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Population Genetics of Raccoons in the Eastern United States

With Implications for Rabies Transmission and Spread

By

Serena Ann Reeder Carroll

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Population Biology, Ecology, and Evolution

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B.S., Oklahoma State University, 2001

M.S., Texas Tech University, 2003

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Abstract

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A central research question in disease ecology concerns how a pathogen moves throughout space and time (Anderson and May 1978). For directly transmitted zoonoses, this process is likely influenced by the biological and environmental characteristics of both the pathogen and its host (Real and McElhany 1996; Hess et al. 2001; Real and Biek 2007). Although these interactions are complex, one can attempt to discern the relative contribution of each component by testing explicit hypotheses systematically. The motivation for this research was to increase our understanding of how a directly transmitted pathogen spreads in a wildlife population, and specifically, to examine host factors that might influence pathogen transmission or spread.

In this dissertation, the raccoon rabies model system was used to explore how social structure and landscape heterogeneity influence pathogen transmission. We used genetic data to generate relatedness estimates of raccoons and tested whether social organization influenced host contact rates or raccoon rabies transmission (Chapter 2). Additionally, we used genetic data to define the historical and contemporary population structure of raccoons throughout the range of the raccoon rabies virus variant (RRV). This information was used to determine whether landscape heterogeneity in the form of historically defined suture-zones (Remington 1968) in the eastern US resulted in limited raccoon dispersal or barriers to gene flow that might also have affected the spread of RRV (Chapter 3). Finally, surveillance data and modeling techniques were used to examine the influence of host biology (home range, incubation, and infectious periods) on the spatial and temporal clustering or aggregation of raccoon rabies in a multi-host system, raccoons and skunks, in the northeastern US (Chapter 4). Each of the chapters discusses the specific hypotheses tested and results in depth, and a summary can be found in Chapter 5.

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CHAPTER 1

Introduction

1.1 BACKGROUND

In 1978, Anderson and May proposed that the host-parasite relationship involved more than just a parasite's impact on its host. Rather, the complex interactions between the two entities influenced the rate of parasite transmission among hosts, with multiple factors leading to unique patterns of parasite (or pathogen) spread. Relevant factors include host behavior, social or genetic population structure, population density or abundance, contact rate, parasite/pathogen virulence, transmission efficiency, and latency period, among others (Schauber et al. 2007; Barton et al. 2010). Of those, population structure is especially interesting in that host population structure can influence pathogen virulence (Boots et al. 2004) and the spread of infectious disease, yet pathogens can similarly influence host genetic variation (Kellam and Weiss 2006). Furthermore, pathogens and parasites may act as moderators of population structure (Chapman et al. 2005), illustrating the fact that host-parasite interactions are often bidirectional.

To complicate the relationship between pathogens and hosts, conflicts between rapidly evolving pathogens and slowly evolving hosts can act to accelerate changes in host behavior and thus create new niches for emerging pathogens (Morens et al. 2004). Local adaptation of pathogens to particular host genotypes can occur and may result in the formation of population structure in the pathogen. This structure can lead to different infectivities in vector populations and subsequently modify the patterns of disease transmission and spread (Joy et al. 2008).

The reality that parasites and their hosts not only interact within populations but also among them (i.e., metapopulation structure) further complicates the question of how parasites move though space and time. Here, at least two levels of host-parasite population dynamics must be taken into account when reconstructing transmission events. Indeed, 30 years later, the question of how parasites/pathogens move throughout space and time remains central to disease ecology (Hudson et al. 2001; Real and Biek 2007).

While elements borrowed from predator-prey models have been combined with elements from conventional epidemiology (Anderson and May 1980) to model infectious disease dynamics and transmission, typical predator-prey models assume that predators and prey are similar entities in terms of their life history characteristics and population size. While this is certainly the case for a multitude of systems, infectious disease is an exception (Vandermeer and Goldberg 2003). In this case, the predators are pathogens (viruses, bacteria, or other microorganisms) and are generally small with short generation times and rapid dynamics whereas the prey (hosts) are much larger and have relatively slow dynamics and longer generation times in comparison (Anderson and May 1991; Vandermeer and Goldberg 2003). In the context of classical epidemiology, SIR (susceptible-infected-recovered) models assume that hosts are homogeneously mixed and pathogen transmission is usually examined against a background of constant hosts (Anderson and May 1980; Vandermeer and Goldberg 2003). One problem with this methodology is that hosts are unlikely to be uniform in their distribution (Anderson and May 1991; Vandermeer and Goldberg 2003), and undetected population structure can

produce potentially misleading results (Marchini et al. 2004). Differences both within and among populations of parasites and hosts can create heterogeneity.

Heterogeneity can be problematic on a variety of levels because of the different forms that it can take. Not only can hosts or parasites be heterogeneous (genetically, spatially, etc.), but the landscape upon which these processes are occurring can also take a heterogeneous form (Real and Biek 2007). A new field, landscape genetics, combines landscape ecology and population genetics approaches to determine the influence of landscape features on microevolutionary processes (Sork et al. 1999; Manel et al. 2003). While the field has been formally recognized only in recent years, scientists have recognized the importance of landscape features on the distribution of organisms for centuries. During the early 1800s, the botanist Augustin Pyramus de Candolle wrote that organism distributions varied across the landscape and depended on physical causes operating on different time scales. Not long afterwards, Alfred Russel Wallace, sometimes referred to as the father of biogeography, described a boundary separating fauna in the Australian Region from the Oriental Region in the Malay Archipelago (the Wallace line) in the 1850s (Manel et al. 2003).

Overlaying genetic differences onto the landscape to detect barriers to gene flow looks to be a promising technique for understanding how these heterogeneities are manifested. As Sork et al. (1999) recently stated: "Regardless of whether landscape heterogeneity is natural or created by recent anthropogenic disturbance, a critical question that researchers can now address is the extent to which the landscape context of populations influences gene movement." These techniques have already been used to describe landscape features that act as barriers to gene flow in a variety of host species

with associated pathogens, especially with the goal of informing disease mitigation and management strategies (Blanchong et al. 2008; DeYoung et al. 2009; Root et al. 2009; Barton et al. 2010).

1.2 GOALS OF THE DISSERTATION

This dissertation represents a body of work aimed at elucidating the factors involved in the transmission and spread of a zoonotic disease in a wildlife population. Specifically, three host-based approaches are taken to gain insight into how raccoon rabies has spread throughout the eastern seaboard. The first examines raccoon relatedness estimates to infer the impact of social structure on disease transmission. The second method uses raccoon population genetics to identify historical and contemporary barriers to gene flow and how that might influence rabies spread. The final method uses surveillance data and modeling techniques to examine the spatiotemporal relationship of raccoon rabies virus in two wildlife species, the raccoon and the skunk, to determine the relative influence of host home range, incubation period, and infectious period on the spatial and temporal clustering or aggregation of raccoon rabies in a multi-host system. After an introduction into raccoons and rabies and their utility as a model system, each of these approaches will be summarized below.

1.3 RACCOONS AND RABIES AS A MODEL SYSTEM

The common raccoon (*Procyon lotor*) is a mesocarnivore distributed throughout North America (Figure 1.1), with few exceptions, from Canada to Panama in Central America (Hall and Kelson 1959; Lotze and Anderson 1979; Wilson and Ruff 1999;

Zeveloff 2002). The earliest known *Procyon* fossil uncovered in North America dates to the Pliocene (Simpson 1945; Lotze and Anderson 1979), approximately 5.5 to 2 million years before the present, indicating the raccoon's long association with the New World. As generalists, they are easily adaptable and can thrive in many diverse habitats, often resulting in high density populations in urban and suburban environments. Their adaptability is further illustrated by their successful introduction to France, Germany, the former Soviet Union, and many Caribbean islands (Zeveloff 2002).

Figure 1.1 Range map of raccoons in North America (Wilson and Ruff 1999)

Traditionally, raccoons have been regarded as solitary animals except during times of high resource availability (Gehrt and Fritzell 1998; Zeveloff 2002); however, recent studies have found that raccoons may be more social than previously thought and

small, non-aggressive groups of 3-4 males may form (Gehrt and Fritzell 1998). Raccoons are highly vagile, and home range estimates vary greatly depending on the habitat (Zeveloff 2002). Raccoons typically occupy smaller home ranges when densities are high, or in urban/suburban areas where food and water resources are concentrated and are readily available (Zeveloff 2002; Prange et al. 2004). Dispersal is predominantly male biased as females are philopatric (Ratnayeke et al. 2002).

Raccoons are hosts to a number of zoonotic diseases including rabies, an acute, progressive encephalitis transmitted primarily via the bite of a rabid animal. Rabies is a nonsegmented, negative-strand RNA virus in the family *Rhabdoviridiae*, genus *Lyssavirus* (Lyles and Rupprecht 2006). Worldwide, rabies remains a significant cause of mortality and accounts for more than 55,000 human deaths each year (World Health Organization 2008). With the elimination of enzootic dog rabies in the United States (US), rabies remains a significant problem primarily in wildlife populations (Velasco-Villa et al. 2008; Blanton et al. 2009). Distinct rabies virus variants (Figure 1.2) have been associated with raccoons, skunks, foxes, coyotes, mongooses, and bats in the US (Rupprecht et al. 1987; Blanton et al. 2009).

The raccoon rabies virus (RRV) variant, which occurs throughout the eastern United States, is of particular public health concern due to frequent human/raccoon interactions. The first documented human fatality due to RRV occurred in Virginia in 2003 (Silverstein et al. 2003). Despite the long evolutionary history of raccoons in the New World, raccoon rabies is a relatively recent development. The first rabid raccoon in the United States was identified in California in 1936 (McLean 1975). After that time, raccoon rabies was reported rather infrequently and was attributed to spillover events

from other rabies virus hosts. In 1947, a rabid raccoon was identified in Brevard County in central Florida (Kappus et al. 1970; Bigler et al. 1973). Rabies in raccoons spread within Florida during the 1950s and throughout Georgia, Alabama, and South Carolina over the next 20 years (Held et al. 1967; Kappus et al. 1970; McLean 1971; Bigler et al. 1973; Niezgoda et al. 2002).

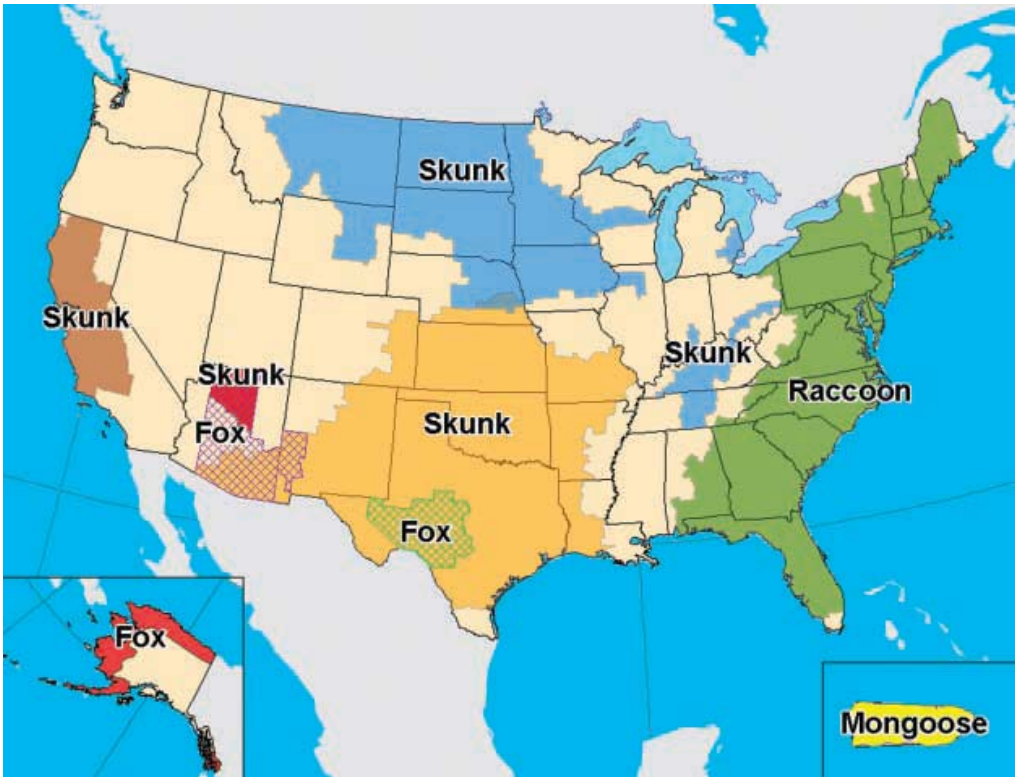


Figure 1.2 Distribution of major terrestrial reservoirs of rabies in the United States and Puerto Rico (Blanton et al. 2009).

During the late 1970s, rabies in raccoons was detected in the Mid-Atlantic States along the border of Virginia and West Virginia in Pendleton County, West Virginia (Nettles et al. 1979; Jenkins and Winkler 1987; Rupprecht and Smith 1994). Antigenic

analysis demonstrated its relatedness to southern cases and indicated that this outbreak was likely the result of long distance translocations of raccoons for hunting purposes (Nettles et al. 1979; Smith et al. 1984). Translocations of raccoons were common practice during this time with over 3,500 raccoons transported from Florida to Virginia between 1977 and 1981 (Jenkins et al. 1988; Rupprecht and Smith 1994). This sparked one of the largest rabies epizootics in history as the outbreak spread both north and south from West Virginia.

During the 1980's, raccoon rabies continued to spread. It arrived in Maryland as early as 1981 and reached Pennsylvania and the District of Columbia by 1982. Delaware first reported rabid raccoons in 1987, and New Jersey followed in 1988. Raccoon rabies reached the western townships of Connecticut (near New York) in 1991 and spread across the state in just 5 years. In 1994/1995, the southeastern epizootic and Mid-Atlantic epizootic foci met in North Carolina and by 1999, the raccoon rabies front reached Canada. Today, raccoon rabies can be found along the eastern seaboard from Florida to Maine, with the western edge occurring along the Ohio/Pennsylvania border in the northern part of its range, extending into eastern Tennessee and south to Alabama (Blanton et al. 2009; see also Figure 1.2). RRV appears to have since been eliminated from Ontario, Canada, with the last reported case occurring in 2005 (Rosatte et al. 2009). Over 50,000 rabid raccoons have been diagnosed to date.

The southeastern and Mid-Atlantic epizootics differed in certain spatial and temporal aspects (Childs et al. 2001). For example, the southern states experienced smaller, less frequent epizootics, without a clear temporal dynamic. The northern epizootics seemed to be larger and more frequent. This is possibly due to the high human

population density from Virginia to Massachusetts, or perhaps, to more favorable habitats for raccoons in the north. Extremely high raccoon densities have been found in urban parks and suburban areas (Riley et al. 1998), indicating a positive association with human population density (Childs et al. 2001). The resulting increased raccoon density could have then contributed to larger rabies epizootics (Childs et al. 2001).

The restriction of RRV to the eastern US is likely due to a combination of natural geographic barriers and barriers created by vaccination. Physical barriers, such as mountains, large rivers, and major highways may restrict or slow raccoon movement or dispersal (Lucey et al. 2002; Smith et al. 2005; Biek et al. 2007; Cullingham et al. 2009). Alternatively, these areas may sustain lower raccoon population densities, resulting in lower contact rates among infected individuals and slower RRV spread. Additionally, oral rabies vaccination (ORV) has been implemented for enhanced restriction of newly infected areas (Smith et al. 2002; Rupprecht et al. 2004; Slate et al. 2005). The ORV campaign involves the vaccination of raccoons against rabies via aerial and ground distribution of fishmeal polymer or coated sachet baits containing a vaccinia-rabies glycoprotein (V-RG) recombinant virus (Wiktor et al. 1984; Rupprecht et al. 1986; Hanlon et al. 1998; Rupprecht et al. 2004; Slate et al. 2005). Effectively, a barrier of vaccinated raccoons is established thus diminishing the pool of susceptible animals and limiting the ability of the rabies virus to spread.

The first release of the ORV bait was in 1990 on Parramore Island, Virginia (Hanlon et al. 1998). New York began baiting in 1995 and by 2001, states participating in the ORV campaign included Florida, Maryland, Massachusetts, New Hampshire, New York, Ohio, and Virginia. Sixteen states currently distribute oral rabies vaccines for

raccoons (http://www.aphis.usda.gov/wildlife_damage/oral_rabies/). As discussed above, this strategy, in combination with the use of natural physical barriers to raccoons, has helped to curb the westward spread of RRV; however, long distance translocations may still pose a significant threat (Rupprecht et al. 2004; Slate et al. 2009).

An added concern for controlling the spread of RRV is the potential involvement of skunks in maintaining and/or transmitting the virus in the northeastern US. While RRV is primarily maintained and transmitted by raccoons, skunks can be infected with RRV and have been shown to be important secondary hosts (Guerra et al. 2003). Independent skunk-to-skunk transmission of RRV has not yet been identified, but previous studies (Guerra et al. 2003) have noted that rabies epizootics in raccoons and skunks are closely coupled. Although skunks may consume ORV baits, this method has not proven efficient in preventing rabies in skunks either due to the vaccine itself or to poor vaccine delivery, whereby the vaccine is lost to the environment as the skunks puncture the vaccine sachets (Charlton et al. 1992; Guerra et al. 2003; Grosenbaugh et al. 2007; United States Department of Agriculture 2007).

Host-switching or host-shift events are also important considerations for RRV control. Recently, Streicker et al. (2010) presented evidence of cross species transmission (CST) of bat rabies viruses and demonstrated that the probability of CST and host shift decreased with increasing phylogenetic distance of host species. The phylogenetic similarity of host species, and to a lesser extent, the geographic overlap between species, played more important roles in predicting CST than did similarity of host ecological traits (Streicker et al. 2010). This has important implications for host shifts involving RRV. Mutation in RRV could result in a variant that readily crosses over

into another host, such as the skunk or another mesocarnivore, and becomes sustained after traversing a flattened fitness valley and adapting to its new host (Streicker et al. 2010). Although this was not previously thought to be common with rabies, potential raccoon to raccoon transmission may have occurred after infection with a fox strain in New York in the late 1940's (McLean 1975; Winkler and Jenkins 1991). Furthermore, recent transmission of a bat rabies virus variant from bats to skunks and foxes in Arizona with sustained carnivore to carnivore transmission highlights the very real possibility of crossing over and adaptation to a new species, even when the phylogenetic distance between host species is great (Leslie et al. 2006; http://www.avma.org/onlnews/javma/nov09/091115m.asp).

The origin of raccoon rabies is somewhat of a mystery (Rupprecht and Smith 1994). The variant is highly adapted to raccoons, and sequence identity of RRVs in raccoons from Florida to Maine is on the order of 99%. In terms of phylogenetics, RRV isolates clearly form a monophyletic clade. It appears that their closest relative is the South Central Skunk variant (Rupprecht and Smith 1994; Smith et al. 1995); however, Szanto et al. (2008) recently suggested that RRV emerged from a North American bat rabies virus variant, either directly or via adaptation of the South Central Skunk variant. Bat-associated origins for RRV have been suggested previously, and three types of transmission from bats to raccoons have been hypothesized: bat bites to the raccoon, aerosol transmission, and consumption of bats as food by raccoons (Winkler and Jenkins 1991). One line of evidence in support of this idea concerns a rabid raccoon trapped in the vicinity of a bat cave in Texas. The raccoon in question was infected with a rabies virus similar to that of a Mexican freetail bat (*Tadarida brasiliensis*) isolate (Constantine

1962; Winkler and Jenkins 1991). In a later study in Florida, however, no evidence was found for bat to raccoon transmission.

1.4 POPULATION GENETICS OF RACCOONS (*PROCYON LOTOR*) CORRESPONDING TO A NEW FOCUS OF RACCOON RABIES IN NORTHEASTERN OHIO: IMPLICATIONS FOR TRANSMISSION

The integration of population genetic and epidemiologic data has the power to provide novel insight into patterns of pathogen transmission within host populations. Recently, these techniques have been used to suggest that relatedness and social structure might play a role in influencing contact rates and disease transmission in a variety of systems (Root et al. 2004; Blanchong et al. 2007). In this study, we test the hypothesis that social structure, inferred through relatedness estimates, influences disease transmission using RRV as a model system.

Female raccoons are philopatric and live in related groups with shared home ranges (Gehrt and Fritzell 1998; Ratnayeke et al. 2002). This social structure implies that females contact other relatives more frequently than non-related individuals. If rabies is spread primarily due to female philopatry, then there should be a sex bias, and rabid raccoons should be more highly related. On the other hand, dispersal is predominantly male biased in this system (Gehrt and Fritzell 1998; Zeveloff 2002). If rabies is spread by highly dispersive, unrelated males then relatedness estimates of rabid males should be lower than expected. Alternatively, raccoon genetic/social structure may not strongly

influence RRV transmission, in which case relatedness estimates should not significantly deviate from zero.

1.5 HISTORICAL AND CONTEMPORARY EVOLUTION ACCOUNT FOR POPULATION SUBDIVISION IN RACCOON (*PROCYON LOTOR*) POPULATIONS IN THE EASTERN UNITED STATES

Landscape genetics, a newly emerging field combining molecular population genetics and landscape ecology, can be used to understand how heterogeneity across the landscape affects population structuring at different geographic and temporal scales (Sork et al. 1999; Manel et al. 2003). Since rabies is a directly transmitted pathogen requiring animal to animal contact for transmission, it is reasonable to assume that landscape factors that act as obstacles to raccoon movement will also act as barriers to RRV progression (Real and Biek 2007; Cullingham et al. 2008; Cullingham et al. 2009). The purpose of this study is to test the hypothesis that the Northeastern-Central and Northern Florida Suture-Zones, areas of geographic overlap between major biotic assemblages with the potential for hybridization, (Remington 1968; Swenson and Howard 2004) have acted as geographic barriers to gene flow in raccoons and their associated pathogen, RRV. Although these areas are presumed to represent historical barriers, it is unclear whether they constitute contemporary barriers or if secondary contact has resulted in gene flow and/or population mixing. By examining a combination of nuclear and mitochondrial markers, we can distinguish between potential historical and contemporary

boundaries. This knowledge should provide insight into how RRV has spread across the landscape and will help to inform on rabies management strategies.

If the defined suture-zones have affected raccoon gene flow historically, then one would expect to find mitochondrial differentiation representing regional population genetic subdivision. If the suture-zones represent current barriers to gene flow, then the observed pattern of genetic differentiation should hold when examined with microsatellite markers; however, if secondary contact has occurred, then raccoons should form single populations (i.e., no genetic sub-structuring based on the microsatellite data) in the suture-zone regions. Alternatively, the suture-zones may not correspond to raccoon subdivision at all, in which case, genetic structure would not be detected at either temporal scale in those areas.

1.6 SPATIOTEMPORAL INTERACTIONS OF ENZOOTIC RACCOON RABIES IN RACCOONS AND SKUNKS

Transmission of disease depends not only on the interaction between the location and timing of cases (Real and Biek 2007) but also on the number of susceptible individuals within a given area considered at risk for infection. Since these factors can change over time and throughout space, disease occurrence is often distributed heterogeneously (Anderson and May 1978). For RRV, the situation is further complicated due to the potential for heterogeneity in a multi-host system. In the northeastern US, RRV infects both raccoons and skunks, and a major concern is that a host-shift will result in sustained skunk to skunk transmission of RRV. Although host

shifts were previously thought to be uncommon for rabies viruses, a bat rabies virus variant in Arizona has recently become adapted to skunks and foxes and is now efficiently circulating within the carnivore populations (Leslie et al. 2006; http://www.avma.org/onlnews/javma/nov09/091115m.asp). If RRV was maintained and co-transmitted via skunks and raccoons, it would greatly complicate RRV control as ORV is relatively ineffective in skunks either due to the vaccine itself or to poor vaccine delivery when skunks consume the ORV baits (Charlton et al. 1992; Guerra et al. 2003; United States Department of Agriculture 2007).

The purpose of this study is to determine whether RRV cases are significantly spatially-temporally clustered (and at what scale) and to determine the involvement of skunks in maintaining RRV in the northeastern US. We examined RRV surveillance records in Massachusetts over a 5 year period and used the spatial-temporal K-function to investigate the influence of host biology on the spatial and temporal clustering or aggregation of raccoon rabies in a multi-host mesocarnivore system. Since rabies is a directly transmitted virus, it requires contact between two animals for transmission to occur. Therefore, host factors such as home range diameter, incubation period, and infectious period likely affect the spatial and temporal clustering of the pathogen due to the transmission process itself (Carslake et al. 2005; Carslake et al. 2006). Clustering of RRV cases on a particular scale could indicate the range of time and/or distance that a particular animal is at risk of infecting other animals. We hypothesized that transmission would most often occur within one home range diameter and one infectious period for each species.

CHAPTER 2

Population Genetics of Raccoons (*Procyon lotor*) Corresponding to a New Focus of Raccoon Rabies in Northeastern Ohio: Implications for Transmission

2.1 INTRODUCTION

Typical predator-prey models assume that predators and prey are similar entities in terms of their life history characteristics and population size. While this is certainly the case for a multitude of systems, infectious disease is an exception (Vandermeer and Goldberg 2003). In this case, the predators are pathogens (viruses, bacteria, or other microorganisms) and are generally small with short generation times and rapid dynamics whereas the prey (hosts) are much larger and have relatively slow dynamics and longer generation times in comparison (Anderson and May 1991; Vandermeer and Goldberg 2003). In the context of classical epidemiology, SIR (susceptible-infected-recovered) models assume that hosts are homogeneously mixed, and pathogen transmission often is examined against a background of constant hosts (Vandermeer and Goldberg 2003). One problem with this methodology is that hosts are unlikely to be uniform in their distribution (Anderson and May 1991; Vandermeer and Goldberg 2003) and failure to incorporate population structure can produce potentially misleading results. For example, undetected human population structure has led to false positive as well as false negative results in large scale genetic association studies of human disease (Marchini et al. 2004).

While host population structure can influence pathogen virulence (Boots et al. 2004) and the spread of infectious disease, pathogens can similarly influence host genetic variation. For example, pathogens have helped to shape the human genome, especially in terms of the major histocompatibility complex (Kellam and Weiss 2006). In addition, pathogens and parasites may act as moderators of population structure; this scenario has been hypothesized in the case of nonhuman primates (Chapman et al. 2005). As an extreme example, outbreaks of Ebola hemorrhagic fever in the Congo Basin are thought to have had severe impacts on gorilla and chimpanzee populations in the 1990s (Huijbregts et al. 2003), potentially resulting in genetic bottlenecks which can alter population structure.

To further complicate the interactions of pathogens and hosts, conflicts between rapidly evolving pathogens and slowly evolving hosts can act to accelerate changes in host behavior and thus create new niches for emerging pathogens (Morens et al. 2004). For instance, the rapid increase of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) cases resulted from a suite of human behavioral changes (increased travel, movement of individuals from rural populations to cities, urban poverty, and a variety of others) that promoted sexual practices conducive for HIV evolution and transmission (Quinn 1994). Another key example is the emergence of the rodent-borne hantaviruses (Morens et al. 2004). An increase in farming and hunting practices has subsequently led to an increase in disturbed habitat patches which are associated with the rodent hosts of hantaviruses. Since disturbed habitats can support high rodent population densities, hantavirus transmission risk increases not only within

the rodent populations, but also for humans due to increased rodent/human contact (Carroll et al. 2005; Mills 2006).

Finally, local adaptation of pathogens to particular host genotypes can occur and may result in the formation of population structure. This structure can lead to different infectivities in vector populations and subsequently modify the patterns of disease transmission and spread as observed with malaria parasites and their mosquito vectors (Joy et al. 2008).

Despite this understanding and appreciation for host-pathogen interactions and host population structure, studies examining host genetics in the context of disease transmission are still relatively rare. The majority of existing studies involve mosquito species that act as vectors for malaria (Braginets et al. 2003; Moreno et al. 2007; Joy et al. 2008; Mirabello et al. 2008) or triatomine insects that transmit Chagas disease (Pizarro et al. 2008). Studies in plants have focused mainly on the population genetics of resistance genes (Rose et al. 2007). The application of population genetic data to epidemiology has the power to provide novel insights into patterns of pathogen transmission within host populations. Recently, these techniques have been used to suggest that relatedness and social structure might play a role in influencing contact rates and disease transmission in rodents associated with hantavirus (Root et al. 2004) and deer affected with bovine tuberculosis (Blanchong et al. 2007). In both of these cases, genetic relatedness estimates were used to show that infected individuals were more related than uninfected individuals. Higher contact rates among family members, reflecting the social organization of the host species (i.e., philopatry), led to increased disease transmission among the groups of interest (Root et al. 2004; Blanchong et al. 2007). Furthermore,

relatedness in the form of inbreeding has been shown to increase the susceptibility of sea lions to certain diseases. Inbred individuals were not only infected by a wider range of pathogens but also took longer to recover from infection (Acevedo-Whitehouse et al. 2003). These responses were likely due to reduced heterozygosity in the major histocompatibility complex associated with inbreeding.

In this study we examine the possibility that social structure, inferred through relatedness estimates, influences disease transmission using raccoon rabies virus (RRV) as a model system. Raccoons (*Procyon lotor*) are highly adaptable mesocarnivores (Lotze and Anderson 1979; Zeveloff 2002) that are hosts to a number of zoonotic diseases including rabies, an acute, progressive encephalitis caused by nonsegmented, negative strand RNA viruses in the family *Rhabdoviridiae*, genus *Lyssavirus*. Worldwide, rabies is a significant cause of mortality and accounts for more than 55,000 human deaths each year (World Health Organization 2008). In the United States (US), rabies remains a considerable problem primarily in wildlife populations. Raccoon rabies is of particular public health concern due to frequent human/raccoon interactions with the first human fatality occurring in Virginia in 2003 (Silverstein et al. 2003). Today, raccoon rabies can be found along the eastern seaboard from Florida to Maine, with the western edge occurring along the Ohio/Pennsylvania border in the northern part of its range, extending into eastern Tennessee and south to Alabama (Blanton et al. 2009).

Ohio has been the subject of increased attention because of its position as a potential gateway for relatively unimpeded westward spread of RRV. The combination of oral rabies vaccination (ORV) and natural physical barriers such as mountains or large rivers likely has contributed to the restriction of RRV to the eastern US (Smith et al.

2002; Rupprecht et al. 2004; Slate et al. 2005; Blanton et al. 2009; Slate et al. 2009). However, in the absence of major geographic barriers to slow its spread, as in Ohio, RRV could become extremely difficult to contain. Russell et al. (2005) predicted that rabid raccoons could move into the central portion of Ohio in as little as 33 months after introduction and that RRV could cover the entire state in 41 months if left unchecked. Raccoon rabies first reached eastern Ohio in 1996 and an ORV program was initiated in 1997 to contain the epizootic (Kostrzewski 2002). Ohio was considered free of RRV by 1999 and it remained unreported, with the exception of sporadic cases along the Ohio and Pennsylvania border, for the next 5 years. In July of 2004, however, a rabid raccoon was identified in Lake County, in an area west of the ORV barrier. As a result, intensive surveillance was conducted to determine the extent of rabies occurrence and additional ORV baiting was implemented to curb raccoon rabies spread. This provided a rare opportunity to examine raccoon population genetic structure in relation to transmission of RRV.

As previous studies have shown (Joy et al. 2008), slowly evolving hosts and rapidly evolving pathogens can interact to create a level of local adaptation that can result in different susceptibilities or infectivity rates in particular host genotypes or populations which may also influence disease spread (Real et al. 2005). We examine this possibility by characterizing raccoon populations and determining whether or not any differences are seen in RRV infection rates. Despite the fact that raccoons are evolving more slowly than RRV, their genetic and social structure could influence patterns of disease transmission due to differences in host contact rates. Female raccoons are philopatric and live in related groups with shared home ranges (Gehrt and Fritzell 1998; Ratnayeke et al.

2002). This social structure implies that females contact other relatives more frequently than non-related individuals. If rabies is spread primarily due to female philopatry, then there should be a sex bias and rabid raccoons should be more highly related. On the other hand, dispersal is predominantly male biased in this system (Gehrt and Fritzell 1998; Zeveloff 2002). If rabies is spread by highly dispersive, unrelated males then relatedness estimates of the rabid raccoons should be lower than expected. Alternatively, raccoon genetic/social structure may not strongly influence RRV transmission, in which case relatedness estimates should not significantly deviate from zero.

Additionally, we examine whether translocated individuals could have played a significant role in this most recent focus of raccoon rabies in Ohio. Henderson et al. (2008) sequenced RRV associated with rabid raccoons from this study and found support for monophyly of the virus sequences. This information could have important implications for managing and preventing the further spread of RRV. By identifying populations at greatest risk for RRV infection and determining how those populations might become infected (repeated translocation vs. establishment in a resident population, for example), one could then design and implement better informed, knowledge-based surveillance and control programs.

2.2 MATERIALS AND METHODS

2.2.1 Raccoon Samples and Collection Localities

Raccoon brain tissue was submitted for rabies diagnosis to the Centers for Disease Control and Prevention (CDC) by the United States Department of Agriculture (USDA)-

Wildlife Services and the Ohio Department of Health. In total, 182 raccoons, including 26 rabid individuals, from an 11 county region in northeastern Ohio were examined (Figure 2.1).

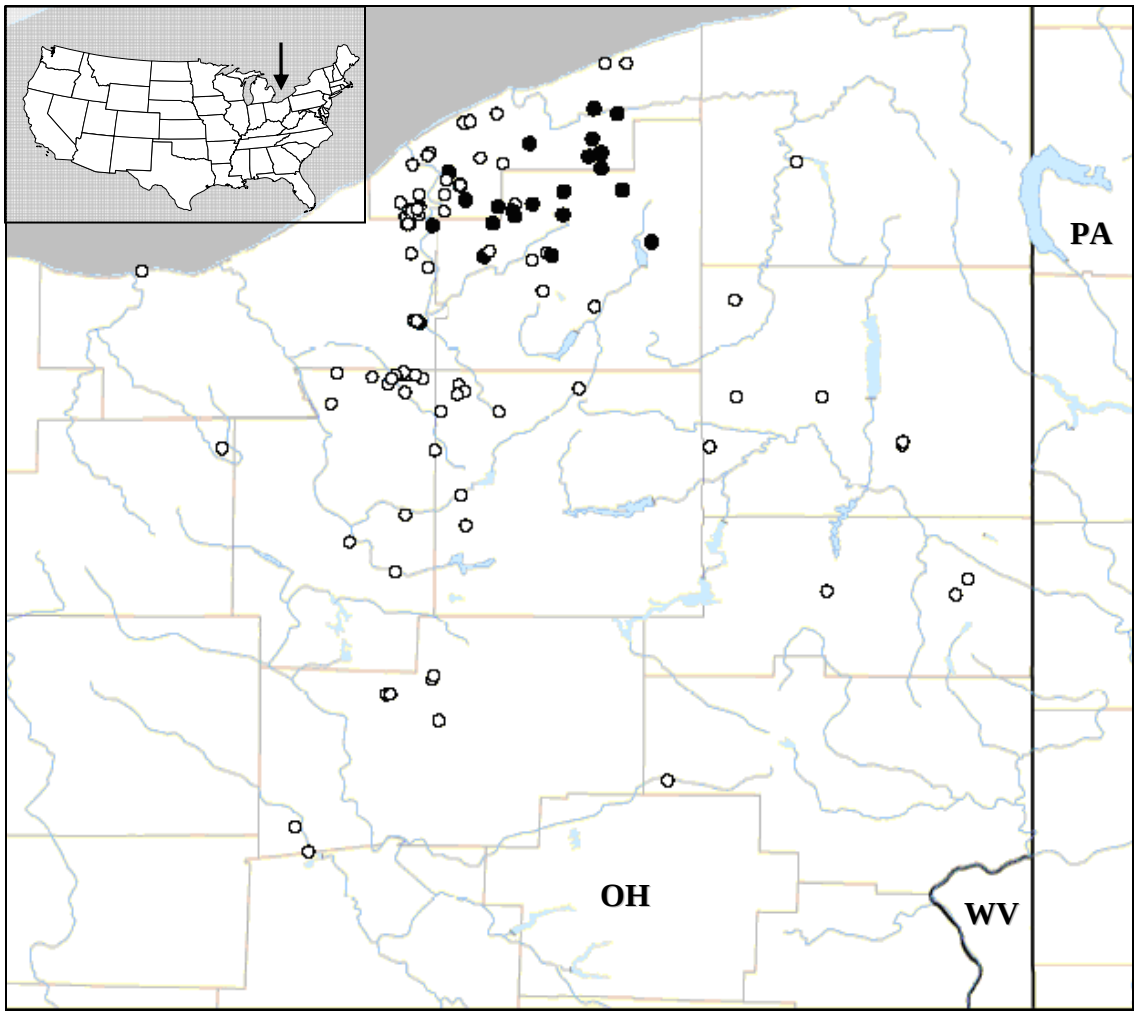


Figure 2.1 Map of collection localities of 182 raccoons sampled in northeastern Ohio. Black circles represent areas where rabid raccoons were found.

The following information was recorded for each sample collected: unique identification number, date collected, locality (latitude /longitude or UTM coordinates

along with county information), sex, age, and results of the direct fluorescent antibody (DFA) test for rabies diagnosis (protocol is available at http://www.cdc.gov/ncidod/dvrd/rabies/Diagnosis/diagnosi.htm).

2.2.2 DNA Isolation and Microsatellites

Whole genomic DNA was isolated from frozen brain tissue using the DNeasy tissue kit (Qiagen®, Valencia, California). Briefly, 10-25 mg of brain tissue was lysed using proteinase K, and DNA was selectively bound to a filter-column membrane. After a series of buffered washes and centrifugation to remove contaminants, the resulting nucleic acid was eluted in water. Seven microsatellite loci were examined, all of which have been previously examined in raccoons and found to be adequate for analysis (Kays et al. 2000; Ary 2003): P135, P140, P161, PFL4, PFL9, PFL11, and G10X (Table 2.1).

Primers were fluorescently labeled with Beckman WellRED Dye D4-PA (Proligo, Boulder, Colorado). Microsatellite reactions were performed in 15 µl volumes, including 9 µl of True Allele PCR Premix (Applied Biosystems, Foster City, California), 2 µM of each primer, and 50-100 ng of DNA. Thermal profile conditions consisted of 1 cycle of initial denaturation at 95°C for 12 minutes; 10 cycles of 94°C denaturation for 15 seconds, 50-55°C annealing for 1 minute, and 72°C extension for 30 seconds; 25 cycles of 94°C denaturation for 15 seconds, 50-55°C annealing for 1 minute, and 72°C extension for 30 seconds; and a final extension of 72°C for 30 minutes. Reactions were then diluted 1:10 in Sample Loading Solution (SLS). Subsequently, 40 µl of SLS, 0.5 µl of Size Standard 400 or Size Standard 600, and 3 µl of the diluted PCR reaction were

loaded into a single well for visualization on a CEQ™ 8000 Genetic Analyzer (Beckman Coulter, Fullerton, California).

Table 2.1 Primers used for amplification of 7 previously described microsatellite loci. All primers have been tested in raccoons (Ary 2003), and original primer descriptions can be found in the studies listed below. A.T. = annealing temperature.

Locus	A.T.	Primer Sequence	Original Study
P135	55	5'-(dyeD4)CTAGGGCATGTGTACTGGAC-3' 5'-CTTCTCCCTCTGACTTCTCC-3'	Ary 2003
P140	55	5'-(dyeD4)ACCAGGCAATGGTAATACAG-3' 5'-CCAGGAGGACTTGTCAGAT-3'	Ary 2003
P161	55	5'-(dyeD4)CTGTCATTCTCCAGTGTGTG-3' 5'-CTAACCCCTAAACATCTCCC-3'	Ary 2003
G10X	50	5'-CCACCTTCTTCCAATTCTC-3' 5'-(dyeD4)TCAGTTATCTGTGAAATCAAAA-3'	Paetkau et al. 1998
PFL4	54	5'-(dyeD4)AGGGAATGTTGCTTCTAATCC-3' 5'-GCAGCCAAACAAACTAAAGTCC-3'	Kays et al. 2000
PFL9	54	5'-(dyeD4)GCCTTCATTTAGTTGAGGTCAG-3' 5'-gCATTCTGTCAGTGGCTTTCAC-3'	Kays et al. 2000
PFL11	55	5'-(dyeD4)CATGCAAATAACACGCAC-3' 5'-CTGAACAAGGTAGGAAAGTCACTC-3'	Kays et al. 2000

A subset of 25 randomly chosen samples was replicated, and genetic profiles were checked to ensure consistency of the allele size-calls. Alleles were scored using the CEQ™ 8000 Genetic Analysis System Software (Beckman Coulter, Fullerton, California) and were proofed by eye. All samples were blinded with regard to rabies

status, and the identities of the replicated samples were blinded as well. Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) was used to adjust allele frequencies to account for potential genotyping errors including null alleles, large allele dropout, and stutter peaks. Arlequin 3.11 was then used to test for Hardy-Weinberg equilibrium as well as linkage disequilibrium between all pairs of loci using 100,000 permutations and 1,000 dememorization steps (Excoffier et al. 2005).

2.2.3 Mitochondrial DNA

An approximately 450 base pair (bp) portion of the mitochondrial control region (D loop) was amplified from the previously isolated genomic DNA using primers H16498 (5'-CCTGAACTAGGAACCAGATG-3') and L15774 (5'-GTAAAACGACGGCCAGTACATGAATTGGAGGACAACCAGT-3') of Shields and Kocher (1991). Polymerase chain reaction (PCR) amplifications were performed in 50 µl volumes containing 500 ng of DNA, 5 µl of 10X Buffer, 4 µl of $MgCl_2$, 2 µl of 10 mM dNTPs, 2.5 µl of each 20 µM primer, and 1.5 U Taq polymerase. Thermal profile conditions consisted of 1 cycle of initial denaturation at 95°C for 10 minutes; 30 cycles of 94°C denaturation for 1 minute, 55°C annealing for 1 minute, and 72°C extension for 1 minute; and a final extension of 72°C for 30 minutes. PCR products were purified with Performa® spin columns (Edge Biosystems, Gaithersburg, Maryland). After cycle-sequencing and additional purification, samples were run on an ABI PRISM® 3100 Genetic Analyzer using BigDye chain terminators (Applied Biosystems, Foster City, California).

Sequencher 3.0 software (Gene Codes, Ann Arbor, Michigan) was used to align fragments and to proof nucleotide sequences, and CLUSTAL X (Thompson et al. 1997) was used for multiple sequence alignments. Unique haplotypes were determined using Collapse 1.2 (available from http://darwin.uvigo.es), and a minimum spanning network of haplotypes was constructed using TCS 1.21 (Clement et al. 2000). Haplotype diversity and nucleotide diversity indices were calculated from the data using Arlequin 3.11 software (Excoffier et al. 2005). To test for isolation by distance, a Mantel test was performed with 1,000 permutations using the genetic and geographic distances calculated between all samples in Microsoft Excel with PopTools version 2.7 (Hood 2006).

2.2.4 Population Genetic Structure

Two Bayesian, model-based approaches were taken to provide basic information regarding raccoon genetic structure, specifically in the context of avoiding undetected population structure that might obscure the results, and to examine potential differences in rabies infection if more than one population was found (i.e. potential local adaptation). The first method used Structure (Pritchard et al. 2000) to identify the most probable number of populations in the study area. Structure can be used to analyze multi-locus genotypic data alone or in conjunction with geographic information, albeit indirectly. In the latter case, the user assigns individuals to "K" populations based on their geographic sampling locations and tests to see if the population assignments reflect geographic structure. Since Structure cannot directly include latitude/longitude coordinates, the analysis was based solely on the genetic data. Simulations were performed in triplicate with 2,000,000 iterations (100,000 dememorization steps) for 1-5 possible populations

(K) under a model of admixture. The average natural log of the probability of the data for each possible number of populations was then used to estimate the posterior probability of the most likely number of populations based on Bayes' Rule, as described in the Structure user guide (Pritchard et al. 2007).

The second approach used Geneland (Guillot et al. 2005) to determine the most probable number of populations in the study area. Like Structure, Geneland uses a Bayesian, model-based clustering algorithm and seeks to minimize deviations in Hardy-Weinberg equilibrium. Only Geneland, however, can incorporate spatially explicit data directly into the analysis. As such, Geneland runs were performed using the multi-locus genotypic data and associated latitude/longitude coordinates for each sample. Simulations were performed in triplicate with 2,000,000 iterations (100,000 dememorization steps) for 1-5 populations. The most probable number of populations was selected based on the posterior density averaged over the 3 runs.

2.2.5 Sex Ratio and Haplotype Distribution

To examine other factors that might be associated with rabies infection, the sex ratio of rabid raccoons was calculated to determine whether infection might be sex-biased. In addition, the distribution of mitochondrial haplotypes associated with rabid individuals was examined in relation to overall haplotype frequency to seek haplotypes that might be more susceptible or resistant to infection. These data were also used to document individuals that may have been translocated.

2.2.6 Relatedness

To assess raccoon relatedness and social structure as well as how that might relate to patterns of rabies infection, average within-group relatedness and pairwise individual relatedness calculations were conducted in Relatedness 5.0.8 (Queller and Goodnight 1989). A matched-pair design was used to look for differences between rabid and non-rabid individuals with similar spatial distributions. The average distance between rabid raccoon captures was calculated for this purpose. Differences in infected vs. non-infected individuals were also examined by sex and were determined using the same design. Additionally, average relatedness was examined as a function of distance. Relatedness estimates can range from -1 to +1, with 0 indicating that individuals are no more or less likely to be related than 2 individuals selected from the population at random. Positive values indicate that individuals are more likely to be related whereas negative values indicate that they are less likely to be related. Standard errors and 95% confidence intervals (CI) were determined via jackknifing over loci.

2.3 RESULTS

2.3.1 Microsatellites

In total, 1,272 loci (2,544 alleles) were genotyped in 182 raccoons. Two individuals failed to amplify at a single locus: one for locus P135 and one for locus PFL4. Micro-Checker indicated the possibility of null alleles at 3 loci (P135 = 0.06, PFL11 = 0.13, G10X = 0.09), and allele frequencies were adjusted to account for possible genotyping errors. Of the 25 replicate samples, 700 alleles were amplified and scored

consistently; genotypes were consistent in replicates with no differences in allele sizes among any of the duplicate samples. Overall, the number of alleles at each locus ranged from 3-25 with an average of 12.9 alleles per locus (Table 2.2).

Table 2.2 Microsatellite allelic diversity for all 7 loci.

Locus	# Alleles	Size Range
P135	11	268-320
P140	11	169-193
P161	10	117-153
G10X	3	141-145
PFL4	25	166-238
PFL9	11	210-230
PFL11	19	144-182
Average	12.9	n/a

Hardy-Weinberg equilibrium could not be rejected ($p > 0.05$) based on the adjusted allele frequencies obtained from Micro-Checker. No statistically significant linkage disequilibrium ($p > 0.05$) was detected among any of the markers (data not shown).

2.3.2 Population Genetic Structure

Each of the replicate Structure runs resulted in similar output values, indicating overall parameter stability. Based on the posterior probability values calculated from the data, the highest support was found for 2 raccoon populations in northeastern Ohio ($Pr(K=2) = 0.82$; Table 2.3).

Table 2.3 Number of populations (K) estimated using Structure. The posterior probability of the number of populations is given by Pr(K), which is determined using the natural log of the probability of the data (X) given the number of populations (K), ln Pr(X|K).

K	ln Pr(X|K)	Pr(K)
1	-4198.8	0.18
2	-4197.3	0.82
3	-4205.3	0.0002
4	-4229.0	1.39×10^{-14}
5	-4285.9	4.95×10^{-39}

One hundred raccoons were assigned to population 1 whereas 82 raccoons were assigned to population 2 (Figure 2.2). Three individuals were equivocal with respect to their population assignment. The two potential populations overlapped geographically in more than 30% of the sampling area.

Alternatively, the Geneland analysis identified a single population in the study area (Figure 2.3) based on the average posterior density of the 3 simulations (0.6413). Geneland indicated that the next most likely scenario was the existence of 2 populations, however, the average posterior density of the 3 simulations was much lower (0.1807) for this situation. In general, average posterior density values declined with increasing values of K (Figure 2.3).

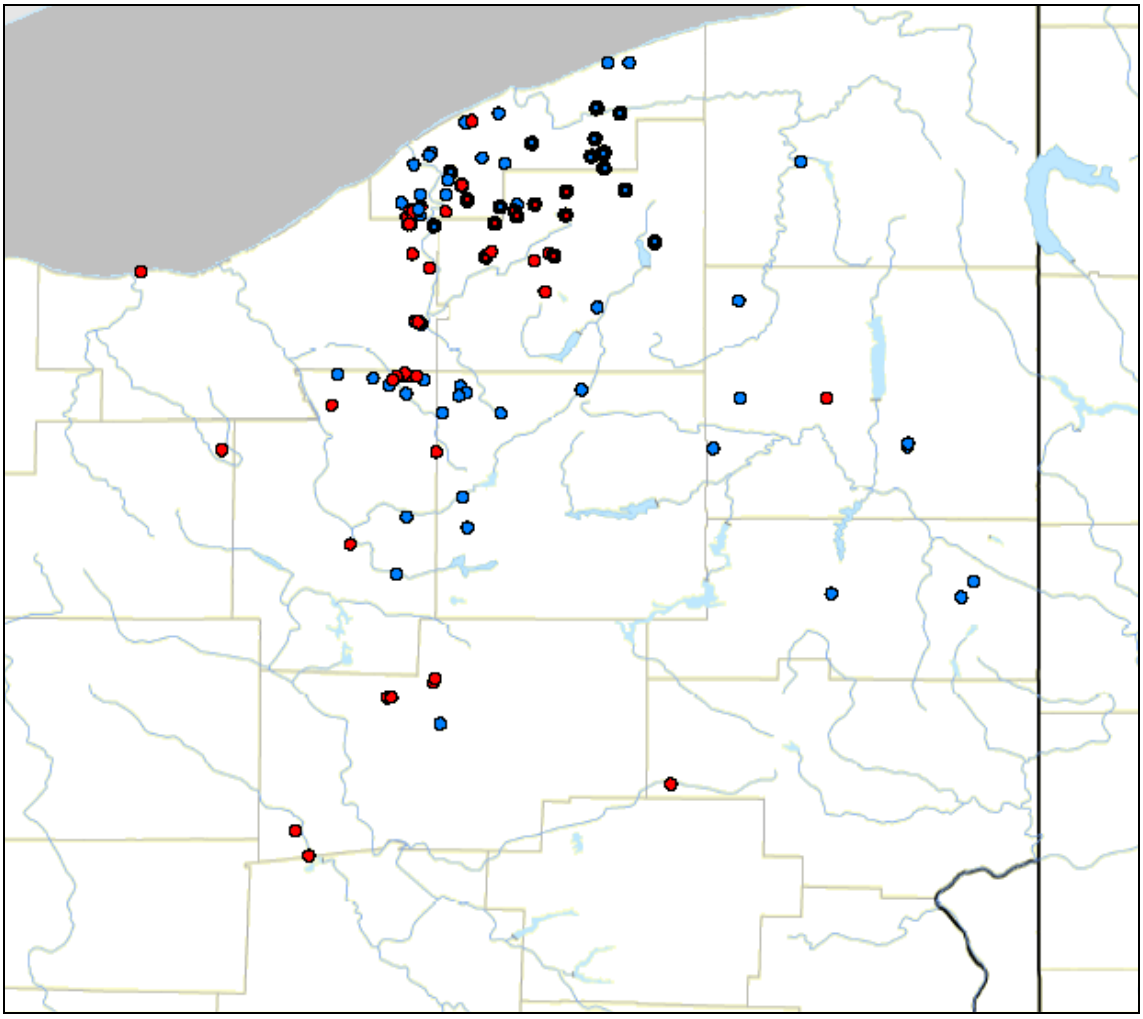


Figure 2.2 Allocation of raccoons into 2 populations, as suggested by the Structure analysis. Population 1 (blue) contained 100 raccoons, and population 2 (red) contained 82. Bold black circles represent areas where rabid raccoons were found.

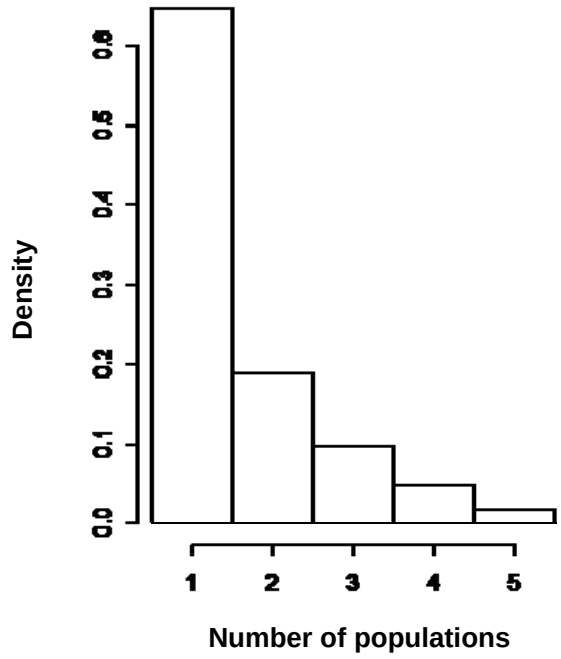


Figure 2.3 Number of populations in northeastern Ohio identified by Geneland. One population was selected as most probable (average posterior density of the three simulations for one population = 0.6413).

Given the results obtained from Structure and Geneland, the prospect of 3 or more populations within the study area was quickly eliminated (Figure 2.3; Table 2.3). Although Structure identified 2 populations as being most probable, the program is known to be sensitive to situations in which the data exhibit a pattern of isolation by distance (Pritchard et al. 2007) and an overestimation of K may result. The Mantel test conducted on the Ohio data indicated that isolation by distance was possible (r = 0.067, p = 0.052). Furthermore, the 2 populations identified by Structure showed a fair amount of

geographic overlap (Figure 2.2). Based on those results, additional analyses were conducted on the conservative and more biologically plausible assumption of a single raccoon population in northeastern Ohio (Figure 2.1). A lone non-rabid individual (OH30665, Summit County) was identified as a potential translocation from outside the study area due to the presence of unique alleles at multiple microsatellite loci.

2.3.3 Mitochondrial DNA

Sixteen unique mitochondrial haplotypes were identified from the 182 raccoons sampled in northeastern Ohio (Table 2.4).

Table 2.4 Definition of 16 unique mitochondrial haplotypes found in 182 raccoons in northeastern Ohio. Polymorphic sites contributing to haplotype definitions are shown with individual site numbers listed vertically.

```
          0001222222222222222222222222222222222222233333333333444
          6693056666666777777777788888888899999999990333477899935 5
          1275023456789012345678901234567890123456789978921201287 27
OH1       AGTCCAAACACCTACATATCAAATACCCACTGCACCACCCCATT-AGGATCGGCAGA
OH2       ..........................................................G
OH3       ..........................................................G
OH4       ...A.....................................................C...T..G
OH5       .....................................................C.....G
OH6       GAC..G...................................................G.T.AT..G
OH7       GA...G...................................................G.T.ATG.G
OH8       GA...G.................C.....................AA..TAAT..G
OH9gap    GA...G-------------------------------------....G....T.AT..G
OH10      GA...G...................................................G.T..T..G
OH11      GA..TG...................................................T.AT.AG
OH12      GA...G...................................................G.T.AT..G
OH13      GA...G...................................................T.AT..G
OH13gap   GA...G-------------------------------------.........T.AT..G
OH14gap   GA...G-------------------------------------...T...G.T.AT..G
OH15gap   GA...G-------------------------------------A.T...G.T.AT..G
```

No double peaks which might be indicative of nuclear copies of mitochondrial DNA (numts) were observed. Of 460 aligned sites, 57 sites were variable. A single nucleotide insertion was found in 2 haplotypes. In addition, 3 haplotypes possessed a 35 bp deletion and another haplotype contained a 36 bp deletion. For the purpose of nucleotide diversity calculations and subsequent analyses, the 35 bp gap was treated as a single evolutionary event.

The most common haplotype in the region, OH12, was identified as the probable ancestral haplotype (Figure 2.4). OH13gap most likely gave rise to the other haplotypes containing insertion/deletion events. Spatial structuring within the area was observed for some of the less frequently sampled mitochondrial haplotypes whereas a large amount of overlap was observed in the distributions of those that were encountered more frequently. Haplotype diversity was moderately high (0.8238 ± 0.0159) whereas nucleotide diversity was relatively low (0.0091 ± 0.0051) suggesting that most of the haplotypes in the region differed only by a few nucleotides (Figure 2.4). If multiple populations had been present, one might have expected to observe spatially segregated, distinct haplotype lineages with varying haplotype frequencies, and perhaps potential differences in RRV infection; however, the results above are consistent with the hypothesis that a single raccoon population exists in northeastern Ohio.

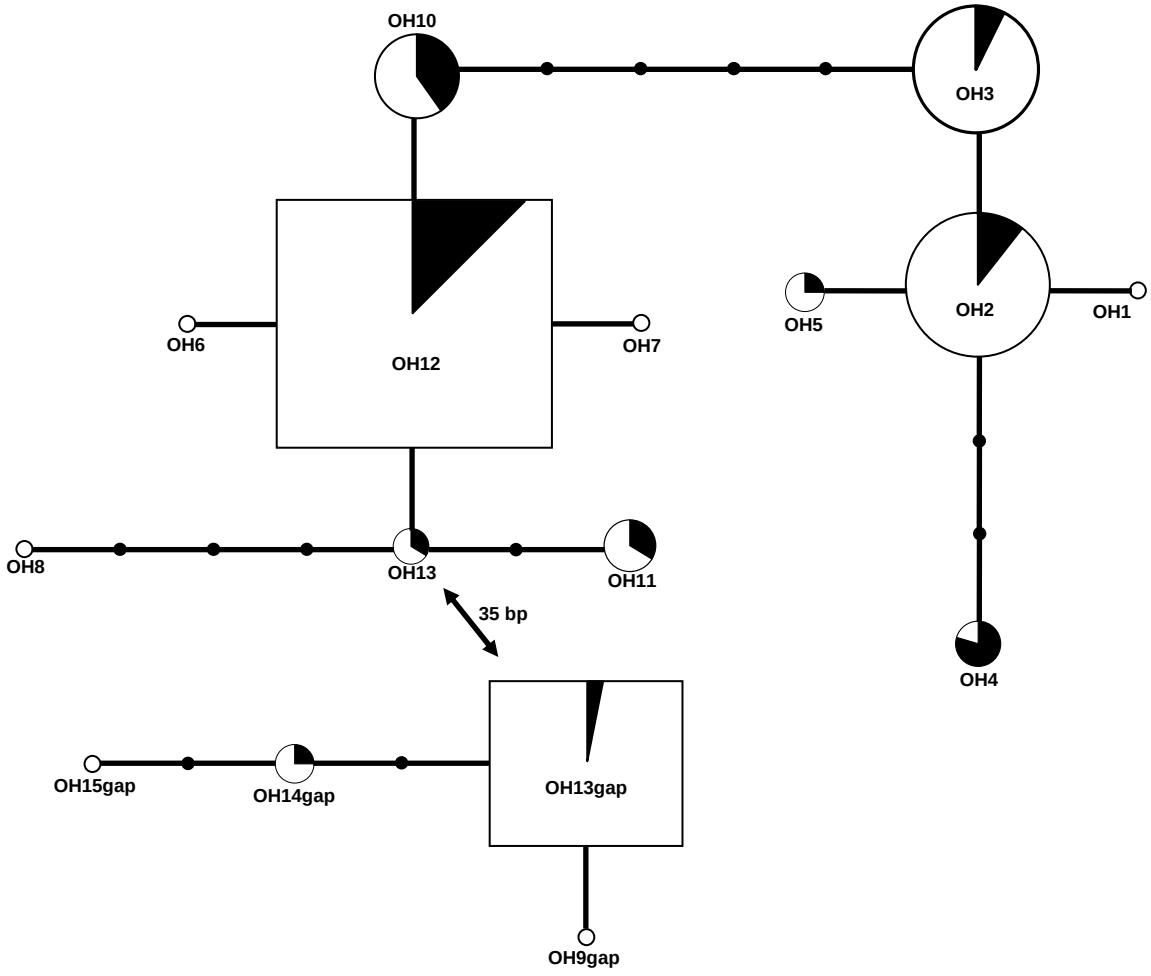


Figure 2.4 Minimum spanning network of mitochondrial haplotypes found in northeastern Ohio. Squares represent probable ancestral haplotypes, and circles represent derived haplotypes. Haplotypes not sampled are represented by black dots. Haplotype frequency corresponds to the size of the square or circle. The proportion of rabid individuals is represented by black shading.

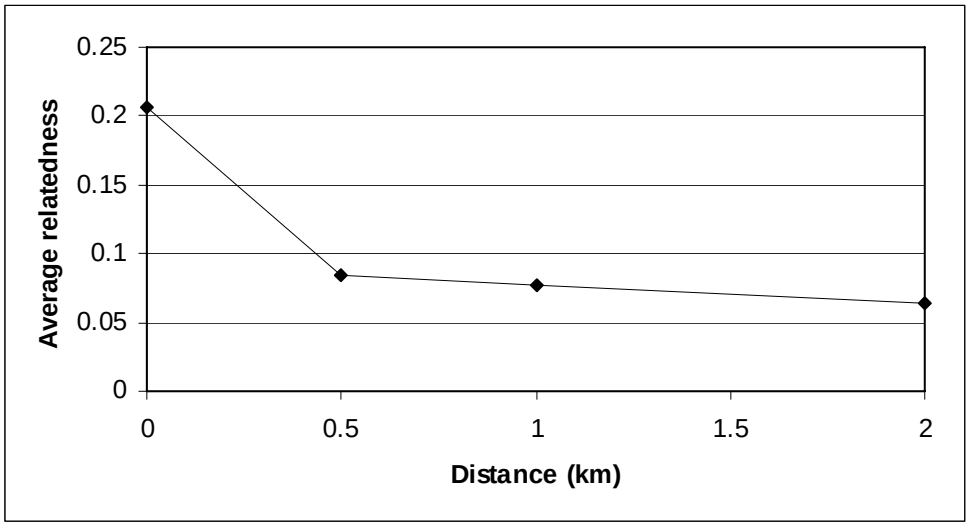
2.3.4 Rabid versus non-rabid raccoons

The overall sex ratio of the population was 1:1 (85 male, 87 female, 10 unknown sex) as was the sex ratio of the 26 rabid raccoons (12 male, 12 female, 2 unknown sex), revealing no bias towards male or female infection. All rabid raccoons possessed mitochondrial haplotypes that were common in the northeastern Ohio region. Of the 16 identified haplotypes, 12 were found in the vicinity of the majority of rabid raccoon captures. Rabid raccoons were sampled from 10 of these mitochondrial lineages (Figure 2.4), 3 of which only have been found in Ohio. OH4 is of particular interest in that 4 of the 5 sampled individuals (80%) were rabid. In another instance, 6/15 individuals (40%) within the OH10 haplotype were rabid. No clear relationship was observed, however, with respect to haplotype distribution and RRV infection. For example, OH4 was sampled only in a 2 county area corresponding to most of the rabid raccoon captures whereas OH10 was found over a much larger 7 county area. For 6 additional haplotypes, all of which were circulating among 2 or more counties, at least 25% of the sampled raccoons were rabid while another 6 low-frequency haplotypes failed to show evidence of RRV infection and were limited in distribution to a single county. Therefore, RRV occurrence does not appear to be heavily influenced by raccoon genetic structure, as inferred from the distribution of mitochondrial DNA haplotypes.

2.3.5 Relatedness

For raccoons in general, average relatedness declined with increasing distance (Figure 2.5).

(2.5a)



(2.5b)

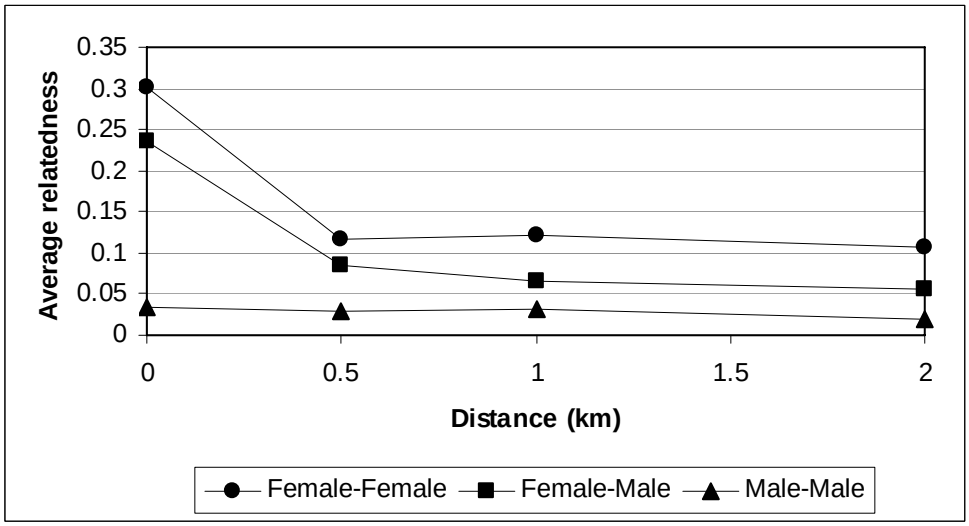


Figure 2.5 Geographic distance vs. average relatedness for (a) all raccoons, and (b) female-female, female-male, and male-male dyads.

Raccoons captured at the same locality exhibited an average relatedness coefficient of 0.2, indicative of second or third-order relatives (Figure 2.5a). Further

resolution was obtained when average relatedness was broken down by sex (Figure 2.5b). Female relatedness was consistently higher than male relatedness, with female-male relatedness falling in the middle. Females captured at the same locality were on average second-order relatives, while the relatedness coefficient of males was an order of magnitude lower. Raccoons trapped 2 kilometers apart were on average third-order relatives or even more distantly related.

All rabid raccoons were caught within a diameter of 43.8 kilometers, with an average distance of 14.31 kilometers between captures. Rabid females were captured from 0 to 27.7 (average = 11.72) kilometers apart whereas rabid males were captured between 2.3 and 43.8 (average = 17.14) kilometers. These capture locations represent point samples, and as a result, cannot account for individual raccoon movements (such as while foraging) or dispersion. Relatedness estimates were calculated on 48 individuals with similar spatial distributions in the rabid vs. non-rabid group and on 24 individuals when relatedness was broken down by sex. Rabid raccoons were no more likely to be related than were non-rabid raccoons (Table 2.5; rabid = 0.0011, 95% CI: -0.0055 – 0.0077; non-rabid = -0.0162, 95% CI: -0.0537 – 0.0213), nor was an association found among males (rabid = -0.0053, 95% CI: -0.0447 – 0.0341; non-rabid = 0.0031, 95% CI: -0.0230 – 0.0292) or females (rabid = -0.0092, 95% CI: -0.0258 – 0.0074; non-rabid = -0.0052, 95% CI: -0.0333 – 0.0229). In fact, none of the mean relatedness values significantly deviated from 0 (Table 2.5); therefore, it does not appear that raccoon genetic or social structure, as inferred through relatedness estimates, strongly influences RRV transmission.

Table 2.5 Relatedness calculations and 95% confidence intervals for rabid and non-rabid raccoons overall as well as rabid and non-rabid raccoons by sex.

Group	Relatedness	95% Confidence Interval
Rabid (overall)	0.0011	-0.0055 – 0.0077
Non-rabid (overall)	-0.0162	-0.0537 – 0.0213
Rabid males	-0.0053	-0.0447 – 0.0341
Non-rabid males	0.0031	-0.0230 – 0.0292
Rabid females	-0.0092	-0.0258 – 0.0074
Non-rabid females	-0.0052	-0.0333 – 0.0229

Individual cases of related, rabid individuals were detected for a handful of dyads. First-order relationships, parent/offspring or full sibling, could be established for 1 rabid male-male dyad and 3 rabid male-female dyads (relatedness coefficient ~ 0.5). Second-order relatives, half-sibling or grandparent/grandchild relationships (~ 0.25), were identified for 12 male-male, 2 female-female, and 6 male-female rabid dyads. Relationships on the order of great grandparent/great grandchild or cousins (~ 0.125) were seen in 12 male-male, 6 female-female, and 20 male-female dyads. The remainder of dyads possessed relatedness measures below that which could be interpreted as third-order family-level relationships.

2.4 DISCUSSION

Although Structure (Pritchard et al. 2000) is an extremely useful tool for identifying population genetic structure, our data appear to fall into the category of datasets for which Structure's algorithm does not perform optimally. Linkage disequilibrium, deviations from Hardy-Weinberg equilibrium, null alleles, and isolation

by distance can all confound the Structure analysis and lead to a potential overestimation of K (Pritchard et al. 2007). A weak signature of isolation by distance was suggested for raccoons in Ohio as was the potential for null alleles. In these situations, the use of a second Bayesian clustering method is suggested for comparison, especially one that can incorporate space explicitly such as Geneland (Guillot et al. 2005). Indeed, in our case, this step was critical for determining the most accurate level of population structure in northeastern Ohio.

Raccoons formed a single population in the study area, eliminating the prospect of separate populations with different susceptibilities to RRV infection or undetected population structure that might distort subsequent results. The fact that rabid raccoons are part of a larger Ohio population adds to the concern that RRV could spread relatively quickly throughout the state and into new areas given that no genetic differences are evident to indicate that raccoons might show some type of resistance to infection. All of the mitochondrial haplotypes were closely related, therefore, one might expect that they could be affected equally by RRV infection. Although certain mitochondrial lineages were proportionately more affected by RRV than others, the small sample size of rabid raccoons makes the generality of this pattern difficult to substantiate. Furthermore, it is unlikely that additional rabid raccoon samples would clarify this pattern given the wide range of haplotypes that were affected by RRV. While it is possible that RRV is still in the process of local adaptation and has not had enough time to result in detectable population structure, it seems more likely that local adaptation is not a factor in RRV transmission.

Given that all rabid raccoons in northeastern Ohio possessed mitochondrial haplotypes and microsatellite alleles common within the study area, it does not appear that translocated individuals played a significant role in the most recent focus of rabies. Only one non-rabid raccoon was identified as a potentially translocated individual based on its genotypic profile. This particular individual belonged to the OH12 mitochondrial lineage, which is widespread throughout the East Coast from Georgia to Ohio. These results correspond well with those of Henderson et al. (2008) who found support for monophyly of the virus sequences in the most recent Ohio raccoon rabies outbreak. Thus, a hypothesis that endemic raccoon rabies activity was present within the area but undetected until 2004 is supported by the combination of viral sequences and raccoon genetic data.

Social organization has been recognized as an important factor in pathogen transmission previously, especially in species that live in large social groups or in dense populations (Altizer et al. 2003; Schauber et al. 2007). For example, parasite prevalence and intensity has been shown to correlate with group size in prairie dogs (Hoogland 1979), and a host density threshold is necessary for establishment of brucellosis in North American bison (Dobson and Meagher 1996). Blanchong et al. (2007) recently discovered that TB-infected deer were more related than uninfected deer and that contact within family groups was an important mechanism of transmission. Similarly, Root et al. (2004) found that deer mice infected with Sin Nombre hantavirus were more closely related than those not infected. Unlike these examples, rabid raccoons in northeastern Ohio were not significantly more related than non-rabid raccoons. Although there does not appear to be a statistically significant relationship between rabies and genetic

relatedness or social structure, individual instances of the involvement of related individuals, perhaps due to social behaviors such as den sharing or common foraging grounds, were observed. Behavioral activities, including denning and breeding, previously have been suggested to influence raccoon rabies transmission (Rosatte et al. 2006; Arjo et al. 2008).

Assuming that female philopatry resulted in more RRV transmission due to shared home ranges and increased contact among relatives, then one would expect to see a higher relatedness coefficient for rabid female-female dyads. This was not the case in Ohio. Overall relatedness tended to decline with increasing distance, and on average, third-order female relatives could be found only at distances up to 1 kilometer. The average distance between rabid females was nearly 12 kilometers, indicating that female family-level associations were operating at a more localized level and thus would not greatly influence RRV transmission. This may have been due to a detection issue, as one of the major limitations of this study was the scale upon which rabid raccoons were sampled, given our reliance on the active and passive surveillance efforts and their inherent biases (as well as available resources) at the state level. However, Dharmarajan et al. (2009) recently suggested that the demographic and behavioral processes affecting raccoon spatial organization are most critical within individual habitat patches in fragmented landscapes. Furthermore, raccoon captures represent single point localities, and dispersion or normal daily movements (such as while foraging) could potentially obscure actual home range areas. Finally, northeastern Ohio, the region examined in this study, is located at the western edge of RRV's current distribution; therefore, it is unclear

whether this area is representative of what might be found in a "core" RRV region or at other RRV "edges".

Male-male interactions appeared equally as prevalent as female-female or female-male interactions, however in terms of dyads, males accounted for more first and second order relationships among rabid individuals. In terms of social structure, the role of males may in fact be greater than previously recognized, especially given their tendency to disperse and the formation of related or non-related male coalitions; however, in terms of RRV transmission, our results suggest that neither social nor genetic structure is responsible for the pattern of RRV spread in Ohio.

Rabies virus emergence has been tied to environmental factors as well as to genetic heterogeneity (Real et al. 2005). Given that the raccoons' genetic and social structure do not influence RRV transmission in Ohio, it is possible that population density alone can account for the increased contact rates. In this case, increased RRV transmission would result regardless of raccoon social structure. Raccoons are known to reach extremely high densities in urban areas (Zeveloff 2002), and northeastern Ohio is home to several large cities including Akron, Canton, Youngstown, and Cleveland. Given Ohio's position as a potential gateway for the westward spread of RRV along with a relatively dense raccoon population and favorable raccoon habitat not only across the state but also in adjacent states, active surveillance will be critical for identifying new cases of RRV and preventing its spread beyond the current vaccination barrier.

CHAPTER 3

Historical and Contemporary Evolution Account for Population Subdivision in Raccoon (*Procyon lotor*) Populations in the Eastern United States

3.1 INTRODUCTION

3.1.1 Landscape Genetics and Relevance to Raccoon Rabies

Landscape genetics, a newly emerging field combining molecular population genetics and landscape ecology, can be used to understand how heterogeneity across the landscape affects population structuring at different geographic and temporal scales (Sork et al. 1999; Manel et al. 2003). While some boundaries to gene flow are evident (mountains, rivers, etc.), others may be cryptic and can result from genetic differentiation in areas without any obvious causes, or from secondary contact in areas where populations were previously isolated (Manel et al. 2003). Since landscape connectivity can change over time, the temporal component has become increasingly important when attempting to interpret the evolutionary processes giving rise to current spatial patterns of genetic differentiation (Zellmer and Knowles 2009). In the past, gene flow was often described based on mitochondrial markers and thus reflected an evolutionary time scale; however, advances in nuclear molecular marker development (microsatellites, etc.) and statistical modeling have permitted the examination of gene flow on an ecological time scale as well (Sork et al. 1999). These methods have recently been used to distinguish between historical and contemporary barriers to gene flow in a variety of study systems

and have a wide range of applicability (Busack and Lawson 2008; Lindell et al. 2008; Paun et al. 2008; Zellmer and Knowles 2009). Furthermore, the application of landscape genetics based studies to disease management is now beginning to gain momentum, especially in the context of examining barriers to host gene flow (Blanchong et al. 2008; DeYoung et al. 2009; Root et al. 2009; Barton et al. 2010).

For raccoons (*Procyon lotor*) that have been affected by a raccoon-adapted variant of rabies virus (RRV) in the Eastern United States (US), these methods have the potential to elucidate raccoon and RRV movement and gene flow patterns, both historic and current, and to inform on management decisions for preventing further spread of RRV. Raccoon rabies was first identified in central Florida in 1947 and has been endemic in the southeastern United States since that time (Kappus et al. 1970; Bigler et al. 1973). Raccoon rabies progressively spread throughout Florida during the 1950s and eventually moved into Alabama, Georgia, and South Carolina over the next 20 years (Held et al. 1967; Kappus et al. 1970; McLean 1971; Jenkins and Winkler 1987; Winkler and Jenkins 1991). The translocation of southeastern raccoons during the mid 1970s likely led to the appearance of raccoon rabies on the Virginia/West Virginia border (Nettles et al. 1979; Smith et al. 1984; Rupprecht and Smith 1994). From this new focus, RRV quickly spread throughout the northeastern United States and progressed into Canada by 1999. The first human fatality attributed to RRV was identified in Virginia in 2003 (Silverstein et al. 2003). Today, RRV can be found throughout the eastern United States from Florida to Maine with its range extending as far west as Ohio, Tennessee, and Alabama (Blanton et al. 2009).

Given that rabies is a directly transmitted pathogen requiring animal to animal contact for transmission, it is reasonable to assume that landscape factors that act as obstacles to raccoon movement will also act as barriers to RRV spread (Biek et al. 2007; Real and Biek 2007; Cullingham et al. 2008; Cullingham et al. 2009). The raccoon, a highly vagile mesocarnivore with a nearly continuous distribution throughout the United States (Hall and Kelson 1959; Zeveloff 2002), is extremely adaptable due to its generalist tendencies (Zeveloff 2002), and can reach high densities, upwards of 140 raccoons per square kilometer (Rosatte et al. 1992; Riley et al. 1998; Smith and Engeman 2002), in urban settings (Prange et al. 2003). For these reasons, it is important to examine potential barriers to raccoon dispersal and gene flow, especially in the context of preventing further spread of RRV. This is highlighted by the fact that supposed physical barriers, such as mountains, large rivers, and major highways are often used in conjunction with oral rabies vaccination (ORV), whereby baits filled with a vaccinia-rabies glycoprotein (V-RG) recombinant virus are distributed to susceptible raccoons creating a buffer zone of vaccinated animals, to prevent RRV from spreading into new areas (Smith et al. 2002; Rupprecht et al. 2004; Slate et al. 2005; Arjo et al. 2008).

3.1.2 Suture-Zones As Historic or Contemporary Barriers to Gene Flow?

In 1968, Remington identified 6 major zones of intraspecific and interspecific hybridization of animals in North America (Figure 3.1). These areas were termed "suture-zones" and referred to areas of geographic overlap between major biotic assemblages with the potential for hybridization (Remington 1968; Swenson and Howard 2004). Within these comparatively localized zones, secondary contact and subsequent

interbreeding has been observed for a number of populations and/or closely related species formerly separated by physical or vegetational barriers. Although instances of hybridization are abundant within these suture-zones (Remington 1968), few examples have been noted outside of these areas.

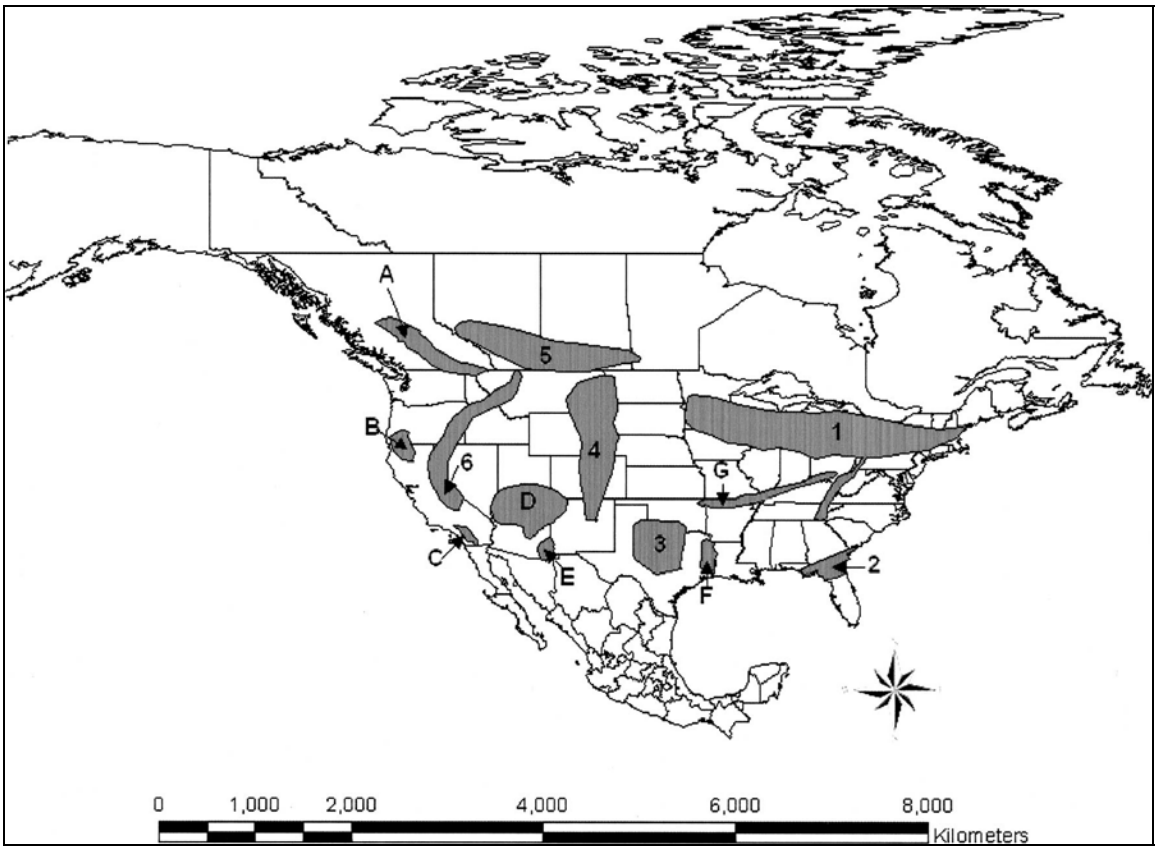


Figure 3.1 Digital version of Remington's 6 major and 7 minor suture zones, taken from Swenson and Howard (2004).

The majority of suture-zones coincide with previously glaciated areas of North America, implicating glacial cycling as a plausible mechanism for both isolation and secondary contact. The Northern Florida Suture-Zone, a relatively narrow strip along the border of Florida and Georgia, is an exception in that it has remained unglaciated and lacks a major physical boundary such as a mountain range. More than 50 possible

hybridizing species are thought to occur in this small area, including plants, insects, reptiles, amphibians, and mammals.

Two of the 6 major suture-zones, the Northern Florida Suture-Zone (Zone 2) and the Northeastern-Central Suture-Zone (Zone 1), a portion of which runs southward along the Appalachian Mountains (Remington 1968), fall within the range of raccoons affected by RRV. The purpose of this study is to test the hypothesis that the Northeastern-Central and Northern Florida Suture-Zones have acted as geographic barriers to gene flow in raccoons and their associated pathogen, RRV. Although these areas are presumed to represent historical barriers, it is unclear whether they constitute contemporary barriers or if secondary contact has resulted in recent gene flow and/or population mixing. By examining a combination of nuclear and mitochondrial markers, we can distinguish between potential historical and contemporary boundaries. If the defined suture-zones have affected raccoon gene flow historically, then one would expect to find population genetic subdivision, based on mitochondrial markers, corresponding to those regions. If the suture-zones represent current barriers to gene flow, then the observed pattern of genetic differentiation should hold based on the microsatellite data; however, if secondary contact has occurred, then raccoons should form single populations (i.e., no genetic sub-structuring) in the suture-zone regions. Alternatively, the suture-zones may not correspond to raccoon subdivision at all, in which case, genetic structure would not be detected at either temporal scale in those areas.

The Northeastern-Central Suture-Zone falls along the Appalachian Mountains and coincides with the western boundary of RRV. In fact, this area is thought to restrict raccoon dispersal across the mountain range or sustain lower raccoon population

densities due to the habitat changes associated with its high elevation (Biek et al. 2007). For years, the Appalachians have been used as a natural barrier in combination with ORV campaigns to restrict RRV spread (Slate et al. 2005; Slate et al. 2009). Recently, however, Cullingham et al. (2008) suggested that raccoon gene flow was not reduced by the Appalachian Mountains but that raccoon density may play a role in limiting rabies spread within this region. If the Northeastern-Central Suture-Zone has acted as an obstruction to raccoon dispersal and gene flow, then raccoons on either side of the Appalachian Mountains should represent genetically distinct populations.

The Northern Florida Suture-Zone lacks an obvious physical boundary for raccoons, yet initial spread of RRV from Florida into the surrounding states occurred at a relatively slow rate over a 20 year period (Kappus et al. 1970; McLean 1971; Jenkins and Winkler 1987; Winkler and Jenkins 1991). Some have hypothesized that landscape shape limited raccoon movement (Cullingham et al. 2008) and thus caused the slow progression of RRV in the southeastern US; however, resource distribution, habitat suitability, and population density are other possibilities that could have affected the rate of RRV transmission and spread. In addition, vegetational barriers have been described for some species within this region (Remington 1968) as have historical physical boundaries.

A deep water channel known as the Suwannee Strait (Figure 3.2) flowed through northern Florida between the Atlantic Ocean and the Gulf of Mexico (Randazzo and Jones 1997) during the Middle Eocene (approximately 56-34 million years ago) effectively isolating Florida from the mainland US. This channel likely influenced a number of unrelated species within the region by preventing genetic exchange between

terrestrial species found on the Florida peninsula and those located on the southeastern mainland while promoting gene flow in aquatic species. Most agree that the strait filled with sediment by the late Oligocene (around 23 million years ago) or during the Miocene (23-5.5 million years ago; Swift et al. 1986), isolating aquatic populations in the Gulf of Mexico from those in the Atlantic Ocean while opening the door for secondary contact in terrestrial species populations.

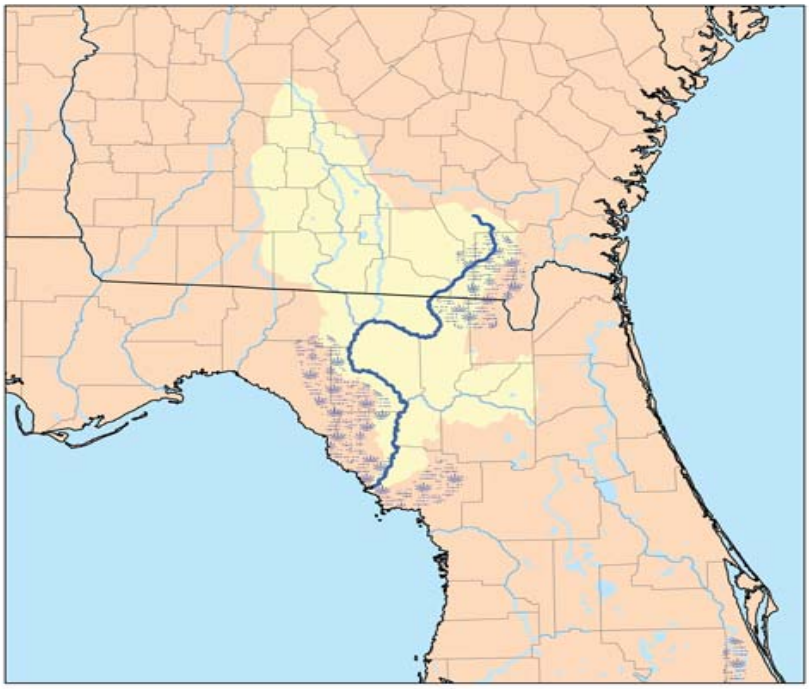


Figure 3.2 Location of the Suwannee River Watershed, site of the prehistoric Suwannee Strait. Map courtesy of Karl Musser

Remington (1968) speculated that the integration of populations within the Northern Florida Suture-Zone was a more recent occurrence. Similar events occurred during the Pleistocene (2 million to 10,000 years ago) interglacial periods, when

moderate to high sea stands turned Florida into a series of islands (Remington 1968). If the Northern Florida Suture-Zone has represented an obstacle to raccoon gene flow, then raccoons in peninsular Florida should be genetically distinct from those found on the southeastern US mainland.

3.1.3 A Common Phylogeographic Boundary for the Southeastern United States Eco-Region?

A secondary purpose of this study is to elucidate the population genetic structure and evolutionary history of raccoons in the southeastern US to test the hypothesized phylogeographic boundary that defines the southeastern US eco-region (Avise 2000) with the highly vagile raccoon as a model system. With the advent of molecular methods, and during the initial rise in popularity of phylogeography, many faunal surveys involved taxa found in the southeastern US (Avise 2000) and facilitated the characterization of Remington's (1968) proposed Northern Florida Suture-Zone. Comparison of these phylogeographic studies resulted in the identification of similar diversification/ differentiation patterns in unrelated species within the southeast, evident from mitochondrial DNA analyses. Broadly, freshwater and marine species tended to separate into eastern and western populations whereas populations of terrestrial species in peninsular Florida often differed from those found on the US mainland. As a result, it has been hypothesized that certain species assemblages in the southeastern US might have been influenced by shared events, glacial cycling for example, throughout their evolutionary histories and that a phylogeographic boundary in the area of the Northern Florida Suture-Zone, perhaps due to the Suwannee Strait or a similar feature during the

Pleistocene, defines the southeastern US eco-region (Avise 1992; Ellsworth et al. 1994; Avise 1996; Walker and Avise 1998; Avise 2000).

Although several examples can be gleaned from freshwater fishes (Philipp et al. 1983; Bermingham and Avise 1986; Swift et al. 1986; Avise 1992; Duggins et al. 1995) and costal-restricted vertebrates and invertebrates (Bert 1986; Avise et al. 1992; Walker and Avise 1998), examples from terrestrial mammalian species have been less abundant. Of note, southeastern pocket gophers (Avise et al. 1979) and eastern woodrats (Hayes and Harrison 1992) have shown patterns of genetic differentiation that could be consistent with the phylogeographic boundary hypothesis. In addition, a distinct line located near the proposed Suwannee Strait separates short-tailed shrews into 2 subspecies (Benedict et al. 2006). Interestingly, an extremely narrow contact zone of the subspecies has been detected less than 2 kilometers from the original dividing line. In all 3 of these cases, small mammals capable of limited dispersal were examined thus making it difficult to definitively document the existence of a broad scale phylogeographic boundary. Rather than common factors influencing an entire eco-region, these divisions could reflect the natural history and biology of species with restricted mobility.

The historical biogeography of a larger mammal, the white-tailed deer, was described based on restriction enzymes (Ellsworth et al. 1994) and like the smaller mammalian species, genetic subdivision was observed in the southeastern region. Given the potential for long distance dispersal in this species, the result might have been surprising had the authors not commented on previous studies showing that white-tailed deer in the southeast were “remarkably sedentary”. As such, it was not unreasonable to

expect some differentiation in the area because of the ecological and behavioral characteristics of the deer (Ellsworth et al. 1994).

Despite these examples, the historical phylogeographic boundary hypothesized to define the southeastern United States eco-region in the area of the Northern Florida Suture-Zone has not been tested in a terrestrial mammalian species capable of moving large distances with no *a priori* assumptions for population subdivision.

3.2 MATERIALS AND METHODS

3.2.1 Raccoon Samples

As part of continuing surveillance for RRV in the eastern US, raccoon brain tissue was submitted to the Centers for Disease Control and Prevention (CDC) by the United States Department of Agriculture (USDA) Wildlife Services or by state Departments of Health for rabies diagnosis. Each raccoon was assigned a unique identification number with an associated collection locality (latitude/longitude or UTM coordinates, as well as county information) and date of collection. Additionally, museum-archived raccoon specimens were used to supplement areas in which raccoon samples were lacking. A total of 625 raccoons from the eastern US, encompassing both the Northeastern-Central Suture-Zone and the Northern Florida Suture-Zone, were sampled from Florida to Ohio (Figure 3.3)

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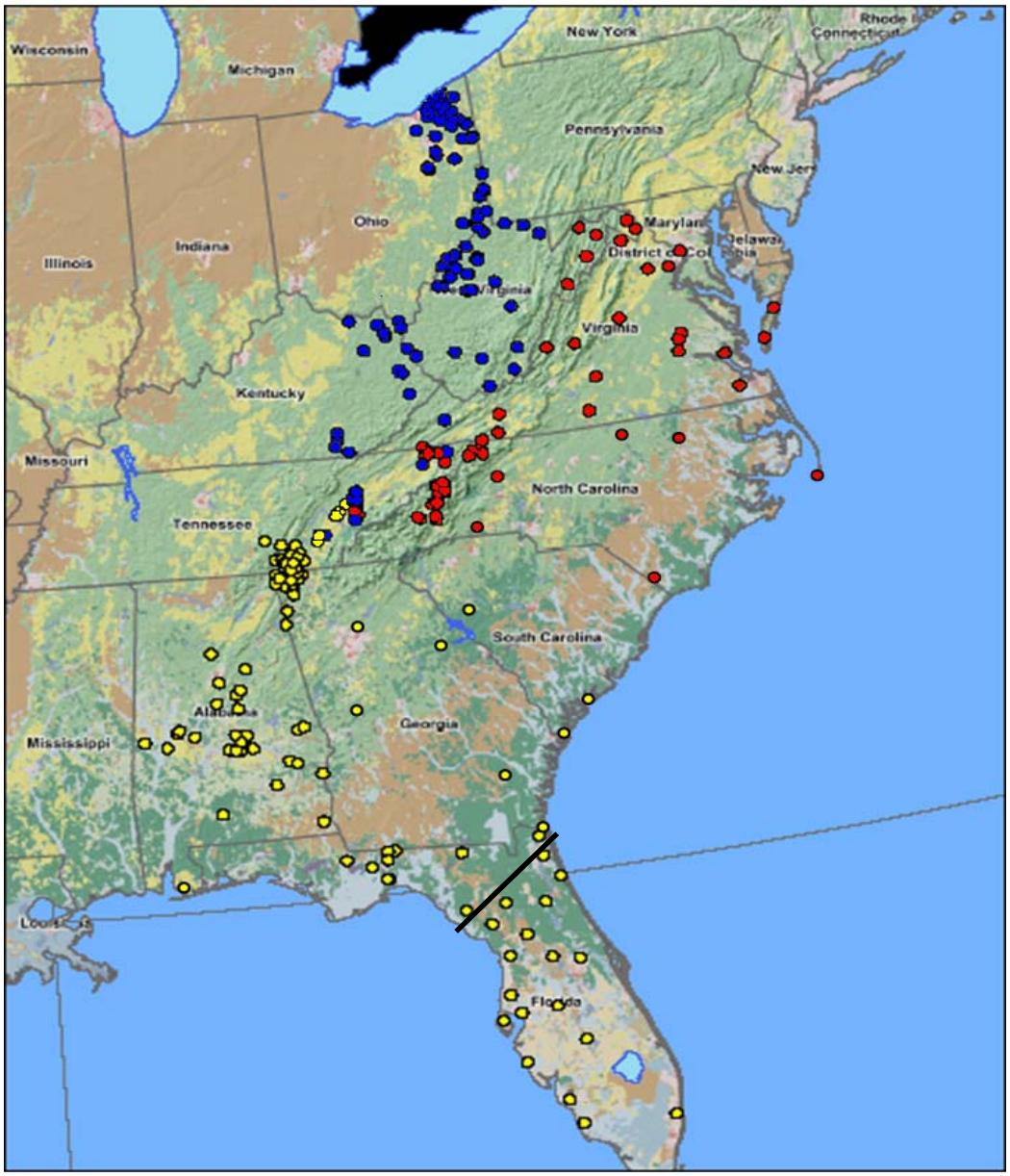


Figure 3.3 Sampling localities of 625 raccoons throughout the Eastern US. Population 1 = blue dots; population 2 = yellow dots; population 3 = red dots. Black line indicates approximate line of mitochondrial differentiation of Florida raccoons.

3.2.2 DNA Isolation and Microsatellites

Genomic DNA was isolated from whole blood, bone, brain, or liver tissue using the DNeasy tissue kit (Qiagen®, Valencia, California) in a biosafety level-2 facility. Briefly, 10-25 mg of tissue, up to 100 mg of ground bone, or 100 microliters of blood was lysed using proteinase K, and DNA was selectively bound to a filter-column membrane. After a series of buffered washes and centrifugation to remove contaminants, the DNA was eluted in water. Aside from overall DNA yield, no differences would be expected due to isolation from different tissues. Seven microsatellite loci previously tested in raccoons (P135, P140, P161, PFL4, PFL9, PFL11, and G10X; Kays et al. 2000; Ary 2003; see also Chapter 2) were used to characterize the population structure of raccoons in the eastern US. Standard methods were used to amplify each of the 7 microsatellite loci, with reactions performed in 15 µl volumes (9 µl of True Allele PCR Premix (Applied Biosystems, Foster City, California), 2 µM of each primer, and 50-100 ng of DNA). Thermal profile conditions consisted of 1 cycle of initial denaturation at 95°C for 12 minutes; 10 cycles of 94°C denaturation for 15 seconds, 50-55°C annealing for 1 minute, and 72°C extension for 30 seconds; 25 cycles of 94°C denaturation for 15 seconds, 50-55°C annealing for 1 minute, and 72°C extension for 30 seconds; and a final extension of 72°C for 30 minutes.

The CEQ™ 8000 Genetic Analysis System Software (Beckman Coulter, Fullerton, California) was used to score microsatellite alleles with subsequent proofing by eye. Microsatellite reactions were repeated for a blinded subset of 63 randomly chosen samples (10% of the overall sample size), and genetic profiles were checked to ensure consistency of allele size calls. Micro-Checker 2.2.3 (Van Oosterhout et al. 2004) was

used to adjust allele frequencies to account for potential genotyping errors including null alleles, large allele dropout, and stutter peaks, which could affect the outcome of subsequent analyses. Tests for linkage disequilibrium as well as Hardy-Weinberg equilibrium were carried out in Arlequin 3.11 software (Excoffier et al. 2005) using 100,000 permutations and 1,000 dememorization steps.

3.2.3 Population Genetic Structure

An accurate representation of raccoon population genetic structure is necessary to discern between alternative hypotheses; therefore, Bayesian model-based clustering algorithms implemented in two programs, Structure (Pritchard et al. 2000) and Geneland (Guillot et al. 2005), were used to determine the most probable number of raccoon populations in the eastern US, in the areas surrounding the hypothesized suture-zones. One potential disadvantage of the Structure program is that in certain instances, linkage disequilibrium, deviations from Hardy-Weinberg equilibrium, null alleles, isolation by distance, and differences in sample size between clusters can all confound the analysis and lead to a potential overestimation of populations or genetic clusters (Pritchard et al. 2007; Hubisz et al. 2009); therefore, use of a second method like the spatially-explicit Geneland is recommended.

Although Structure can incorporate geographic information indirectly, it is a crude measure whereby the user assigns individuals to K populations based on their geographic location, and the resulting output is compared with what one might expect if there was indeed geographic structure. Therefore, Structure is generally regarded as a nonspatial method. As a result, simulations solely on the genetic data were performed in

triplicate with 5,000,000 iterations (500,000 dememorization steps) for a possibility of 1 to 10 populations (K) under a model of admixture. The average natural log of the probability of the data for each possible number of populations was then used to estimate the posterior probability of the most likely number of populations based on Bayes' Rule, as described in the Structure user guide (Pritchard et al. 2007).

Geneland, on the other hand, can simultaneously incorporate spatially explicit data (i.e. latitude/longitude coordinates) and genetic data during computations. Therefore, Geneland runs were based on the microsatellite data and an associated spatial location (latitude/longitude) for each sample. As above, simulations were performed in triplicate with 5,000,000 iterations (500,000 dememorization steps) for a possibility of 1 to 10 populations (K). The most probable number of populations was then chosen based on the posterior density averaged over the 3 Geneland runs.

After determining the most probable number of populations, an Analysis of Molecular Variance (AMOVA) was used to examine levels of genetic differentiation between the populations and to confirm validity of the suggested population structure (Excoffier et al. 2005).

3.2.4 Mitochondrial DNA

Sequence data was generated for an approximately 450 base pair (bp) portion of the mitochondrial control region (D loop) from genomic DNA using the primers H16498 and L15774 (Shields and Kocher 1991; see also Chapter 2). Polymerase chain reaction (PCR) amplifications were performed in 50 µl volumes with 500 ng of DNA, 5 µl of 10X Buffer, 4 µl of $MgCl_2$, 2 µl of 10 mM dNTPs, 2.5 µl of each 20 µM primer, and 1.5 U

Taq polymerase. Thermal profile conditions consisted of 1 cycle of initial denaturation at 95°C for 10 minutes; 30 cycles of 94°C denaturation for 1 minute, 55°C annealing for 1 minute, and 72°C extension for 1 minute; and a final extension of 72°C for 30 minutes. After cycle-sequencing reactions and additional purification, samples were run on an ABI PRISM® 3100 Genetic Analyzer using BigDye chain terminators (Applied Biosystems, Foster City, California).

For analyses based on mitochondrial DNA sequencing, nucleotide fragments were aligned and proofed with Sequencher 3.0 software (Gene Codes, Ann Arbor, Michigan), and a multiple sequence alignment was generated using CLUSTAL X (Thompson et al. 1997). Unique haplotypes were distinguished by Collapse 1.2 software (available from http://darwin.uvigo.es), and a minimum spanning network of haplotypes was constructed using the median-joining method (Bandelt et al. 1999) in Network 4.2.0.1 (available from http://www.fluxus-engineering.com). Basic descriptive indices, including haplotype and nucleotide diversity, were calculated from the data in Arlequin 3.11 software (Excoffier et al. 2005). In addition, a hierarchical AMOVA analysis was conducted on the nucleotide sequence data in the same fashion as for the microsatellite data to further confirm the population structure and to quantify the amount of differentiation between populations.

3.2.5 Tests of Selective Neutrality

Fu's (1997) and Chakraborty's (1990) tests of selective neutrality were performed to evaluate possible population expansion or mixing events that might contribute to the observed patterns of raccoon genetic differentiation. For results indicative of expansion,

mismatch distributions and Harpending's raggedness index were calculated to assess potential deviations from a null model of sudden expansion (Rogers and Harpending 1992).

3.3 RESULTS

3.3.1 Microsatellites

The total number of loci amplified and genotyped in 625 raccoons was 4,333 (8,666 alleles) with an overall missing data percentage of 0.96%. Complete microsatellite profiles were generated for 583 raccoons. The remaining 42 raccoons were missing data at one locus. Micro-Checker indicated the possibility of null alleles at 3 loci (PFL9 = 0.18, PFL11 = 0.16, G10X = 0.07), and allele frequencies were adjusted to account for possible genotyping errors. Microsatellites were amplified and scored consistently as no differences were observed in genotypes for the subset of 63 individuals examined twice (882 loci; 1,764 alleles).

Table 3.1 Allelic diversity and size range for all 7 loci.

Locus	# Alleles	Size Range
P135	15	266-320
P140	13	169-193
P161	14	111-153
G10X	10	129-147
PFL4	29	166-238
PFL9	17	190-232
PFL11	20	126-182
Average	16.857	n/a

The number of alleles at each locus ranged from 10 to 29, with an average of 16.857 alleles per locus (Table 3.1). No statistically significant ($p > 0.05$) instances of linkage disequilibrium were identified (data not shown).

3.3.2 Population Genetic Structure

Three raccoon populations (Figure 3.3) were deemed most likely by the spatial method implemented in Geneland with the average density of the 3 simulations = 0.5975 (Figure 3.4).

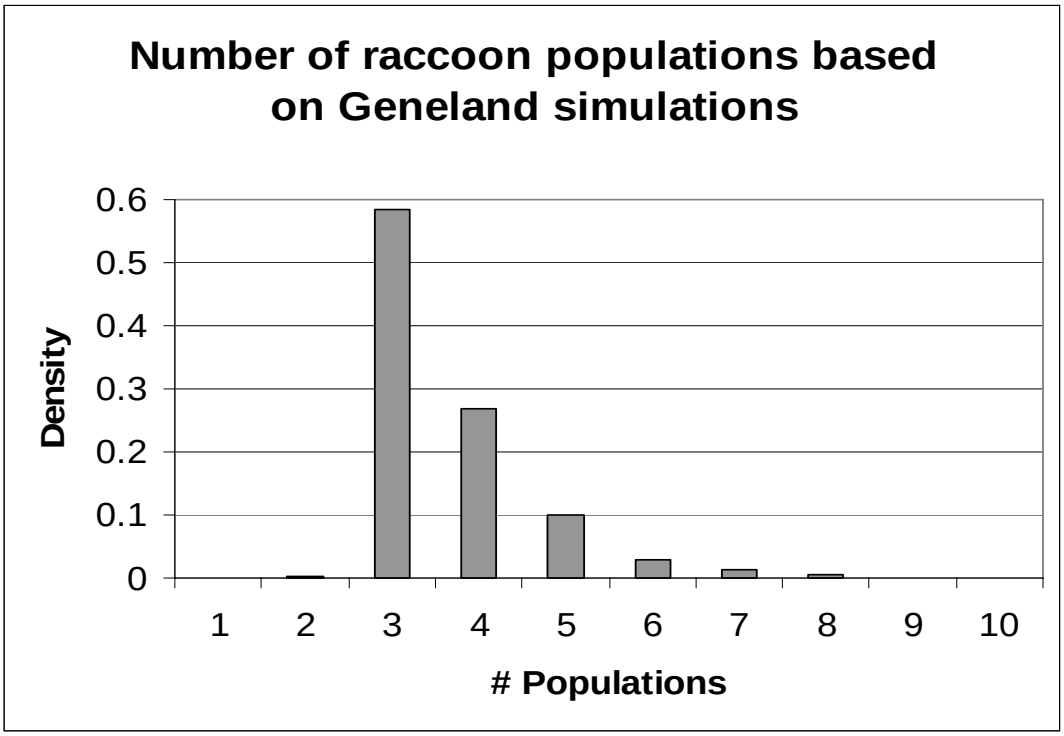


Figure 3.4 Number of raccoon populations in the Eastern US identified by Geneland. Three populations was selected as most probable (average posterior density of the three simulations for one population = 0.5975).

Population 1 consisted of 218 raccoons from Kentucky, Ohio, West Virginia, extreme western Virginia, and Tennessee; population 2 was comprised of 228 raccoons from Alabama, Georgia, South Carolina, Tennessee, and Florida; and population 3 included 179 raccoons from northeastern West Virginia, Virginia, North Carolina and eastern Tennessee (Figure 3.3).

Four populations (Table 3.2) were identified by the Structure analysis (Pr(K=4) = 0.99) with the additional population occurring where the 3 populations tended to overlap in Tennessee. Most individuals assigned to this population were highly admixed, however, and support for the population was based on low individual assignment probabilities. Therefore, subsequent analyses were based on the more conservative 3-population structure.

Table 3.2 Number of populations (K) estimated using Structure. The posterior probability of the number of populations is given by Pr(K), which is determined using the natural log of the probability of the data (X) given the number of populations (K), ln Pr(X|K).

K	ln Pr(X|K)	Pr(K)
1	-7022.6	0.0
2	-6943.5	0.0
3	-6753.8	4.74×10^{-22}
4	-6704.7	0.999999081
5	-6785.1	1.21×10^{-35}
6	-6845.4	7.85×10^{-62}
7	-6718.6	9.19×10^{-7}
8	-6874.7	1.48×10^{-74}
9	-7086.9	0.0
10	-7207.6	0.0

The AMOVA based on the bi-parentally inherited microsatellite data indicated low to moderate levels of subdivision ($F_{ST} = 0.05593$, $p < 0.01$) whereas the overall φ_{ST} value, based on the maternally inherited mitochondrial data, signified moderate to high amounts of subdivision ($\varphi_{ST} = 0.16820$, $p < 0.01$). Pairwise F_{ST} and φ_{ST} values (Table 3.3) showed that population 3 was most divergent. Further support for the 3-population structure came from an analysis of Hardy-Weinberg equilibrium. When raccoons were analyzed as a single population, a deficiency in overall heterozygosity was detected; however, no deviations from Hardy-Weinberg equilibrium were observed when raccoons were assigned to 3 separate populations (data not shown). Since population assignment algorithms seek to minimize deviations from Hardy-Weinberg and linkage disequilibrium when constructing population boundaries and individual assignments, this can be taken as additional evidence in support of the 3 described raccoon populations.

Table 3.3 Population pairwise F_{ST} and φ_{ST} values: F_{ST} and φ_{ST} values are located above the diagonal with the associated p-values below the diagonal. First line corresponds to F_{ST}, and second line corresponds to φ_{ST}.

	Population 1	Population 2	Population 3
Population 1	--	0.02996	0.06823
		0.09535	0.16213
Population 2	0.00000	--	0.04938
	0.00000		0.18554
Population 3	0.00000	0.00909	--
	0.00000	0.00000	

3.3.3 Mitochondrial DNA

Ninety-two distinct mitochondrial haplotypes were identified (Figure 3.5). No double peaks, which might be indicative of nuclear copies of DNA (numts), were observed. Of 440 aligned nucleotides, 71 sites were polymorphic. Haplotype diversity was relatively high (0.8692 ± 0.0304) whereas nucleotide diversity was low (0.0139 ± 0.0073; Table 3.4). The majority of haplotypes differed by a small number of substitutions, although a 35 bp deletion was present in 4 separate haplotypes found in the northeastern part of the study area. Furthermore, one haplotype not containing the deletion event contained an additional 36 bp insertion. For all population genetic analyses, these insertion/deletion events were represented as single evolutionary events.

Three main haplotype groups were identified (Figure 3.5), roughly corresponding to the populations described based on the microsatellite results: one consisting of samples from all localities except Florida (I); another group restricted to Ohio, Tennessee, Kentucky, and West Virginia (II); and a final group containing samples from Florida, Georgia, Alabama, and Tennessee (III). All 3 haplotype groups were represented in Tennessee. Most haplotypes were limited in range, although a single nearly cosmopolitan haplotype belonging to haplotype group I was found in every location sampled with the exception of Florida. This particular haplotype was widely distributed and occurred at the highest overall frequency (Figure 3.5), resulting in its identification as the likely ancestral haplotype.

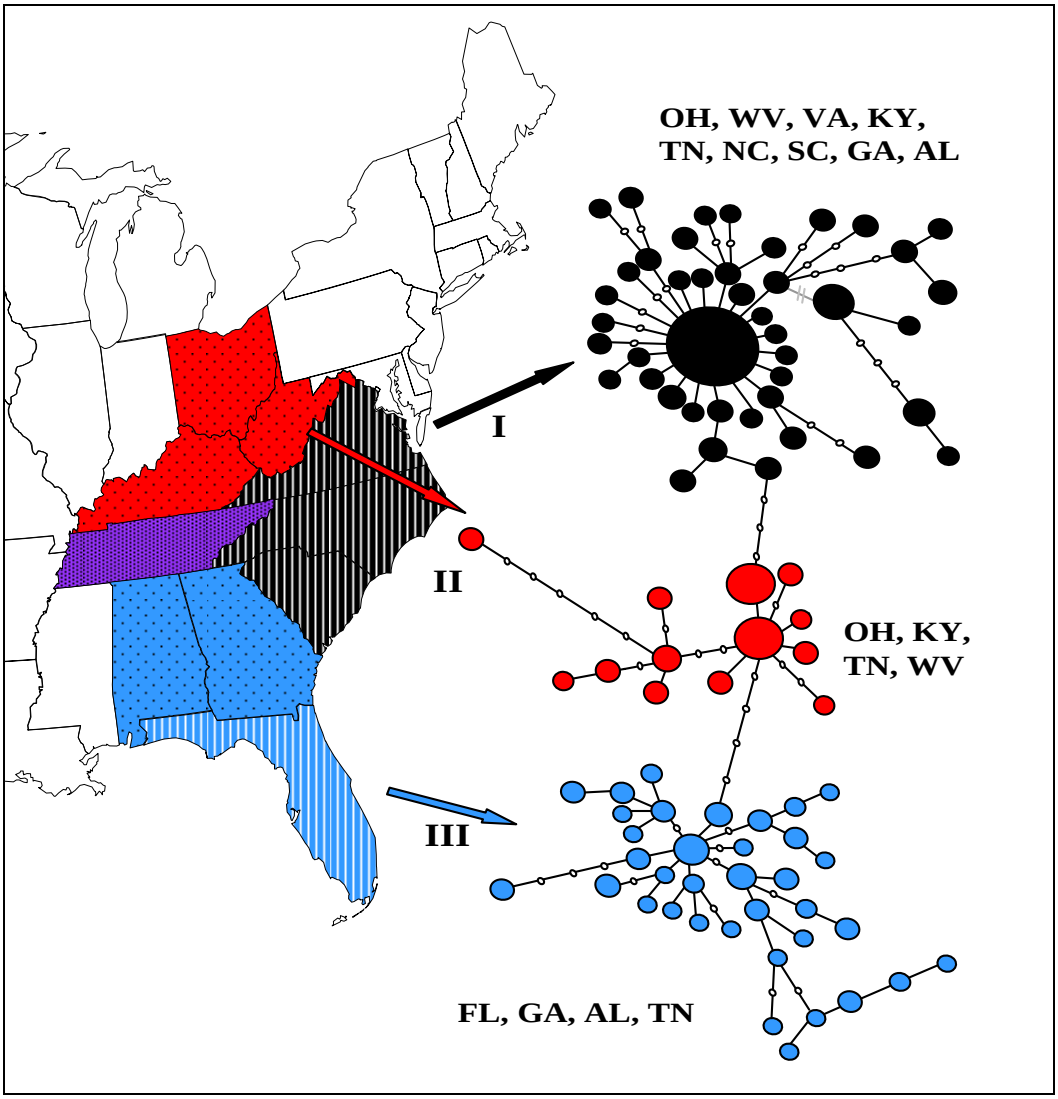


Figure 3.5 Distribution of the 3 haplotype groups—states with a single haplotype group are shown with vertical lines; states where 2 haplotype groups occur are shown with dots; all 3 haplotype groups are found in Tennessee, shown in purple. Node size corresponds to relative haplotype frequency. Unsampled haplotypes are shown as small, open circles. Branch lengths correspond to the number of mutational steps between haplotypes. The 4 haplotypes in group I containing a 35 bp deletion event are indicated via a light gray line with a break.

Table 3.4 Haplotype and nucleotide diversity indices and neutrality tests. Sample size (n), haplotype diversity ($\hat{H}$), and nucleotide diversity (π). For Chakraborty's test, the number indicates number of observed alleles (for this test, $p < 0.02$ is equivalent to $p < 0.05$).

Population	1	2	3	Avg
N	218	228	179	208
$\hat{H}$	0.8437±0.0146	0.9633±0.0077	0.8007±0.0689	0.8692±0.0304
π	0.0183±0.0094	0.0177±0.0091	0.0057±0.0035	0.0139±0.0073
Fu's Fs	0.00377	-24.58216	-1.90560	n/a
p-value	0.57800	0.00000*	0.16000	
Chakraborty's	28	67	9	n/a
p-value	0.00634*	0.00323*	0.26513	

Despite grouping with individuals from Alabama, Georgia, and a number of Tennessee raccoons based on microsatellite data, raccoons from peninsular Florida were mitochondrially distinct (Figure 3.3) and retained a high haplotype diversity index when analyzed as a separate population (data not shown). Five haplotypes found in northern Florida were shared with individuals in Georgia and Tennessee; however, 3 of these cases involved single individuals in Florida.

3.3.4 Tests of Selective Neutrality

Chakraborty's test of population amalgamation (Chakraborty 1990) signified population mixing in populations 1 and 2 (Table 3.4), and Fu's Fs test (Fu 1997), which is particularly sensitive to detecting population expansion events, provided support for expansion of population 2 (Table 3.4). The mismatch distribution ($p = 0.295$) and

calculation of Harpending's raggedness index (HRI = 0.009, p = 0.589) also suggested that the null hypothesis of population expansion could not be rejected for population 2.

3.4 DISCUSSION

3.4.1 Contemporary Population Structure and Barriers to Gene Flow

Three distinct, contemporary raccoon populations were identified using Bayesian clustering methods based on the microsatellite data: one to the west of the Appalachian Mountains ranging from Ohio to Tennessee (population 1), another to the east of the Appalachians incorporating a 4-state area (population 3), and the last population extending over a large portion of the southeastern US (population 2). Although Cullingham et al. (2008) suggested that raccoon gene flow was not reduced by the Appalachian Mountains, our study found that populations were divided along the northern and middle portions of their range, thus confirming that the Appalachian Mountain range does act as a formidable barrier to raccoon dispersal in the absence of human-mediated long distance translocation events. This is not surprising since the area corresponds to part of the Northeastern-Central Suture-Zone where a variety of species have been divided historically (Remington 1968). Moreover, very little RRV activity has been detected to the west of the Appalachians (Biek et al. 2007), providing additional support for distinct populations on either side of the range.

In a recent study revisiting raccoon subspecific designations, Cullingham et al. (2008) observed lineage mixing between the historically defined subspecies with the exception of *P. l. elucus*, the subspecies supposedly restricted to Florida. Despite that

study, which examined mitochondrial data only, and historic evidence for a possible phylogeographic boundary in the area, the nuclear data generated in the current study suggests contemporary gene flow between Florida and "mainland" raccoons and highlights the importance of using both maternally and bi-parentally inherited markers when studying population structure. Raccoons in Alabama, Georgia, southern Tennessee, and Florida formed a single population (population 2, Figure 3.3), indicating that the Northern Florida Suture-Zone does not constitute a contemporary barrier to raccoon gene flow. In fact, a probable dispersal corridor for raccoons within this region runs along the Cumberland Plateau, and landscape connectivity is supported by the large area encompassing population 2. Just north of that region, raccoons from all 3 populations could be found, perhaps indicative of a former refugium whereby raccoons later dispersed north along the Allegheny Plateau. Further support for dispersal along the Allegheny Plateau can be gleaned from the recent study by Root et al. (2009) who found that raccoons in the ridge-and-valley system in Pennsylvania comprised a panmictic population with a signature of isolation by distance.

These results could have important implications for management of the oral rabies vaccination campaign, where mountains are used in conjunction with vaccine-laden baits to prevent the westward spread of RRV. Based on this study, rabies management officials should continue to use the Appalachian Mountains as a natural physical barrier in conjunction with ORV baits. In addition, increased attention should be given to the region along the Cumberland Plateau when thinking about areas of relatively unimpeded raccoon movement and gene flow as well as the potential for additional RRV spread.

3.4.2 Historical Population Structure and Barriers to Gene Flow

Despite the fact that Florida and southeastern US "mainland" raccoons form a single, modern population based on the microsatellite data generated in this study, raccoons from the Florida peninsula are mitochondrially divergent from their southeastern US counterparts. These results support the observation of Cullingham et al. (2008) that *P. l. elucus* is a mitochondrially distinct subspecies and that it has been historically isolated from other southeastern US raccoons. Mitochondrial DNA based analyses also indicate that raccoons further north, on either side of the Appalachian Mountains, are divergent. This implies that both the Northeastern-Central and Northern Florida suture-zones served to limit raccoon gene flow historically, yet the two regions have influenced raccoons in very different manners since that time.

According to fossil evidence, raccoons have been present in North America since the Pliocene (Simpson 1945; Hibbard et al. 1965; Lotze and Anderson 1979). Furthermore, raccoons were abundant throughout the US during the early Pleistocene with a range from the Atlantic Ocean to the Pacific coast (Goldman 1950). Similar to other previously examined mammals (Avise et al. 1979; Hayes and Harrison 1992; Ellsworth et al. 1994), raccoons appear to have been influenced by the Pleistocene glacial cycles. Despite a lack of ice cover, even unglaciated areas experienced climatic changes, resulting in altered habitat locations and dispersal routes (Robison 1986; Starnes and Etnier 1986; Lomolino et al. 2006). Raccoons most likely shifted their range southward during the cooler temperatures and altered climates that occurred during this time. The area with the highest amount of haplotype diversity corresponds to population 2, consistent with the notion that genetic diversity tends to remain highest where potential

refugia may have occurred. Lower genetic diversity values were observed in the northern area, also supporting this scenario. Once the climate warmed again, raccoons could have moved northward. This argument is supported by the apparent expansion of population 2. In addition to expansion, population mixing was identified for both populations 1 and 2, especially in Tennessee. This was rather surprising because of the mountainous terrain in some parts of the state; however, the Cumberland Plateau could have served as a corridor allowing raccoons from each population to mix. Evidence of population mixing has been described from this area previously as the center of diversity and largest assemblage of freshwater fish species in North America, with the exception of the Mississippi River drainage, occurs in the Tennessee and Cumberland River drainage areas (Starnes and Etnier 1986).

3.4.3 Implications for a Southeastern US Eco-Region

Overall, raccoons do share similar patterns of subdivision as do other unrelated taxa within the southeastern US eco-region based on the mitochondrial data although nuclear data fail to support Florida as a biologically distinct entity. This pattern is reflective of a historical, temporary vicariance event (gathered from mitochondrial DNA) and ongoing contemporary gene flow (based on microsatellites).

As noted above, raccoons from the Florida peninsula are mitochondrially distinct from other areas along the eastern seaboard, with the division occurring roughly along the same line in Florida as has been documented for short-tailed shrews (Benedict et al. 2006). This line falls within the proposed Northern Florida Suture-Zone (Remington 1968), lending evidence to a common phylogeographic boundary for multiple species in

the southeastern US eco-region. In fact, Remington (1968) previously included the raccoon on his list of more than 50 possible hybridizing species in the suture-zone.

An isolating mechanism during the moderate to high sea stands of the Pleistocene may be responsible for the mitochondrial differentiation in Florida raccoons. During this time, Florida was described as a series of small islands separated by water (Remington 1968) during interglacial periods. In particular, the St. Johns River in northern Florida functioned as an extension of the Atlantic Ocean and may have acted in a similar fashion as the much older Suwannee Strait. Furthermore, Swift et al. (1986) noted that peninsular Florida south of the Suwanee and St. Johns drainages served as a distinct faunal province for fish, lending additional evidence to an isolating mechanism in that particular area. It is unlikely that Florida raccoons underwent any bottlenecks or founder effects given the high amount of haplotype diversity present in the population whether analyzed as part of a larger southeastern group or alone.

3.4.4 Factors Influencing Raccoon Rabies Spread During the 1950s-1970s

Raccoons are clearly heterogeneous throughout the RRV endemic area. Since the limitation to raccoon dispersal appears to have occurred much earlier than the period of time when RRV spread across the southeastern US, other ecological factors such as population density or resource distribution may have contributed to its relatively slow progression during the 1950s–1970s. Cullingham et al. (2008) claimed that there is little contemporary gene flow into Florida despite the absence of an obvious barrier and suggested that landscape shape is responsible for raccoon dispersal and the slow spread of the RRV in the southeast. Cullingham's study (2008) was based solely on

mitochondrial DNA, however, and highlights the importance of examining both mitochondrial and nuclear markers to gain a complete understanding of a population's evolutionary history. In the current study, we found evidence of historical isolation of Florida raccoons but recent gene flow among raccoons in the southeastern US. This would argue against limited gene flow in this region and would not fully explain landscape shape as a factor in the 20+ years it took for RRV to spread from Florida into Alabama, Georgia, and South Carolina.

Raccoon density is another possibility for the slow progression of the virus during the 1950s-1970s; however, raccoon densities vary considerably and are often due to differences in habitat quality or resource distribution. While a lower raccoon population density in the southern US has been hypothesized to explain the variation in RRV spread, exceptions to this generality are easy to find. For example, raccoon density at an urban park in Florida (Smith and Engeman 2002) is 2.5 times higher than the raccoon density in northern Ohio (Ramey et al. 2008). Although raccoons are generalists and are capable of exploiting a variety of habitats (Zeveloff 2002), differences in resource distribution and/or habitat quality may cause spatial aggregation and could affect population density estimates, contact rates, and the potential for RRV transmission.

Interestingly, raccoons in northern Florida share haplotypes with individuals in Georgia and Tennessee but fail to share any mitochondrial haplotypes with raccoons in Alabama, the southwestern edge of RRV's current range. Future studies should examine additional raccoons in Alabama, Mississippi, and in other western localities to determine the significance of this trend. Given the range of raccoons in population 2, it is interesting to speculate that without translocation, RRV may never have moved out of the

southeastern US. Regardless, for the purposes of rabies management and control, identifying additional barriers to gene flow among raccoons and RRVs will be of critical importance.

CHAPTER 4

Spatiotemporal Interactions of Enzootic Raccoon Rabies in Raccoons and Skunks

4.1 INTRODUCTION

The question of how a pathogen moves in space and time is of fundamental importance for disease ecologists (Anderson and May 1978). Transmission of disease depends not only on the interaction between the location and timing of cases (Real and Biek 2007) but also on the number of susceptible individuals within a given area considered at risk for infection. Since these factors can change over time and throughout space, disease occurrence is often heterogeneously distributed. For multi-host systems, this heterogeneity becomes even further complicated because the hosts will have different biological or natural history characteristics that can result in different patterns of aggregation. For example, the host distribution of a solitary species will look much different than the distribution of a social species.

Methods to examine spatial clustering patterns were developed (Ripley's K-function; Ripley 1976) to account for the heterogeneous distribution of a particular event. In practice, the average number of events within a distance "h" of a randomly chosen event is divided by the average number of events per unit area. Deviations from the null hypothesis of complete spatial randomness (CSR), where an event is equally likely to occur at any location within a given study area, indicate either clustering or inhibition (Waller and Gotway 2004). The first spatial-temporal clustering method is attributed to

Knox (1963), but his method was dependent on events occurring within a threshold value for distance and time. Diggle et al. (1995) later developed a method for estimating spatial-temporal clustering as a function of the spatial and temporal separation in the data.

Although K-function techniques have been used to investigate disease dynamics in human and livestock populations (French et al. 2005; Levy et al. 2006), only recently have these methods been extended to studying disease in wildlife populations. As a novel application, Carslake et al. (2005) adapted Diggle's K-function (Diggle et al. 1995) to examine spatial-temporal interactions of cowpox virus infection in two rodent hosts, wood mice and bank voles. Transmission risk was most important locally for both species, but key differences were noted. Multiple discrete, short-lived outbreaks that appeared to be seasonal were detected in wood mice based on the temporal clustering pattern of cowpox cases. This indicated that the wood mice might not be able to sustain cowpox infection year round. Rather, multiple reintroductions from other host species or environmental sources might be responsible for the continued maintenance of cowpox in wood mice. Bank voles, on the other hand, had the potential to transmit cowpox year round and cases tended to rise with population increases.

In a subsequent study, Carslake et al. (2006) used the same methods to examine "who acquired infection from whom" in different classes (i.e., males and females) of the same rodent species. Different modes of transmission were evident based on the space-time interactions observed within and between sexes for each species. Wood mice transmitted primarily between opposite sexes whereas females seemingly were more important for infection of both sexes in bank voles. Aside from these studies, wildlife

populations have not been widely considered when examining the spatial and temporal scale of transmission risk based on K-function methods.

Raccoon rabies virus (RRV) has proven to be a particularly well-suited model system to examine infectious disease dynamics and is significant due to its obvious public health importance (Coyne et al. 1989; Lucey et al. 2002; Smith et al. 2002; Guerra et al. 2003; Gordon et al. 2004; Real et al. 2005). Enzootic in the southeastern states from the 1950s onward (Kappus et al. 1970; McLean 1971; Bigler et al. 1973), raccoons presumably incubating virus were translocated to the West Virginia/Virginia border in the late 1970s (Nettles et al. 1979; Rupprecht and Smith 1994). This sparked a new focus of raccoon rabies in the Mid-Atlantic States that subsequently spread along the eastern seaboard (Jenkins and Winkler 1987). RRV can now be found from Florida to Maine, with the leading edge now extending into Ohio, Tennessee, and Alabama (Blanton et al. 2009). RRV is the predominant variant in the eastern United States and has already resulted in at least one human fatality (Silverstein et al. 2003).

In the northeast, the situation is particularly interesting because of the potential involvement of skunks in the maintenance and transmission of RRV. With a two-host system, like RRV in the northeastern US, host-switching or host-shift events are also important considerations. Mutation in RRV could result in a variant that readily crosses over into another mesocarnivore host, such as the skunk or fox, and becomes sustained in a new species. Although this is not thought to be common with rabies, potential raccoon to raccoon transmission may have occurred after infection with a fox strain in New York in the late 1940's (McLean 1975; Winkler and Jenkins 1991). Furthermore, transmission of a bat rabies virus variant from bats to skunks and foxes in Arizona (Leslie et al. 2006;

http://www.avma.org/onlnews/javma/nov09/091115m.asp) with suspected sustained carnivore to carnivore transmission highlights the very real possibility of rabies virus crossing over from one host and subsequent adaptation to a new species. Recently, Streicker et al. (2010) demonstrated cross species transmission (CST) of several bat rabies viruses and highlighted phylogenetic similarity and geographic overlap as two key determinants for CST events and potential host shifts.

Although RRV is primarily maintained and transmitted by raccoons, skunks can be infected with the raccoon variant and have been shown to be important secondary hosts (Guerra et al. 2003), although the duration of clinical signs is shorter in skunks infected with RRV as opposed to skunk rabies virus variants (Charlton et al. 1988). Epizootics of rabies in raccoons and skunks moved in a similar direction from 1990-2000 in the eastern United States, and the number of rabid skunks could be predicted based on the number of rabid raccoons identified one month prior (Guerra et al. 2003). More recently, in 2008, 47.1% of rabies cases in skunks, in states where RRV is enzootic, could be attributed to spillover from raccoons (Blanton et al. 2009). Guerra et al. (2003) were unable to provide evidence for independent cycling among skunks in RRV enzootic areas but did establish that rabies epizootics in raccoons and skunks are closely coupled. Given the differences in host biology, however, it is reasonable to expect variation in patterns of transmission for raccoons and skunks. Importantly, although skunks may consume oral rabies vaccine (ORV) baits formulated for raccoons, this method has not proven to be efficient in preventing rabies in skunks in the field (Charlton et al. 1992; Guerra et al. 2003; Grosenbaugh et al. 2007; United States Department of Agriculture 2007) which could be extremely problematic for controlling the spread of RRV.

The purpose of this study is to determine, for both raccoons and skunks, if where a RRV case occurs is independent of when it occurs (i.e., whether the cases are spatiotemporally linked) and at what spatial and temporal scale an individual is a risk to others in an enzootic area. Clustering of RRV cases can be expected given that rabies virus is directly transmitted from animal to animal via biting, meaning that individuals must come into close contact with one another for transmission to occur. Given that raccoons and skunks both occupy home ranges with some degree of overlap with others, these home range sizes likely affect contact rates and thus the spatial range (distance) of transmission risk. Similar to Carslake et al. (2005), we hypothesize that infection risk is highest within one home range diameter and one infectious period. Previous studies have suggested an incubation period of 3-8 weeks for raccoon rabies (Charlton et al. 1991; Guerra et al. 2003) but may range from 10 days to 3 months or more (Rupprecht and Smith 1994). Although home range size can vary, it is used only as an a priori reference for the spatial scale of infection risk. If an infected individual's movement is altered after RRV infection then spatial clustering might occur at scales larger or smaller than the expectation of one home range diameter. This knowledge regarding the scale of transmission and risk could have implications for rabies management as well as oral rabies vaccination planning strategies. Furthermore, it is important to determine the degree of involvement of skunks in the RRV endemic area.

4.2 MATERIALS AND METHODS

Rabies is a reportable disease in the United States, and state health departments conduct routine surveillance, particularly related to exposed humans and domestic animals. This surveillance data is a tremendous resource and can be used to address a variety of questions. For this study, RRV surveillance data from Massachusetts was selected for the following reasons. First, RRV was detected in Massachusetts in September of 1992 and has remained enzootic in the state for over 15 years. Between 1992 and 2002, 2,136 raccoons were diagnosed rabid (Kostrzewski 2002); therefore, the number of cases is sufficient for analysis. In addition, detailed surveillance records are available for the state from 2000-2005. Finally, the involvement of raccoons and skunks as vectors of RRV in Massachusetts provides an opportunity to test for interspecific interactions and to determine the potential importance of skunks relative to raccoons in the maintenance of RRV.

A total of 4,024 captures were recorded between 2000 and 2005 (Table 4.1). Raccoons accounted for 1,990 records and skunks for 2,034. Each record had an associated date of capture, spatial location, and rabies virus infection status. Rabies results were unknown for 170 captures which were subsequently excluded from further analysis. Cape Cod, located in Barnstable County (Figure 4.1), remained free of RRV until March of 2004; however, as a result of heightened surveillance, 103 rabid raccoons were subsequently identified. Since the incursion of RRV onto Cape Cod indicates a potential wavelike expansion of the virus, all records from Barnstable County (1,091) were removed from consideration so that no artificial influences like an advancing wave front would bias the analysis. This left a total of 2,764 records for analysis (Figure 4.2).

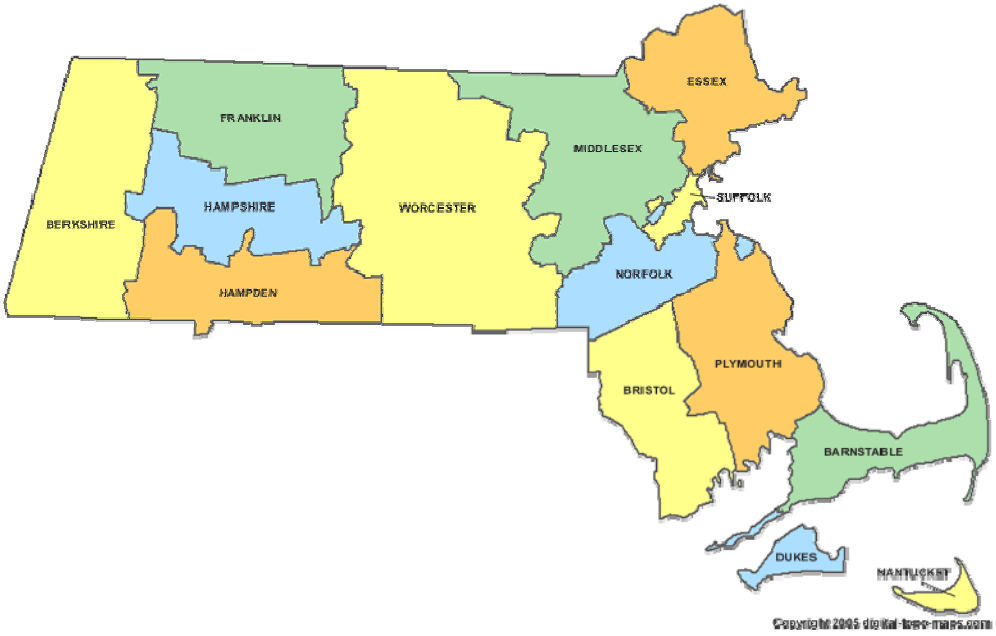


Figure 4.1 County map of Massachusetts showing Barnstable County

Table 4.1 Number of captures recorded between 2000-2005 in Massachusetts. After excluding individuals of unknown rabies status and those found in Barnstable County, a total of 2,764 records were analyzed.

Species	Rabid	Non Rabid	Unknown	Total
Raccoons	700	1243	47	1990
Skunks	668	1243	123	2034
Total	1368	2486	170	4024

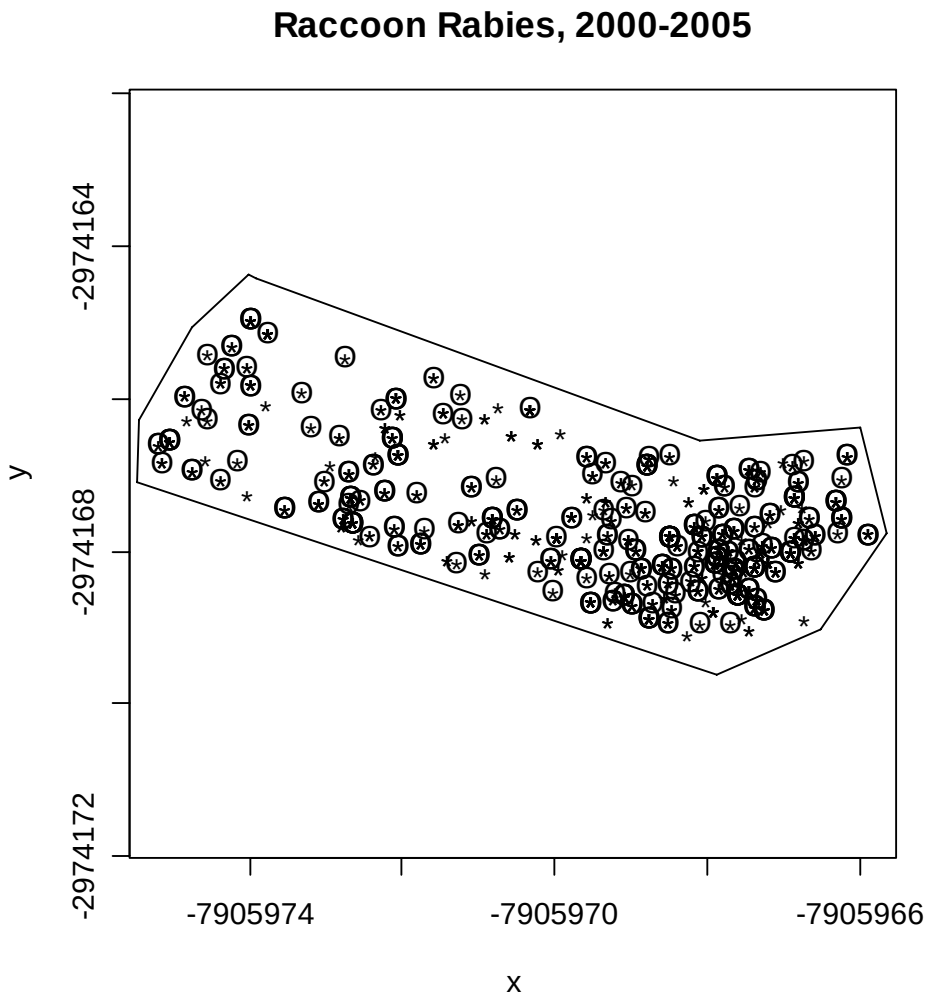


Figure 4.2 Raccoon rabies in Massachusetts, 2000-2005. Localities where rabid raccoons or skunks were sampled are circled.

The general equation for the spatial-temporal K-function is $K(s,t) = E / \lambda$, where E is the number of additional events occurring within distance s and time t of a random event and λ is the number of events per unit space per unit time following Diggle et al. (1995). In practice, it can be calculated as $\hat{K}(s,t) = |A| T \{n(n-1)\}^{-1} \Sigma \omega_{ij} \upsilon_{ij} I(d_{ij} \leq s)$

$I(u_{ij} \leq t)$, where $|A| \times \{n(n-1)\}$ represents the number of pairwise comparisons in a given spatial-temporal region, d_{ij} is the spatial distance between cases i and j, u_{ij} is the temporal distance between cases i and j, $I(d_{ij} \leq s)$ is an indicator function equal to 1 if the spatial distance $d_{ij} \leq s$ and 0 otherwise, $I(u_{ij} \leq t)$ is 1 if $0 \leq u_{ij} \leq t$ and 0 if otherwise, ω_{ij} is a weighting term to correct for spatial edge effects, and υ_{ij} is a weighting term to correct for temporal edge effects (Diggle et al. 1995).

Simulations were performed using the splancs library (Rowlingson and Diggle 1993; most recent version available at http://www.maths.lancs.ac.uk/~rowlings/Splancs/) in R or ClusterSeer2 for (1) purely spatial, (2) purely temporal, and (3) spatial-temporal clustering to determine the relative impact of each component on the overall pattern of clustering. Each simulation was conducted separately on raccoons and skunks. Rabid individuals were termed cases whereas non-rabid individuals were termed controls. Purely spatial and purely temporal clustering was examined separately using the K-function method under a random labeling hypothesis. The random labeling hypothesis assumes an equal probability of case-control assignment at all locations and can be used to account for a heterogeneous population distribution (Waller and Gotway 2004). To assess the statistical significance of the difference function, 500 Monte Carlo simulations were performed. In addition, the Cuzick and Edwards (1990) nearest neighbor test was used as a comparison to look at spatial clustering in terms of neighboring individuals rather than by distance.

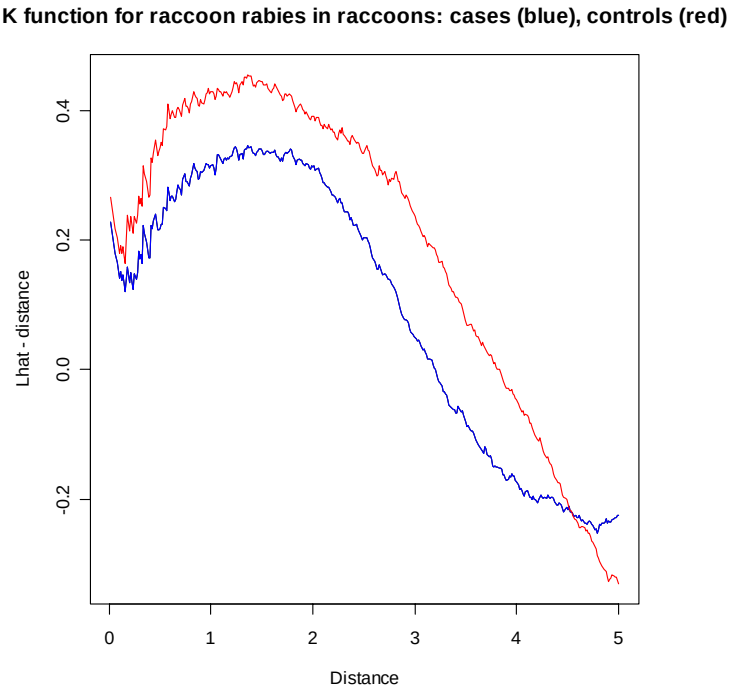
Spatial-temporal clustering of cases was also analyzed using a random labeling type of approach but here the temporal data was randomly reordered while holding the locations of cases constant. As before, 500 Monte Carlo simulations were performed to

assess significance. Additional analyses were conducted based on Knox's method (Knox 1963) and Cuzick and Edwards (1990) nearest neighbor statistic extended to incorporate both spatial and temporal data.

4.3 RESULTS

Purely spatial and purely temporal interactions—Purely spatial interactions were observed for distances within 20 kilometers for raccoons and skunks using the spatial K method as implemented in R (Figure 4.3a and 4.3b); however, Monte Carlo simulations indicated that no spatial structuring was detected beyond what would be expected under the null hypothesis. Rabies cases and controls generally showed the same degree of clustering with controls actually slightly higher than cases.

(a)



(b)

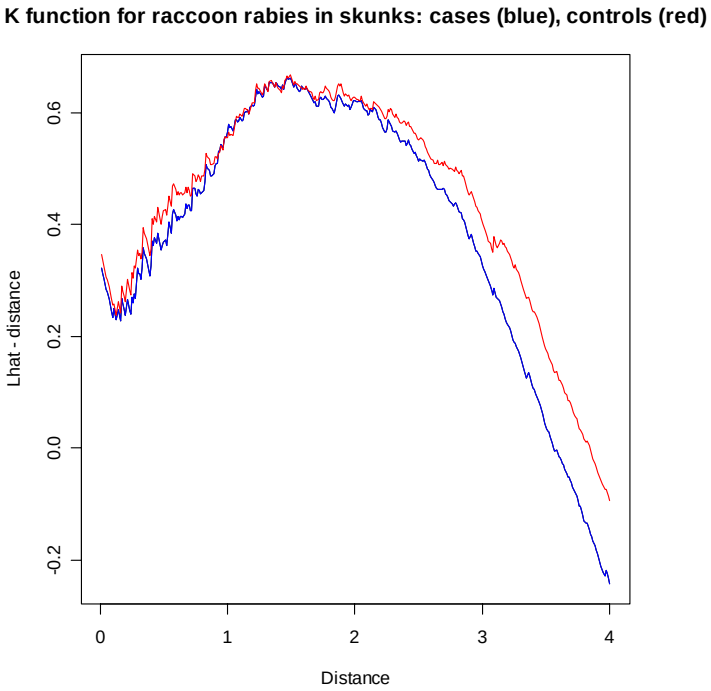


Figure 4.3 Spatial clustering of raccoon rabies virus in raccoons (a) and skunks (b). Each distance class represents 5 kilometers.

The Cuzick-Edwards (1990) test examining the 10 nearest neighbors also confirmed the lack of purely spatial structure for both raccoons ($p = 0.253$) and skunks ($p = 0.253$) based on Monte Carlo simulations. Raccoons showed no significant evidence of purely temporal clustering ($p = 0.41$), consistent with similar numbers of sampled rabid raccoons across the 12 months in a year. Skunks, on the other hand, demonstrated significant temporal clustering at a 4 week scale between weeks 12 and 16 ($p = 0.02$) and weeks 32-48 ($p = 0.006$). The elevated values appeared to be higher during weeks 32-48 than weeks 12-16.

Spatiotemporal interactions—Significant spatiotemporal interactions were observed for both raccoons and skunks. Raccoons showed the highest significant space-time interaction at 6 kilometers and 8 weeks, with significant interactions beyond that expected under the null hypothesis of space-time independence occurring from 0-12 kilometers and 5-8 weeks ($p < 0.01$). Skunks similarly showed the highest significant space-time interaction at 6 kilometers and 8 weeks, however in this case, significant interactions occurred from 0-14 kilometers and from 3-8 weeks ($p < 0.01$). Using Knox's (1963) method with a critical distance of 6 kilometers and 8 weeks, areas in which cases were close in both space and time were identified for raccoons (Figure 4.4) and skunks (Figure 4.5). While patterns were similar for raccoons and skunks, skunks showed more areas with significant space-time interaction across the range than did raccoons.

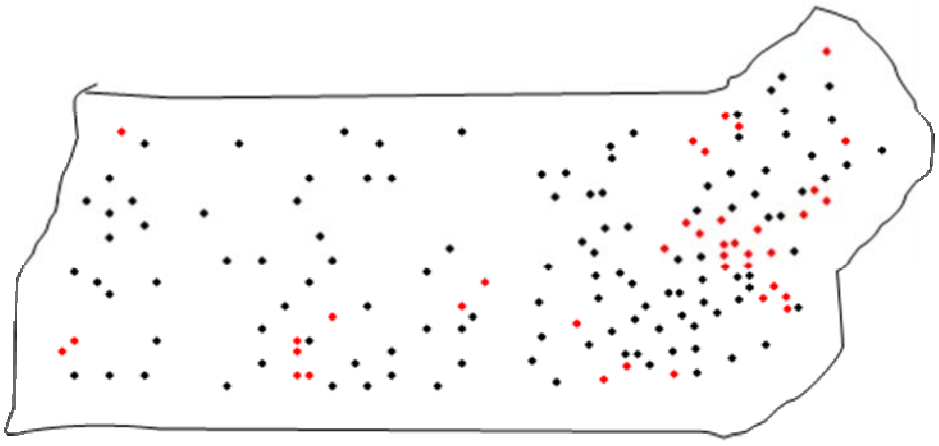


Figure 4.4 Spatial-temporal interaction of raccoons infected with raccoon rabies at a critical distance of 6 kilometers and 8 weeks. Cases are shown in black and those with significant space-time interaction are shown in red.

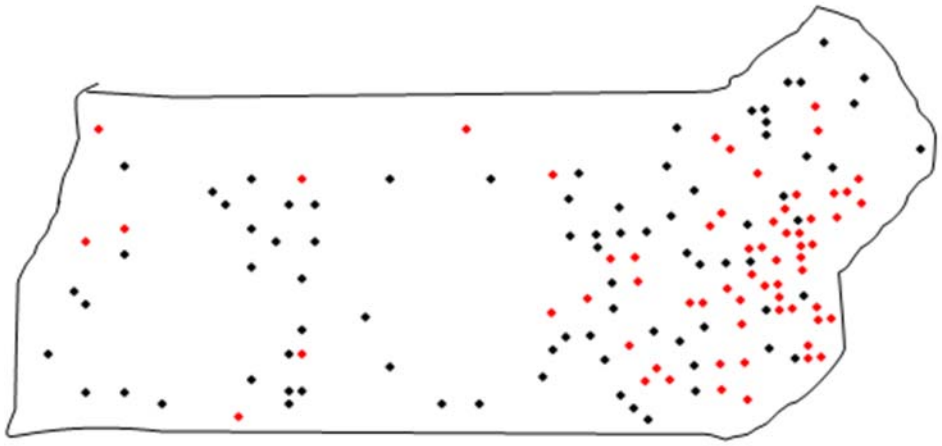


Figure 4.5 Spatial-temporal interaction of skunks infected with raccoon rabies at a critical distance of 6 kilometers and 8 weeks. Cases are shown in black and those with significant space-time interaction are shown in red.

4.4 DISCUSSION

No evidence was found for purely spatial clustering of cases or controls for raccoons or skunks, meaning that the space-time interaction observed is likely to be indicative of the transmission process itself rather than aggregation of the hosts. Raccoons did not show any evidence of increased infection at different temporal scales, however skunks appeared to cluster at 4 week intervals during the spring (March-April) and again on a larger scale in the fall (August-November). Guerra et al. (2003) similarly found that skunks maintained a seasonal component and saw elevated rabies cases in the last quarter of the year. This might correspond to the time of juvenile dispersal when

skunks have increased contact with one another and perhaps with raccoons as well. Interestingly, raccoons failed to show the same pattern as skunks even though raccoon juveniles are also known to disperse.

Similar to Carslake et al. (2005), our data suggest that for both species there is a significant space-time interaction at the hypothesized spatial and temporal range corresponding to one home range diameter and one incubation period, indicating that the risk of transmission is most important locally. This is consistent with the notion that raccoon rabies is directly transmitted and depends on rabid animals coming into contact with susceptible animals while they are showing clinical signs. Since home ranges likely overlap for both raccoons and skunks, there are ample opportunities for this to occur; however, Schauber et al. (2007) caution that factors such as the composition and size of social groups must also be considered in conjunction with home range overlap when examining contact rates for directly transmitted pathogens. The risk of transmission is likely to be a decreasing function of distance, and skunks appeared to pose a risk to others at a slightly larger spatial scale than raccoons, although the difference in the two species was only 2 kilometers. Skunks also showed a wider temporal range, with significant interactions occurring as early as 3 weeks post-infection. This might suggest that the incubation period in skunks is slightly shorter than in raccoons, similar to the observation by Charlton et al. (1988) that skunks infected with RRVs exhibit shorter periods of clinical signs relative to skunks infected with skunk rabies virus. While these results support the concept that skunks are an important component in the circulation of RRV in the northeastern United States, they do not provide definitive evidence for independent circulation of RRV within skunks.

There are many similarities among the spatial and temporal interactions of raccoons and skunks evident in this study, and previous authors (Guerra et al. 2003) have noted that rabies epizootics in raccoons and skunks are closely coupled. Numbers of rabid raccoons and skunks were similar in Massachusetts between 2000 and 2005 as were the overall numbers of raccoons and skunks that were tested indicating that no bias in sampling was encountered beyond that which is inherent to normal surveillance.

In general, these data are beneficial for determining the scale at which rabid animals might pose an infection risk to another and thus might aid in developing or revising ORV strategies. If skunks are indeed playing a large role in the maintenance of raccoon rabies, it will be important to develop an improved vaccine delivery system for ORV. Although skunks may consume the baits formulated for raccoons, this method has not proven efficient in preventing rabies virus infection in skunks. While this was previously thought to result from the vaccine itself, recent evidence suggests that the vaccine delivery system may be the culprit, as skunks are unable to obtain a protective dose from the vaccine sachets (Charlton et al. 1992; Guerra et al. 2003; Grosenbaugh et al. 2007; United States Department of Agriculture 2007). The higher prevalence of raccoon rabies cases in skunks may indicate that the ORV campaign preferentially targets raccoons. Given that the ORV bait is not taken up well by skunks (Grosenbaugh et al. 2007; United States Department of Agriculture 2007), the number of susceptible individuals might be higher for skunks than for raccoons. This could potentially facilitate a host switching event if the path of least resistance is for RRV to cycle through skunks. Clearly, there is a large amount of geographic overlap in the two species' distributions in the areas enzootic for RRV, a key factor for host shifts, according to Streicker et al.

(2010). Additionally, the amount of phylogenetic similarity between the two mesocarnivores might be such that a CST or host shift is easily facilitated if the fitness valley is not too much for RRV to overcome. Unfortunately, these data do not allow one to say whether or not skunks are independently maintaining RRV in the northeastern US, although the shorter duration of clinical signs, i.e. time to death, observed by Charlton et al. (1988) and the shorter incubation period found in this study suggest that RRV still exhibits some host specificity and has not yet become as efficient in skunks as other skunk rabies viruses, which is what one might expect if RRV had become adapted to the new species. Further study, which might include sequencing the RRV variants found within skunks to look for consequential genetic changes, will be needed to clarify the situation in skunk and raccoon populations; however, it is also possible, though less likely, that independent circulation could occur in the absence of significant genetic changes.

CHAPTER 5

Summary and Conclusions

The field of population genetics holds an enormous amount of promise in regard to unraveling questions relating to how pathogens spread within and among host populations (Archie et al. 2008); however, developing testable hypotheses is essential for this type of work. This dissertation represents an attempt to elucidate the factors involved in the transmission and spread of a zoonotic disease in a wildlife population.

Chapter 2 explored the hypothesis that host social structure influences pathogen transmission. Previous studies have demonstrated that the social organization of a species can have a large impact on disease transmission, especially in species with dense populations or in those that exhibit philopatry (Root et al. 2004; Blanchong et al. 2007). Female raccoons are philopatric and live in related groups with shared home ranges (Gehrt and Fritzell 1998; Ratnayeke et al. 2002) which would imply that females contact other relatives more frequently than non-related individuals and would provide an increased opportunity for rabies transmission.

This study identified a single raccoon population in northeastern Ohio, ruling out local adaptation or populations with differing susceptibilities to RRV infection. No sex bias was observed, and none of the raccoon relatedness estimates significantly deviated from zero. Average genetic relatedness declined with increasing geographic distance. No support was found for increased transmission among related individuals, and the scale of female philopatry was more localized than expected. These data indicate that raccoon social structure does not influence RRV transmission. Instead, contact rates are likely

determined by other factors, which may include population density, regardless of social structure. Future studies should incorporate additional rabid animals collected on a finer spatial scale to try to determine whether or not scale was truly a limiting factor of the current study. Additionally, it would be interesting to examine this particular question in a "core" area of RRV distribution for comparison with the current study, which was conducted at the western edge of RRV's distribution.

Chapter 3 explored the hypothesis that landscape heterogeneity in the form of the historically defined suture-zones (Remington 1968) in the eastern US results in limited dispersal or barriers to gene flow in raccoons and their pathogen, RRV. This hypothesis was tested at two temporal scales to determine which barriers were historical and which might still be operational. Three main populations were identified: one east of the Appalachian Mountains, another west of the Appalachian Mountains, and a third across a large portion of the southeastern US. Given that raccoons on either side of the Appalachian Mountains represented distinct populations based on nuclear and mitochondrial markers, the Northeastern-Central Suture-Zone appears to represent a barrier to raccoon gene flow both historically and currently.

Peninsular Florida raccoons were mitochondrially distinct from raccoons on the US mainland; however, microsatellite data identified a single population in the southeastern US. These results suggest that while the Northern Florida Suture-Zone prevented gene flow of raccoons in the past, raccoons have been moving around the area freely in more recent times. The St. Johns River in northern Florida functioned as an extension of the Atlantic Ocean during the moderate to high sea stands during the Pleistocene interglacial periods and could have served as an isolating mechanism for the

raccoons as well as other terrestrial species, with secondary contact occurring once the waters receded.

Rabies management officials should continue to incorporate the Appalachian Mountain range as a natural barrier in their ORV baiting strategies and should pay close attention to potential raccoon and RRV corridors along the Cumberland and Allegheny Plateaus. Since the limitation to raccoon dispersal appears to have occurred much earlier than the period of time when RRV spread across the southeastern US, other ecological factors such as population density, or even more likely resource distribution or habitat quality, may have contributed to its relatively slow progression in the 1950s–1970s. Future studies should examine raccoons in Alabama, Mississippi, and other western localities to determine what landscape features might influence gene flow among the raccoons as well as to determine how connected raccoons in those areas are to the raccoons in the current study area, in the southeastern and eastern US. This could be important for determining connectivity and thus relative risk of RRV infection for uninfected raccoons in areas west of the current RRV edge.

Finally, Chapter 4 tested the hypothesis that RRV spread is heterogeneous and is closely tied to host biological characteristics including home range size, incubation and infectious periods. Given a mesocarnivore two-host system in the northeastern US, we hypothesized that the significant space-time interaction would occur within one home range diameter and one infectious period for each species. Results indicate that the risk of transmission is most important locally and occurs at the hypothesized spatial and temporal scale for each species. These results are consistent with the notion that the directly transmitted RRV depends on rabid animals coming into contact with susceptible

animals while they are showing clinical signs. The risk of transmission is likely to be a decreasing function of distance, and skunks appear to pose a risk to others at a slightly larger spatial scale than raccoons. Skunks also showed a wider temporal range, with significant interactions occurring as early as 3 weeks post-infection. While skunks are certainly involved in the maintenance of RRV, there is not enough evidence to determine whether RRV is cycling independently within skunk populations given the amount of overlap in spatiotemporal interactions in skunks and raccoons. Future studies might consider sequencing the RRVs found in skunks to look for potential consequential genetic changes; however, this is not an absolute requirement for a host shift event with sustained transmission in a new species.

Each of the three chapters was designed to elucidate host factors that might be important for pathogen transmission in a wildlife population; however, many additional factors related to both the host and pathogen remain to be tested. At the very least, the studies represented here provide a foundation for further examination into the complex interactions between the host, pathogen, and the environment.

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