

### Study Area and Sample collection

The NIA region is characterized by rich agricultural land where the environment is favorable for fruit production while the STL is characterized by non-agricultural rural landscape; it is not agriculturally productive due to the Canadian Shield, a nutrient poor substrate for vegetative growth. Samples were obtained from trappers employed by the Ontario Ministry of Natural Resources (OMNR) and the United States Department of Agriculture – Wildlife Services (USDA-WS) over an area of approximately 7600 km<sup>2</sup> in NIA and 4200 km<sup>2</sup> in STL as part of their rabies control programs (Figure 7.1). Hair samples were collected from live animals and brain tissue from public health control samples. Locations of individuals were obtained using handheld GPS units (5 m resolution); for the majority of samples sex and age class were also recorded.

## DNA Extraction and Genotyping

Dry hairs were immersed in 1X lysis buffer (2 M urea, 0.1 M NaCl, 0.25% n-lauryl sarcosine, 5 mM 1,2, cyclohexanediaminetetraacetic acid and 0.05 M Tris HCl pH 8) and incubated overnight at 37°C with 25 uL proteinaseK (600 U/mL). Brain tissue was preserved in 1X lysis buffer; digestion was carried out using 25 uL proteinaseK incubated for 2 hrs at 65° C and an additional 25 uL left overnight at 37° C. Using 50 uL of tissue

lysate DNA was extracted using an automated magnetic bead procedure and quantified following procedures outlined in Cullingham *et al.* (2006). Ten highly polymorphic microsatellite loci developed specifically for the raccoon were used to profile samples using three multiplex reactions described in Cullingham *et al.* (2006). Samples were analyzed using the MegaBACE 1000 (GE Healthcare), raw data was processed in Genetic Profiler 2.0 software (GE Healthcare) to assign locus-specific genotypes.

# Genetic Diversity

Tests of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were performed, and number of alleles, and expected and observed heterozygosities for each region were estimated. All calculations were conducted using GENEPOP (web version, Raymond and Rousset 1995), and significance levels were adjusted for multiple comparisons (Zar 1999).

#### Population Assignment

STRUCTURE version 2.1 (Pritchard *et al.* 2000, Falush *et al.* 2003) was implemented to estimate the number of population clusters; the model assuming admixture with correlated allele frequencies was used. To estimate the number of clusters, ten independent tests were conducted of K = 1-10 with  $10^6$  MCMC cycles each for burn-in and data collection. Prior and posterior probabilities were analyzed at each K to ensure enough MCMC cycles were used to reach stability in the parameters (Excoffier and Heckel 2006). The algorithm developed by Evanno *et al.* (2005) was used to infer the optimal number of K clusters; this calculations looks at the greatest change in the LnP(D) (Pritchard *et al.* 2000) for all tests of K. To visualize the clusters, all individuals were considered a member of a cluster if their probability exceeded 0.6 and this data was

visualized using ArcView3.3 (ESRI). To determine the number of potential migrants, individuals with assignment values exceeding 0.8 were looked at that were not spatially located within their cluster.

The Niagara and St. Lawrence rivers were expected *a priori* to represent significant barriers to raccoon movement and because of this ON and NY samples were analyzed separately for each region. Using the same parameters as above, STRUCTURE was used to infer clusters for each of the four data sets to look for more regional effects on population structure.

### Spatial Genetic Structure

Spatial genetic structure was analyzed for each region using Spatial Pattern Analysis of Genetic Diversity (SPAGeDi ver 1.2 -Hardy and Vekemans 2002). SPAGeDi estimates pairwise genetic relatedness between individuals and associates those estimates with spatial distances; permutations of individual locations results in an equivalent to a Mantel test (Hardy and Vekemans 2002). Distance classes were assigned for both NIA and STL to be the same to allow for direct comparison, the selection of those classes was made after allowing the program to maximize the pairs compared between classes. Ritland's (1996) kinship co-efficient was used; this value is calculated using a correlation-based estimator that does not make assumptions about the degree of relatedness in the population (Ritland 1996). It also shows low sampling variance and high power when highly polymorphic markers are used (Lynch and Ritland 1999; Vekemans and Hardy 2004). Significance testing was performed for each region using 10 000 permutations.



Figure 7.1. Study sites, Niagara (lower left) and St. Lawrence (upper right) and spatial distribution of samples (▲ represent sample from Ontario and ● represent New York samples)

#### Results

#### Samples

Genetic profiles were obtained for 744 individuals in NIA (ON = 412, NY = 332), of these 563 (76%) individuals had complete profiles, 73 (10%) were missing one locus, 30 (4%) were missing two loci, and 78 (10%) were missing three loci. For STL, 802 individuals were genotyped (ON = 733, NY = 69), of these 752 (94%) individuals had complete profiles, 32 (4%) were missing one locus and 18 (2%) were missing two loci. *Genetic Diversity* 

Genetic diversity estimates including number of alleles, and observed and expected heterozygosities are presented in Table 7.1 for each region. One locus (PLO2-123) tested significant for HWE in both regions, and two pair-wise comparisons for LD were significant in both NIA and STL after correction for significance testing (PLOM20 with PLO3-71 and PLO2-14 with PLO2-123).

#### Population Assignment

For NIA two clusters were identified as the most probable (Figure 7.2a), the spatial separation follows the Niagara River (Figure 7.3a). The first cluster consists of 333 individuals from ON and 15 from NY; the second cluster consists of 279 individuals from NY and 62 from ON; 55 individuals (37 - ON; 18 - NY) were unassigned (probability < 0.6). To identify potential migrants, stringency was increased to > 0.8; seven individuals were found in the ON cluster but they assigned to NY, and 23 individuals were found in the NY cluster but assigned to ON.

Three clusters were identified as most probable for STL (Figure 7.2b), the spatial spread of the three clusters does not correspond to geography and they co-occur across

	Niagara			St. Lawrence		
Locus	Alleles	H <sub>obs</sub>	H <sub>exp</sub>	Alleles	H <sub>obs</sub>	H <sub>exp</sub>
PLOM15	19	0.79	0.81	16	0.79	0.84
PLO2-117	33	0.89	0.92	28	0.88	0.90
PLOM2	16	0.86	0.87	14	0.85	0.86
PLOM20	13	0.83	0.86	13	0.86	0.89
PLOM3	7	0.76	0.78	7	0.73	0.75
PLO2-14	32	0.88	0.89	31	0.83	0.85
PLO3-71	14	0.86	0.88	11	0.71	0.72
PLOM17	8	0.77	0.81	7	0.78	0.76
PLO2-123	12	0.81	0.87	12	0.82	0.83
PLO3-86	40	0.81	0.88	28	0.85	0.90

Table 7.1. Diversity measures for microsatellites in Niagara and St. Lawrence study areas. Allele numbers, observed and expected heterozygosities for all loci in each region calculated in GENEPOP (Raymond and Rousset 1995)



Figure 7.2(a) Niagara probabilities for K using Evanno *et al.* (2005) calculation indicating the most likely K as being two and (b) St. Lawrence probabilities for K indicating the most likely K being three.



Figure 7.3a) Raccoons assigned to clusters in Niagara based on probabilities >0.6,  $\bullet$  represent individuals belonging to cluster 1,  $\blacktriangle$  belong to cluster 2 and  $\circ$  are unassigned individuals and b) Raccoons assigned to clusters in St. Lawrence,  $\bullet$  represents cluster 1,  $\blacksquare$  represents cluster 2,  $\Delta$  represents cluster 3, and  $\circ$  represents unassigned individuals as assigned in STRUCTURE

the study area (Figure 7.3b), of the 802 individuals, 507 (473 - ON; 34 - NY) were unassigned (probability < 0.6); this indicates there does not exist any significant population structure.

Since there was no detectable structure in STL using the entire data set, a regional analysis was performed only for NIA. Neither NY or ON datasets showed significant population structure. Two clusters were identified as most likely for both, but the spatial distribution of both clusters was not spatially structured for either ON or NY; in addition, the assignment probabilities for all individuals were distributed around 0.5 indicating no population structure.

### Spatial Genetic Structure

Spatial analysis of the two regions differed considerably. NIA displayed higher genetic relatedness than expected up to 20 km (p < 0.001 for all classes < 20 km) and over 45 km genetic relatedness values were less than expected (p < 0.001 for distances of 50 km-115 km). For STL only distances < 6.5 km had genetic relatedness higher than expected (p < 0.001) and over 30 km relatedness values were less than expected (p < 0.001) and over 30 km relatedness values were less than expected (p < 0.001) from 30 km-60 km). Distance classes beyond 60 km could not be evaluated due to the lack of statistical confidence since the number of comparisons included was not sufficient (Hardy and Vekemans 2002). The relatedness values were also higher in NIA than in STL at smaller distances and were lower at greater distances (Figure 7.4).

## Discussion

Genetic analysis of raccoon samples in ON and NY across the Niagara River indicate significant genetic structure concordant with the river. Both STRUCTURE and the spatial genetic analysis supported this finding. Samples with high assignment



Figure 7.4. Relatedness values calculated using Ritlands (1996) estimator between pairs of individuals for Niagara and St. Lawrence separated by their respective distance classes calculated in SPAGeDi (Hardy and Vekemans 2002).

probabilities were found on opposite sides of the Niagara River and a number of potentially admixed individuals were found suggesting the river is not an absolute barrier to gene flow. The region displays strong genetic structuring with genetic similarity decaying only at distances over 45 km indicating that other than the river, raccoons do not have barriers to dispersal in either ON or NY.

The same river effects were not found in the STL region. STRUCTURE was not able to detect any significant clusters with spatial consistency and the spatial genetic structure was weak with only similar genotypes occurring at distances up to 6.5 km. The lack of river effects in the STL could be the result of sampling; the majority of samples were located in ON whereas the NIA region had almost equal number of samples in ON and NY. Serre and Paabo (2004) and Rosenberg *et al.* (2005) showed that sample size will have a significant effect on the number of inferred clusters. Larger sample sizes in NY might have give a better indication of the degree at which the St. Lawrence River acts as a barrier to gene flow.

The effect of rivers on raccoon movement has been previously implicated in genetic studies. Mitochondrial DNA sequence analysis of raccoon populations identified the Mississippi River as a historical barrier to gene flow and the Niagara and St. Lawrence Rivers correlated significantly with genetic distance (see Chapters 4 and 5). River effects have also been found in rabies incidence modeling (Smith *et al.* 2002) where the Connecticut River had an effect of decreasing the spread of rabies. While the effect was significant in all these cases, it was not absolute and our data also suggest raccoons are able to cross major rivers. They are able swimmers (Gehrt 2003; Sanderson 1987) and if widths and velocity are not excessive they may cross willingly. It is also possible a

number of migrants could be due to "hitch-hiking" effects by means of transports and railway cars (Smith *et al.* 2002; Rosatte *et al.* submitted).

Although these data do implicate the Niagara River as being a barrier to raccoon movement, they have not shown the St. Lawrence River restricting movement to the same degree. Rabies incidence data suggests there are differences in the permeability of the Niagara and St. Lawrence rivers since raccoon rabies was anticipated to enter ON across the Niagara River years before entering across the St. Lawrence River (Rosatte *et al.* 1997); in fact, rabies still has not been detected in the NIA region of ON and was first reported in the STL region in 1999 (Rosatte *et al.* 2001).

Differences in the clustering results between the NIA and STL could be attributed to unbalanced sampling. However, differences in the spatial genetic structure cannot be attributed to sampling skew because they are at the individual level. These differences suggest there are population dynamic differences between the two areas. The importance of resource distribution affecting population genetic structure has been noted in a number of species (Hoelzel *et al.* 19981; Carmichael *et al.* 2001; Dalen *et al.* 2005; Pilot *et al.* 2006) and is a major difference between the two study areas that could explain differences in their genetic structure. Niagara is a highly productive area; raccoons are opportunistic and may be able to take advantage of this and as a result will be produce larger litters and have smaller home ranges (Rosatte 1991, 2000; Prange *et al.* 2003, 2004). Large and dense populations will have higher effective population size and will not be as subject to genetic drift (Hedrick 2000) which will maintain genetic relatedness over greater distances. The STL region has less productive land due to the Canadian Shield; raccoons in habitats with fewer resources will have larger home ranges and

populations will not be as dense (Seidensticker *et al.* 1988; Rosatte 1991; 2000; Prange *et al.* 2003, 2004). Lower numbers and greater distances traveled will result in genetic relatedness at only small distances.

The occurrence of raccoon rabies in the STL region, while no cases have been reported in the NIA region, imply the St. Lawrence River is not as effective a barrier as the Niagara. Continued control actions will be necessary to prevent further spread of this disease in this region. However, although the NIA region does not have reported rabies cases, the spatial genetic data suggest that if it were to invade the area, it would spread rapidly, making the need for control actions in this region even more important so long as rabies is a threat from NY.

### Conclusions

There have been considerable advances in the sensitivity of methods to detect interbreeding genetic clusters without the use of priors (Pritchard *et al.* 2000; Falush *et al.* 2003; Guillot *et al.* 2005; Francois *et al.* 2006). More recent developments (Guillot *et al.* 2005; Francois *et al.* 2006) have been shown to detect cryptic genetic structure when the more traditionally employed program, STRUCTURE, could not (Coulon *et al.* 2006). While these methods were considered, they were inappropriate for the objectives of the analysis. The aim was to detect significant breaks in raccoon genetic structure and associate these with geographic barriers to gene flow. This information is to be used to inform management decisions for placement of vaccination barriers to prevent spread of raccoon rabies. Had methods been utilized that are sensitive to small genetic differences recommendations would be made that do not represent significant barriers to movement and therefore would not be effective for rabies management. While the use of more

sensitive methods should be supported their application should be carefully considered based on the question(s) being addressed.

## Chapter 8

#### **General Discussion**

The analysis of both mtDNA control region and microsatellite nuclear markers in raccoons has resulted in a number of significant findings about population structure across geographic scales. What follow is a syntheses of these data and comments on raccoon ecology that would generate the defined structure.

Investigation of raccoon mtDNA across eastern North America indicates significant genetic structure; while this structure does not correlate to subspecific lineages (Hall 1981) it is partly explained by geographic distance (see Chapter 4). This is not surprising as isolation-by-distance is an often observed pattern in population genetics (Lougheed *et al.* 1999; Purdue *et al.* 2000; Laikre *et al.* 2005; Ortero-Arnaiz *et al.* 2005; Broquet *et al.* 2006; Kelly *et al.* 2006; Purcell *et al.* 2006) and is the null model for a number of population models (Wright 1951; Hedrick 2000; Hardy and Vekemans 2002). Although geographic distance has a significant effect at the broad scale there remains a large portion of variation left unexplained. This implies there are regional ecological factors additionally affecting population structure.

Rabies incidence data suggest that the Appalachian Mountain range is an effective barrier to movement (Niezgoda *et al.* 2002). However, analysis of mt lineages does not suggest the mountain range has an appreciable affect on population structure. While this was only analyzed with mtDNA data, microsatellite data may lead to alternative conclusions. Yet, mtDNA has a four-fold lower effective population size and would therefore be more susceptible to effects of genetic drift (Hedrick 2000) and should therefore display stronger genetic structure than microsatellites rather than weaker.

Alternatively, mtDNA may show little genetic structure where dispersal is female biased (Prugnolle and de Meeus 2002; Hammond et al. 2006), but as has been shown from both ecological data (Seidensticker et al. 1988; Gehrt and Fritzell 1998b) and genetic dispersal analysis (Chapter 6) female raccoons are more philopatric than males. Based on genetic population theory and raccoon ecology, the mtDNA data do not suggest the Appalachian range is an effective barrier to movement. This result leaves the cause for the lack of rabies movement to the west unexplained. An alternative explanation could be a result of density differences associated with mountain habitat; Seidensticker et al. (1988) found that densities of raccoon populations in the foothills of the Appalachians were not high enough to support a rabies epizootic. Numerous models have demonstrated the importance of a minimum host density to maintain an epizootic (Barlow 1993; Mollison et al. 1994; Dwyer and Elkinton 1995; Van Buskirk and Ostfeld 1998; Deal et al. 2000), and this density is increased for a disease like rabies, where the host will succumb in a relatively short period of time (Jackson 2002). Therefore the lack of west-ward progression of the disease is more likely due to the host's density rather than the host's inability to transverse the mountain range.

While the Appalachian Mountains were not implicated as a barrier to movement, the genetic data indicate rivers as an important barrier in restricting raccoon movement. The current distribution (Figure 5.2) of the mtDNA lineages indicates there were historically three lineages and the Mississippi River was the most likely barrier that separated raccoon populations which formed lineage III. Additionally, analysis of both mt and microsatellite data at a regional scale also support rivers as having a significant effect. Interestingly, the regional analysis of mt structure in the STL and NIA region, while

implicating the rivers as a significant correlate to population structure, geographic distance did not explain any of the variation. This is an important finding that suggests at a fine scale IBD is not a factor in causing population structure while it was demonstrated to be a significant explanatory variable at the broad scale.

The analysis of microsatellite data across the Niagara and St. Lawrence Rivers also indicate that IBD is not having a major effect on population structure as there was no detectable structure in the STL region, or on either side of the Niagara River. The river effect in creating population structure was again evident for the NIA region where STRUCTURE detected two population clusters, one located in ON and the other in NY (Figure 7.3). Interestingly, STRUCTURE analysis of the STL region did not result in any detectable clusters. While this could be attributed to a skew in sample size (Serre and Paabo 2004; Rosenberg et al. 2005), a post-hoc analysis was conducted on the STL data where ten random sub-sets of 69 individuals from ON were analyzed with the 69 individuals from NY. The findings from this support the initial analysis of no population structure across the river. The sample size is considerably smaller than the NIA analysis, and accuracy of clustering is affected by sample size (Rannala and Mountain 1997; Cornuet et al. 1999; Pritchard et al. 2000; Falush et al. 2003; Evanno et al. 2005). Further analysis of the NIA data using a random sub-set of 70 individuals from each ON and NY still resulted in the identification of two clusters on either side of the Niagara River. These additional analyses suggest the NIA and STL regions have different population dynamics and the nature of rivers will differ in how effectively they prevent raccoon movement.

Spatial genetic analysis of NIA and STL also indicate differences in population dynamics. In NIA, relatedness values are higher (Figure 7.4), but Vekemans and Hardy (2004) do not recommend this as a robust method for comparison; rather, they suggest the Sp statistic  $(-b/(1 - F_{(1)}))$ , where b is the slope of the line and  $F_{(1)}$  is the average relatedness value for the first distance class). Again, the Sp statistic is higher for NIA (0.004) than STL (0.003); this is surprising for a number of reasons. First, NIA has higher genetic diversity than STL (both alleles/locus and heterozygosities: Table 7.1), it is expected that lower genetic diversity would give increased relatedness values since individuals are more probable to share alleles than when diversities are higher (Ritand et al. 1996; Taylor et al. 1997; Vekeman and Hardy 2004). Second, density is an important factor in determining relatedness across the landscape as less dense populations will be more prone to genetic drift (Hedrick 2000). The Sp statistic is inversely proportional to density (Heywood 1991; Vekemans and Hardy 2004), therefore, based on the Sp statistics for NIA and STL; their proportional densities should be 238 and 400, respectively. However, estimates of densities in NIA are greater than STL (Rosatte 2000; Rosatte et al. in press; Rees *et al.* submitted). Based on the genetic and ecologic data for raccoons in these regions the Sp statistic should be larger for the STL region.

The discrepancy between the Sp statistic and what would be expected could be a result of differences in dispersal. In the STL, restricted dispersal of raccoons would result in a lower Sp value than the NIA region. However, the parentage analysis of these regions does not suggest that raccoons move considerably more in the NIA region, or less in the STL region. Additionally the lack of detectable population structure in STL suggests dispersal is not restricted in this area. Again, the primary difference between these two

regions that may result in this discrepancy is the differences in the rivers as effective barriers. The Sp statistic assumes the null model of population structure is IBD (Vekemands and Hardy 2004), where both mt and microsatellite data have shown that for both of these regions, IBD is not an important factor in causing genetic structure. The difference in the Sp statistic is therefore reflective of differences in the effects of the river in these regions, where individuals in NIA are more related if they are on the same side of the river in relation to individuals on opposite sides of the river. The notion that the river is causing this difference is also supported by the spatial analysis of the parentage data. The calculated Sp statistic for NIA (0.004) and STL (0.004) adults are the same, where the effect of the river is not apparent as these samples are only on the ON side.

The difference in the effect of the rivers in inhibiting raccoon movement is evident from these data; where the NIA is a barrier and the STL is not. However, the mtDNA analysis at the regional level indicated that the St. Lawrence River explained the majority of variation in the genetic differences from the partial Mantel test (r = 0.730, p = 0.025). The conflict between the mt and microsatellite data could have resulted from differences in effective population size. Mitochondrial DNA haplotypes are more prone to genetic drift than microsatellite markers, and it would therefore take longer to reach mutationdrift equilibrium (Karl *et al.* 1992; Hedrick 2000). This may be the cause of the differences, but, dispersal analysis (Chapter 6) has shown differences between the sexes. Although the differences were not strong between male and female dispersal, males will disperse greater distances, and have lower relatedness values than females at close distances suggesting females are more philopatric. Male raccoons may be more likely to cross the St. Lawrence River than females which would homogenize nuclear genetic

differences while having much less effect on mt structure (Baker *et al.* 1998; Goudet *et al.* 2002; Blundel *et al.* 2002; Milligan 2003). Field studies in this region also found no significant differences between male and female dispersal, but males were more likely to travel greater distances (Rosatte *et al.* in press). These dispersal differences together with the differences in effective population size between the genetic markers are enough to explain the disparity between the mt and microsatellite data.

Analysis of raccoon populations using two genetic markers and different scales has resulted in a better understanding of raccoon population dynamics. At the largest extent, the overall structure fit an IBD model, but, a large portion of variance was left unexplained. This variation is possibly explained by factors affecting structure at more regional extents. Fine-scale analysis in the NIA and STL regions using both markers demonstrates that ecological features can have different effects on population structure. Unfortunately, because of these differences, the ability to generalize effects on population structure to regions not considered in this study is not possible. However, after the effects of the river have been accounted for in the NIA region, the actual differences in genetic structure are minimal in comparison to the STL, despite there being differences in both population densities (Rosatte 2000; Rosatte *et al.* 2001; Rosatte *et al.* in press; Rees *et al.* submitted) and landscapes. Thereby, without major ecological barriers, panmixia can be inferred at least to the extents that have been analyzed in the NIA and STL regions.

#### Applications to Management

There are a number of important messages from the genetic analysis of raccoon populations that can be applied to management and control of raccoon rabies. Most

importantly, the Appalachian Mountains have been assumed to be a barrier to raccoon dispersal, based on the rabies incidence data (Niezgoda *et al.* 2002). However, the mtDNA data do not implicate the mountains as a barrier; therefore it is a region that should have management priority and continual surveillance, especially in regions where densities are higher and more likely to support an epizootic. Another key point is the permeability of rivers to raccoon movement has been shown to vary, where the St. Lawrence does not pose much of a barrier in comparison to the Niagara River. This result suggests that when a region of rabies management importance has a river transecting, further analysis should be conducted to determine if it can be utilized in the control strategy. Finally, while the dispersal distribution indicates most raccoons move <3km, it shows a small portion of raccoons will travel up to, and over 20km each year. These distances should be taken into account for PIC type operations to ensure all raccoons within a 20km radius of the infected individual receive vaccination, either by TVR or baiting.

## Chapter 9

#### Conclusion

The use of a variety of genetic analysis methods including phylogeography, parentage, spatial genetic analysis and landscape genetics have contributed to a better understanding of raccoon dispersal and how this has shaped their current distribution. Since raccoons are very successful habitat generalists characterizing their population genetic structure is a challenge. Broad scale analysis of genetic diversity indicated genetic differences were not indicative of subspecies lineages rather they correlated to geographic distance; however, a large amount of variation was left unexplained suggesting regional factors have an important effect on genetic diversity. Analysis of both mtDNA and microsatellites revealed the effects of major rivers as barriers to gene flow. Regional analysis of two regions, NIA and STL, with different habitats suggested differences in their population structure are attributed to differences in river permeability's. These results suggest management of raccoons for purposes of rabies prevention and eradication will have to consider each area separately as regional effects will impact their movement dependent on their habitats.

While the use of genetics has been able to provide a thorough description of dispersal behaviour in raccoons than have field studies, the importance of field work is significant and management of species should not focus on only one method, rather they should take an interdisciplinary approach. Without field work, sample information could not be reliably obtained, which can have impacts on genetic data interpretation. For instance, if the spatial genetic analysis to characterize dispersal was only conducted for all individuals rather than having the information to looking at the age and sex class, the

impact of time of juvenile dispersal could not be addressed. Not only is the merging of field and genetic methods important, the addition of other areas including landscape ecology and wildlife management are also crucial to conduct a thorough and inclusive study.

This comprehensive analysis of raccoon population genetic structure not only demonstrates the importance of merging methods across disciplines but also the approach taken by an investigator does have a number of options for data analysis and not all are appropriate for their study system. Both the phylogenetic and landscape genetic analysis of raccoons could have been analyzed differently, but the choices were defined based on the data available and the questions that needed to be addressed.

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