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The Use of Genetic Tools to Assist in White-tailed Deer (*Odocoileus virginianus*) Management in West Virginia

Darren M. Wood

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The Use of Genetic Tools to Assist in White-tailed Deer (*Odocoileus virginianus*) Management in West Virginia

Darren M. Wood

**A Dissertation submitted
to the Davis College of Agriculture, Natural Resources, and Design
at West Virginia University**

in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy in
Forest Resources Science**

**Amy B. Welsh, Ph.D., Chair
James M. Crum, Ph.D.
Stephen P. DiFazio, Ph.D.
John E. Edwards, Ph.D.
Michael P. Strager, Ph.D.**

School of Natural Resources

**Morgantown, West Virginia
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**Keywords: chronic wasting disease, dispersal, genetic assignment, genetic database,
poaching, PRNP, white-tailed deer**

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ABSTRACT

The Use of Genetic Tools to Assist in White-tailed Deer (*Odocoileus virginianus*) Management in West Virginia

Darren M. Wood

Although nearly extinct historically, the current population of white-tailed deer (*Odocoileus virginianus*) in West Virginia serves as a plentiful resource, however; currently there are two uncertainties to the socio-economic benefits of white-tailed deer in West Virginia: poaching and chronic wasting disease (CWD). While infection with the disease invariably results in a 100% fatality rate, previous studies have indicated that nucleotide polymorphisms in the 285th and 286th position of the prion precursor gene (*PRNP*) have been associated with the delay of clinical disease symptoms. To determine the association with CWD positive individuals in a CWD-affected population in West Virginia and determine temporal differences in polymorphism frequency before and after the first detection, 513 CWD negative female white-tailed deer were sampled in Hampshire County, WV as well as 146 CWD positive male and female white-tailed deer. Female white-tailed deer (CWD positive and negative) were also selected to determine any changes associated with *PRNP* polymorphisms and disease status as well as temporal changes before disease detection within a high density area of CWD detections ($>1/\text{km}^2$). While polymorphisms at *PRNP* 285 (Adenine to Cytosine) were not statistically associated with disease detection at either sampling, polymorphisms at *PRNP* 286 (Guanine to Adenine) were statistically associated with the prevalence of disease detection at both samplings ($P=0.000$, $P=0.0478$). Comparisons between observed allele frequencies at *PRNP* 286 following CWD detection and expected allele frequencies found a significant decrease in frequency of the beneficial allele in both the high density sampling as well as the Hampshire County sampling. This result is counter to the expected increase in frequency if selection was occurring and may be due to migration. To determine the dispersal distance of 40 white-tailed deer (20 male, 20 females) testing positive for CWD, genotype profiles were generated using 16 microsatellite loci and the control region of the mitochondria (D-loop) for each individual was sequenced. Pairwise relatedness was calculated between 559 females and a focal individual testing positive for CWD and interpolated across the study region (Hampshire County). Although dispersal distances of females (14.6 km) were not statistically different from males (16.1 km; $P=0.57$), the dispersal pattern of females contrasted that of males. Females dispersed into a management area in which deer density was reduced through “special collections” whereas males dispersed through an area of high CWD density ($>1 \text{ km}^2$). Using the same microsatellite and mitochondrial control region, broad-scale genetic differentiation of white-tailed deer was detected across the 22 counties of West Virginia. Using the Bayesian genetic program STRUCTURE, a total of four genetic populations were determined to be the most likely. However, only 2% of genetic variation could be explained within the groups identified by STRUCTURE using an AMOVA. Additionally, after grouping haplotypes into mitochondrial clades, the majority of neighboring sampled counties had similar mitochondrial clade frequencies. Although a county by county genetic assignment could not be determined, the results of this study indicate that geographic assignment can be used to assign purportedly poached individuals to regions within West Virginia. However,

exposure of evidence to environmental factors including changing temperatures and UV radiation may degrade evidence below qualities and quantities suitable for DNA analysis. Analysis through a one-way ANOVA indicated that both exposure to high temperatures and substrate significantly affected DNA quantity ($P=0.000$, $P=0.0119$, respectively); however exposure time had no influence on DNA quantity ($P=0.444$). Samples exposed to UV radiation were not significantly affected by exposure ($P=0.785$). Additionally, although substrate type did not significantly influence DNA quantity ($P=0.258$), an increase in exposure time significantly decreased DNA quantity ($P = 0.018$). Results from this study indicate that exposure to the environment can reduce the quantity of DNA; however, these quantities were large enough to be amplified through qPCR. Overall, the results of these studies provide a suite of management tools for assisting with the current problems facing a valuable resource in West Virginia.

Table of Contents

<i>Section</i>	<i>Page</i>
Chapter 1: Literature Review and Objectives	1
• Abstract	1
• Introduction.....	2
• Literature Cited	35
Chapter 2: Spatio-temporal analysis of genetic diversity associated with extended pre-clinical symptoms of Chronic Wasting Disease	
• Abstract	49
• Introduction.....	50
• Methods.....	53
<i>Sample collection</i>	53
<i>DNA extraction and amplification</i>	55
<i>Statistical analysis</i>	56
• Results.....	57
• Discussion.....	58
• Tables.....	65
• Figures.....	67
• Literature Cited	69
Chapter 3: Creating a genetic database to understand the genetic structure of white-tailed deer in West Virginia	
• Abstract	77

• Introduction.....	78
• Methods.....	81
<i>Sample collection</i>	81
<i>Laboratory procedures</i>	82
<i>Genetic analysis</i>	83
• Results.....	85
• Discussion	87
• Tables.....	92
• Figures.....	95
• Literature Cited	104

Chapter 4: Using genetic relatedness to measure dispersal distance of white-tailed deer with Chronic Wasting Disease

• Abstract.....	111
• Introduction.....	112
• Methods.....	116
<i>Study area</i>	116
<i>Sampling methods</i>	117
<i>Laboratory procedures</i>	118
<i>Genetic analysis</i>	120
• Results.....	121
• Discussion	122
• Tables.....	127
• Figures.....	128
• Literature Cited	134

Chapter 5: Environmental and temporal variables that contribute to DNA degradation in white-tailed deer blood

• Abstract	142
• Introduction	143
• Methods	146
• Results	148
• Discussion	148
• Tables	153
• Literature Cited	155

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Dedication

To Dr. Amy Welsh, thank you for providing me the opportunity to be a graduate student and work on the same species that inspired me to study wildlife and fisheries. You have served as a great mentor and friend.

To the many friends at I made during my years at West Virginia University, thank you for the great times fishing, hunting, and showing me how to live life to the fullest.

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To my parents, thank you for supporting me throughout my many years in college. You are the ones who introduced me to wildlife and fisheries through camping, hunting, and fishing. You raised both of us boys in the perfect “classroom” to have successful careers teaching and inspiring students.

To my beautiful wife Michelle, thank you for believing that I was the best person for the job even though you never knew fully understood what method I was using to complete the job. Completing a Ph.D. has been the toughest thing I have ever done in my life, but having you by my side made it that much easier.

Chapter 1

Literature Review

Abstract

Although nearly extinct historically, the current population of white-tailed deer (*Odocoileus virginianus*) in West Virginia serves as a plentiful resource, supporting 350,000 license holders (roughly the equivalent of one hunter per family) with the majority of hunters pursuing white-tailed deer. Estimates of the economic contribution of hunting activities to the state's economy are \$270 million annually, supporting 5,000 local and state-funded jobs. However, currently there are management problems to white-tailed deer in West Virginia: poaching and chronic wasting disease (CWD). Poaching, defined as the intentional taking of illegal flora and fauna, is a concern for white-tailed deer as an appreciable economic and aesthetic value exists. According to the West Virginia Division of Natural Resources, from 1999 – 2014, there have been 5,368 prosecutions of illegal possession of wildlife and an additional 1,798 cases of illegal killing or taking of white-tailed deer, turkey, or boar. Although authorities may not witness the poaching event, collecting biological evidence including hair, blood, and tissue that may be produced during the crime can be used to determine the sex, match the identity of an individual white-tailed deer, and genetically designate individuals to geographic regions. CWD is a transmissible spongiform encephalopathy (TSE) first described in mule deer (*O. hemionus hemionus*) and has currently been detected in free-ranging populations of white-tailed deer throughout the mid-western United States (Wisconsin, Michigan, Minnesota, Missouri, Illinois, Iowa) and eastern states including Maryland, Virginia, West Virginia, Pennsylvania, and

New York. Once contracted, survival from CWD averages two years, but is nevertheless fatal. While disease prevalence is greater in males and increases with age, the probability of infected females is not influenced by the number of nearby infected males, but rather by close relationships with female kin. Therefore dispersal of infected males is believed to be responsible for natural spread of CWD across a geographic area. While both of these threats is inherently different, a better understanding of white-tailed deer ecology through genetic techniques can help to provide tools for both management and law enforcement.

History and management of white-tailed deer in West Virginia

Since the European settlement of West Virginia in 1719, populations of white-tailed deer have fluxuated between near extirpation to state-wide population expansions. Pre-colonial deer numbers were high, likely because of total utilization practices harvest by Kanawha, Mingo, Delaware, and Shawnee Native American tribes. Market hunting and selling of white-tailed deer goods became popular with the expansion of European settlers westward; however, historic numbers of deer could not withstand market pressure due to the high price of deer hides (C. Ryan, West Virginia Division of Natural Resources, personal. communication.). Although signs of localized extirpation were seen as early as the mid-1800's, numbers of white-tailed deer state-wide reached their lowest numbers in the early 1900's (Allen and Cromer 1977), prompting the first management actions for white-tailed deer in the state's history.

Responding to low numbers of game, legislation made harvesting doe illegal and prohibited the exportation of white-tailed deer outside of the state in 1909. These two actions, paired with an antler-restricted buck season (>4 inches), triggered natural re-population by the late 1920's. Stocking of white-tailed deer was initiated in 1920 by sportsman's groups and was

continued by the state in 1923 to supplement natural repopulation; however, deer hunting ceased statewide until November 1, 1927 (C. Ryan, West Virginia Division of Natural Resources, personal communication.). Money raised under the Pittman-Robertson Act continued to facilitate stocking in parts of the state through 1977 (Allen and Cromer 1977), with the majority of deer being released between 1936 and 1945.

In 1945, legislation was passed for the establishment of an antlerless season and distribution of crop damage permits for lost harvest. A year later, the first antlerless shoot occurred at Watoga State park, making it the first harvest of does since legislation made it illegal in 1909. By 1951, harvesting of antlered deer including spikes were made legal, producing a harvest of nearly 22,000 deer and in 1956 the state's first management zones were created, grouping all 55 counties into one of three different classes based on the deer harvest per square mile of forest land. From 1957 until 1965, regulations shifted between "hunter's choice" and bucks only; however, from 1968 through the 1973 season, only bucks could be legally harvested. A year later, legislation created the Class N Special antlerless deer license, making a 2-day antlerless permit available in six counties for the harvesting of one antlerless deer. From 1978 until 1982, an additional 19 counties (26 total) adopted the two-day, Class N antlerless license (C. Ryan, West Virginia Division of Natural Resources, personal communication).

Growth of the white-tailed deer population resulted in increased harvest of does through the early 1990's. Between 1982 and 1988, an additional day was added to antlerless season (3 day season), as well as the number of counties with antlerless licenses (37 total) and unlimited antlerless permits on private lands (four total). A year later, 22 counties in total had adopted unlimited antlerless permits on private land and by 1992, unlimited permits were issued for West Virginia residents. During this time, 40 counties adopted an antlerless season and non-

resident permits were issued in 33 counties (C. Ryan, West Virginia Division of Natural Resources, personal communication)

Shifts in management legislation in the mid-1990s resulted in expansions of both antlerless season length and the beginning of harvest on wildlife management units. Through the early 2000's, the growth of county antlerless harvest increased to 51 of 55 counties (93%). However by 2006, antlerless season was closed in 18 counties and portions of three other counties. Additionally, antler restrictions (14-inch spread) to preserve older deer were placed in four Wildlife Management Areas and Cooper's Rock State Forest. A year later, antlerless hunting was re-established in eight counties (41 total) and currently occurs in 51 counties. Currently the present population of white-tailed deer in West Virginia serves as a plentiful resource, supporting 350,000 license holders (roughly the equivalent of one hunter per family) with the majority of hunters pursuing white-tailed deer. Estimates of the economic contribution of hunting activities to the state's economy are \$270 million annually, supporting 5,000 local and state-funded jobs (Southwick Associates 2012).

Wildlife Poaching

Poaching, defined as the intentional (Muth and Bowe 1998) and illegal taking of flora and fauna (Musgrave et al. 1993) is both an international and domestic concern. In Russia, organized black market networks are common sources of illegal trading of furbearer species as well as falcons (Wyatt 2009). In south African countries, rhinoceroses, cheetahs, reptiles, birds, as well as abalone on coastal countries are top targets for both consumption and the pet trades (Warchol et al. 2003). Additionally, an estimated 22,000 African elephants were illegally harvested across the African continent in 2012 (Wittemyer et al. 2014). Within the United States,

poaching occurs in both terrestrial and aquatic species. In Washington state, nearly 2,000 white sturgeon (*Acipenser transmontanus*) were illegally killed for caviar between 1985 – 1990. (Cohen 1997). Additionally, Haines et al. (2012) identified a total of 67 individual poaching events of white-tailed deer occurring from 2000 – 2009 in Fayette County, Iowa. Furthermore, Kindall et al. (2011) reported that 12.9% of mortalities of a reintroduced Rocky Mountain Elk (*Cervus elphus*) population in Tennessee were due to poaching.

Although wildlife crimes including poaching are often “victimless” as they do not immediately affect individuals or groups of humans, the socio-economic importance wild fauna bring to a local area, region, or country is important. Poaching of both game and non-game in the United States is viewed as theft of public resources due to the economic value hunting contributes. In 2012, hunting and related activities contributed over 6.3 billion dollars to the United States economy and in West Virginia, the annual economic contribution of all hunting related activities in 2012 was estimated over \$421 million USD, which accounted for over 5,000 jobs within West Virginia. Deer hunting alone contributed nearly \$3 billion USD to the economy (Southwick Associates 2012) and remains a viable tool to control white-tailed deer populations across the United States (Brown et al. 2000). However, a financial incentive exists as millions of dollars are earned annually from poaching in the United States (Musgrave et al. 1993), which is creating an international problem with an estimated costs of hundreds of millions of dollars (World Wildlife Fund 2017).

Although a financial incentive exists as a motivation to poach wild species, Muth and Bowe (1998) outlined ten motivations for poaching that include commercial gain, household consumption, recreational satisfactions, trophy poaching, thrill killing, protection of self and property, rebellion, traditional right of use, disagreement with regulations, and gamesmanship.

For these many reasons, poaching continues to persist in an advanced, industrial society (Muth 1998), especially where larger individuals contributed to an increased social status (Eliason 2012). In a study of poacher motivations in the Atchafalaya Basin in Louisiana, Forsyth et al. (1998) interviewed 41 individuals who were arrested or identified by anonymous informants as poachers of alligator, deer, or waterfowl. Of these individuals, 90% stated that the primary purpose for poaching was to provide food or products for family or nearby residents. However, while 22% of individuals stated that money was a primary purpose of poaching, 27% of persons responded that they were motivated by the tradition of taking game species and nearly 20% stated that they sought an exhilarating experience (Forsyth et al. 1998). Using a similar study design, Kahler and Gore (2012) explored perceptions of poaching, motivations, and knowledge of rules in Namibia. Although the large majority of individuals interviewed were aware of the rules (93.1%) and believed that these rules were right (94%), individuals still poached. Similar to the results of Forsyth et al. (1998), income generation and food were the top two motivations for poaching; however, wildlife removal to protect agricultural land and lives became important motivations (Kahler and Gore 2012).

Although there are a large number of motivations to poach, protecting species of flora and fauna has an equal number of motivations to guard against illegal activities. Citizens who witness an event may be influenced to report illegal activity for several reasons. McSkimming and Berg (2008) interviewed 36 individuals who reported poachers through Pennsylvania's "Turn In A Poacher" (TIP) program, which directly connects the citizen with a conservation officer to report a possible crime. The majority of citizens reported these crimes as they viewed the activities as a threat to the future of the resource. Other reported incentives including loss of

stream access in private areas, loss of local economic benefit, and safety from those individuals who possessed weapons during these activities (McSkimming and Berg 2008).

With the ease of white-tailed deer harvesting and transport, poaching of white-tailed deer is a massive problem that is currently being faced by the West Virginia Division of Natural Resources (WVDNR) Law Enforcement Section. According to the WVDNR, from 1999 – 2014 there have been 5,368 prosecutions of illegal possession of wildlife and an additional 1,798 cases of illegal killing or taking of white-tailed deer, turkey, and or boar. While authorities may not witness the poaching event, collecting biological evidence including hair, blood, and other tissue that may be produced during the crime can be used to determine the sex (Shaw et al. 2003, Lindsay and Belant 2008), match the identity of an individual white-tailed deer through genetic analysis (Anderson et al. 2002), and genetically designate individuals to geographic regions (Green et al. 2014).

While microsatellite markers can generate individual genetic profiles of wild individuals for forensic analysis (e.g. *Sus scrofa*; Lorenzini 2005), a comprehensive genetic database that represents potentially affected areas is needed to assign individuals to geographic regions. Manel et al. (2002) evaluated two assignment tests, a full Bayesian assignment as well as a partial Bayesian exclusion test, as tools to identify or exclude the population of origin. The results indicated that assignment can be accurate (99.9%) when populations are highly differentiated and the number of loci is greater than 10. In a case study in Italy, Lorenzini et al. (2011) utilized a microsatellite genetic database of Sardinian mouflon (*Ovis musimon*) along with Bayesian assignment testing to genetically identify a poached individual and confirm its geographic location. However, while genetic assignment has become a potential tool to identify the geographic location of a white-tailed deer by designating individuals to distinct geographic

locations, this technique requires a database of discrete genetic neighborhoods that corresponds to geographic locations and thus an understanding of both the organism's ecology and power of the genetic marker to delineate a continuously distributed population at a very fine scale (Ogden et al. 2009).

White-tailed Deer Female Ecology

Social structuring and behavior of mammals including white-tailed deer has significant implications for population management. Hawkins and Klimstra (1970) aged, marked and released 465 deer in the Carb Orchard National Wildlife Refuge in Southern Illinois and identified 49 family groups most commonly comprised of an adult doe, a yearling doe (daughter) and two fawns. Additionally, the second most common group only lacked the yearling doe, suggesting strong matrilineal social organization among females composed of multiple generations. Similarly, Tierson et al. (1985) used both radio-collars and tags to track movement of 408 deer within a 116 km² area of the Adirondack Mountains (Huntington National Forest). Using data from 366 tagged and radio-collared deer, nine social groups were identified, each with discrete home ranges. Although female-fawn relationships were not established, Tierson et al. (1985) noted movements of fawns followed that of the apparent parent and that the home range of 1 – 2 year – old females overlapped with either female parents or of other social group members. The overlapping home ranges of related females suggested a social structure analogous to that of a rose petal (Porter et al. 1991). Furthermore, Ozega et al. (1982) visually observed and radio-tracked prepartum females and postpartum mother and fawn pairs to detect changes in behavior and credits the matriarchal social structure to mother-fawn social isolation from four to six weeks post-partum.

Furthermore, Aycrigg and Porter (1997) collected location data using ear tags and radiotelemetry to further partition the socio-spatial patterns within and among social groups to female age through principal component analysis (PCA). While the ranges of females 0 – 2 and 3 – 5 years old overlapped with themselves and with females >5 years old, the ranges of females >5 did not overlap. This result suggests that females ≤ 5 may be unable to maintain range exclusivity to other females. In addition, Aycrigg and Porter (1997) found that female dispersal was low (<3%), supporting strong philopatric behavior in females. However, Oyer et al. (2007) documented movement of a single female in southern Wisconsin and found dispersal and establishment of ≥ 2 new home ranges beyond that of her natal range.

In order to test the hypothesis of female philopatry influencing the genetic structure, Mathews and Porter (1993) used >15,000 radiotelemetry relocations for 41 yearling and adult female white-tailed deer in the Huntington Wildlife Forest (New York) to successfully assign individual deer to eight core areas based on 50% minimum convex polygons. Average summer home ranges for both yearling and adult females was 2.4 ± 4.69 km with ranges of young females (< 2 years old) being fully encompassed by the ranges of older females. In addition, genetic analysis (starch-gel electrophoresis) of 95 deer in the population revealed that the social structure created through matrilineal relationships influences genetic differentiation among the groups. Additionally, Miller et al. (2010) used visual observations, radiotelemetry and 14 microsatellite loci to examine the relationship between social and genetic structure of a high density white-tailed deer population within the central Appalachian Mountains. Using telemetry and visual observation of only adult females (≥ 1.5 years old), 102 female white-tailed deer were assigned to 28 social groups. These females were never found to change social groups. Genetic analysis corroborated the telemetry and observation data by revealing a very fine (<1 km) spatial

genetic structure. A similar result of fine-scale genetic variation was also found in a study of deer populations inhabiting western Maryland (Sheffield et al. 1985).

Hunted regions may show different patterns of population structure. In the southeastern United States (Savannah River Site, South Carolina), Comer et al. (2005) radiocollared 17 female deer consisting of fawns (≤ 1 year old), yearlings (1.0 – 2.0 years old), adults (2.0 – 4.5 years old) and older adults (> 4.5 years old) and collected tissues from an additional 21 deer (38 total). Relatedness of female groups as well as sibships were calculated using 14 microsatellite loci. A Mantel test revealed a weak correlation between relatedness and distance for both genetic (correlation = -0.06) and geographic (correlation = 0.036) analyses indicating an absence of spatial structure within females for both measures. Conversely, a study in the same Savannah River Site (South Carolina) found genetic sub-structuring within the area using the sorbitol dehydrogenase locus; however, the genetic clusters were found to overlap and vary both spatially and temporally, therefore indicating the possibility of a random level of genetic clustering (Scribner et al. 1997).

Dispersal

While studies of female home range have noted low levels of dispersal, resulting in fine-scale genetic sub-structuring (Aycrigg and Porter 1997), male dispersal has been found to be contrasting to females. Hawkins et al. (1971) marked 687 white-tailed deer in a mixed habitat landscape (27% cropland, 17% pasture, 27% brush, 29% timber) in the Crab Orchard National Wildlife Refuge consisting of age classes 0 – 10 of both males and females. Analysis of visual observations revealed a dispersal rate of 13% for yearling does and $> 80\%$ for yearling males. Percentages of deer hunter-harvested outside of the refuge were highest in yearling males (22%)

and two-years old bucks (8%) compared to only 3% of yearling does. A similar trend of dispersal was observed in 11 doe-fawn groups consisting of a total of 10 males and six females. While all six yearling females continued to use their natal range, 70% of males 1 – 2 years old established both summer and winter ranges outside their birth range (Nelson and Mech 1984). However, in another dispersal study using radiocollared white-tailed deer (Nelson 1993), a dispersal rate (64%) similar to that of the Nelson and Mech (1984) was observed; however, 20% of females 12 – 18 months old dispersed. Other studies of white-tailed deer dispersal throughout the United States have found dispersal rates of 70% for yearling males in Maryland (Rosenberry et al. 1999) and 68% and 44% for two sites in southern Texas (McCoy et al. 2005), 65% in Illinois (Nixon et al. 2007), and 77% in Pennsylvania (Long et al. 2010).

To characterize influences of dispersal rate and distance on temporal and spatial distributions of white-tailed deer, 308 juvenile (7 to 10 months old) white-tailed deer were radio-tracked to assess dispersal in two regions in Pennsylvania with varying levels of forest cover and deer densities. While dispersal rate (74%) was not statistically related to either deer density ($P = 0.143$) or forest cover ($P = 0.687$), average and maximum dispersal distance was significantly related to the percentage of forest cover ($r^2 = 0.94$, $P < 0.001$; $r = 0.826$, $P = 0.006$, respectively) (Long et al. 2005).

White-tailed deer inhabiting landscapes dominated by agriculture exhibit patterns of dispersal different than that of forested environments. Vercauteren and Hygnstrom (1998) radio-tagged 30 female white-tailed deer (20 adult, 10 juvenile) in Nebraska. While 53% were found to be residents, 47% dispersed from the study area, either on an annual basis or permanently. Additionally, reduced female dispersal was observed in forested habitats in Illinois compared to intensively farmed regions (Nixon et al. 2007). However, Brinkman et al. (2005) studied

seasonal and land cover influences of dispersal in the northern range of white-tailed deer (Minnesota) and found that temperature and depth of snow affected dispersal rates and distance rather than land cover. Additionally, yearly differences in acorn mast production was found to drive the increase of home ranges from summer to fall of females during good mast years compared to years of low mast-fall (McShea and Schwede 1993). Hunting's influence on female dispersal has been found to be only temporary. Vercauteren and Hygnstrom (1998) found that 90% of females that were displaced from their range during hunting activities returned back to their original location.

While female white-tailed deer display natal homing, differences in individual male white-tailed deer have been observed. Dispersal rates and distances of orphaned ($n=15$) and non-orphaned males ($n=19$) were compared through radio-collaring and visual observations (ear tags) (Hölzenbein and Marchinton 1992a). While 78% of non-orphaned males extended their range beyond that of their mother's, only 31% of yearling or adult male orphans made an extension of their natal range, suggesting that males disperse to avoid inbreeding. This trend was also observed in an additional study by Hölzenbein and Marchinton (1992b) who observed rates of natal dispersal of 86.5% for male fawns that matured with their mothers and 9.1% for orphans. Additionally, a temporal pattern of avoidance with related females during the breeding season has been observed as a cause of natal dispersal (Long et al. 2008). Differences in young male behavior between dispersers and non-dispersers during the breeding season have also been observed (Rosenberry et al. 2001). Dispersers participate in breeding behaviors more often than non-dispersers ($P = 0.005$); however, when dominance was tested, dispersers were statistically more submissive to other males than non-dispersers ($P = 0.095$).

Dispersal of male white-tailed deer has also been found to be influenced by other landscape features. Long et al. (2010) found that dispersal of males was non-random and paralleled ridgelines. Additionally, roads served as a semi-permeable barrier to dispersal with males establishing home ranges on the near side of those features. However, the direction of roads had no apparent effect on the orientation of deer movement (Feldhamer et al. 1986).

Genetic Markers and White-tailed Deer Population Studies

Understanding the microevolutionary processes that influence the genetic population structure is essential for species management. Identifying and quantifying contemporary and historical patterns of population gene flow requires genetic markers that have the ability to delineate populations at a very fine-scale. Neutral markers are found on the non-coding portions of DNA and therefore are not influenced by selection. Instead, they are influenced by the evolutionary processes of migration and genetic drift. Neutral markers have emerged as a tool for both identifying and quantifying influences of genetic variation within a population (Schlötterer 2000). The two neutral markers that are typically applied in recent population genetics studies include the control region of mitochondrial DNA, (mtDNA), and microsatellites. While these markers are both neutral, they differ in their inheritance patterns, which can have important implications for population genetic studies.

The mitochondrion is an energy conversion organelle found within the structure of a eukaryotic cell. DNA specific to the mitochondria's function is coded within the 15,000 to 20,000 bases (16,600 bp in white-tailed deer; Carr et al. 1986) of its circular genome which consist of 13 protein coding genes, 22 transfer RNA's, 2 ribosomal RNA's and a highly conserved area known as the control region. The control region itself consists of three domains,

which contain initiation sequences (right domain), a conserved region (central domain), and termination sequences (left domain). Spanning all three domains is the displacement loop or “D-loop” that includes the initiation site of heavy strand replication and sequences responsible for termination (Clayton 1982, 1991). Variability within the D-loop region is attributed to base substitutions (Brown et al. 1982), resulting in rates of evolution estimated to be between 2.8 (Cann et al. 1984) to 5 (Aquadro and Greenberg 1983) times higher than other regions of the human mitochondria. This rapid evolution combined with maternal transmission of mtDNA results in a highly efficient marker to study inter- and intra-population gene flow between populations (Moritz 1994), especially when gene flow is sex biased (Avice 1995).

Sex-biased gene flow occurs when a single sex of an organism displays philopatry to their natal range (Greenwood 1980). In cases where organism dispersal is male dominated, as is the case of white-tailed deer, a geographic genetic population structure can be delineated at a scale determined by the female’s range using maternally-inherited genetic material (Avice 1995) and may provide finer resolution of sub-population structuring compared to nuclear inherited genetic markers like microsatellites (Karl et al. 1992). Purdue et al. (2000) examined mtDNA variability in populations of white-tailed deer within the coastal regions of Georgia and South Carolina and found 15 different haplotypes from six geographically different sampling locations. Samples that were adjacent to each other were the only ones to share haplotypes, with the exception of one instance, indicating strong spatial heterogeneity between sites and the potential to distinguish white-tailed deer populations with mtDNA. Additionally, white-tailed deer populations in Texas had a similar pattern of multiple mtDNA haplotypes within the same population (Carr et al. 1986). However, a study in northeastern Minnesota found that mtDNA

markers were not able to distinguish genetic sub-populations of white-tailed deer, likely due to colonization events (Cronin et al. 1991).

While these studies utilized mtDNA to examine gene flow between populations, the mutation rate limits the utility of the marker to selected population genetic questions that are focused on historical patterns of gene flow. However microsatellites, which were discovered in the 1980's, have become a widely applied neutral genetic marker in population genetic studies (Oliveira et al. 2006). Also known as short tandem repeats (STR) or simple sequence repeats (SSR), microsatellites are regions of bi-parentally inherited, non-coding DNA that have variable number of tandem repeats (VNTR) with repeat motifs ranging between 1 – 6 nucleotides.

Variability of microsatellite loci is attributed to slip-strand mispairing during DNA replication (Eisen 1999) resulting in a mutation rate of $10^{-2} - 10^{-6}$ per locus per generation (Li et al. 2002). This mutation rate, resulting in high genetic variability, combined with transmission from both parents, results in a marker that can distinguish contemporary gene flow patterns. However, unlike mtDNA which has hundreds to thousands of copies of the genome per cell, the nuclear genome only has one copy per cell (Birky et al. 1989).

The name microsatellite is a term combined from two separate discoveries. The first was the density gradient centrifugation of heterochromatin in the 1960's resulting in the detection of circular bands or "satellites". Twenty years after the discovery of bands, DNA satellites were found to contain repeat motifs of various repeats between 10 – 30 nucleotides (minisatellites; Jeffreys et al. 1985); upon further examination, these motifs were found to be shorter in length, eventually leading to the term microsatellites (Ellegren 2004).

Microsatellite loci have been used to study population genetic structure in white-tailed deer. Miller et al. (2010) used 14 microsatellite loci to delineate the genetic structure of a high density white-tailed deer population within the central Appalachian Mountains and revealed a very fine (<1 km) spatial genetic structure. Additionally, in a study of white-tailed deer in Wisconsin and Illinois, fine scale-population structure of white-tailed deer was delineated using 15 microsatellite loci (Robinson et al. 2012b).

In addition to mtDNA and microsatellite genetic markers, single nucleotide polymorphism (SNPs) have the ability to delineate populations through single base-pair changes within the genome. These changes are detected through low cost, high-throughput sequencing of the targeted gene or desired locus. However, unlike the mtDNA control region and microsatellite markers which are neutral, SNPs can be found in the coding and non-coding regions of the genome and occur in most species every 200 – 500 base pairs. In white-tailed deer, Seabury et al. (2011) found 10,476 SNPs within the mitochondrial and nuclear genome. Mutation rates of SNPs are low and range between 10^{-8} - 10^{-9} per generation (microsatellites; 10^{-4}) and since these single base pair changes can occur within the genetic code, they can potentially change the corresponding amino acid sequence. However, mutations directly affecting the amino acid sequence have been crucial in understanding chronic wasting disease detection in white-tailed deer.

Chronic Wasting Disease Pathology

Transmissible spongiform encephalopathy's or prion diseases (Prusiner 1982) are a family of fatal neurodegenerative disorders associated with the abnormally shaped cellular glycoprotein (PrP^c) lacking a nucleic acid (Prusiner 1997). Members of this family include transmissible mink encephalopathy (Burger and Hartsough 1965, Hartsough and Burger 1965),

bovine spongiform encephalopathy, (BSE; Wells et al. 1987), Creutzfeldt-Jacob Disease (Creutzfeldt 1920, Jakob 1921), Kuru (Gajdusek and Zigas 1957), scrapie (Comber 1772), and chronic wasting disease (Williams and Young 1980). Normal cellular isoforms, PrP^c, are converted from an α -helical sheet into a proteinaceous β -pleated sheet conformer (Pan et al. 1993). The misfolded isoform of prion proteins, PrP^d, acts as a template for the conversion of the normal PrP protein isoform into the irregular and infectious shape known as a prion (Prusiner 1982).

This mechanism for prion perpetuation has been demonstrated as the “seeded model”. The seeded model proposes that the conversion of PrP^c (normal state) to PrP^d (disease state) involves PrP^d host mediated transmission of epigenetic information to PrP^c, likely occurring on the cell surface (Caughey and Raymond 1991) and subsequent nucleation of PrP^d protein conformers (slower step of the model) (Jarrett and Lansbury 1993). Seeding of the polymerized nucleus diseased isoform acts as a template for the conversion of the normal PrP host protein for new PrP^d formations (faster process). The dependence on nucleus formation emphasized in the seeded model may explain the virus-like features of prions as fragmentation of fibrils as aggregate division exposes new areas for growth (Jarrett and Lansbury 1993). According to the model, variability within prion protein, encoded by the *PRNP* gene, will change the tertiary and quaternary structures of the PrP^c, therefore reducing the efficiency of conversion.

CWD: the early years

Although scrapie, the first described TSE, has been observed in European populations of sheep since the 1700's (Comber 1772), CWD was not characterized until 1967 when it was first detected in a Colorado captive mule deer (*Odocoileus himionus hemionus*) herd and shortly

thereafter observed in a similar Wyoming facility. In 1978, the disease was established as a spongiform encephalopathy (SE) through histopathology of brain and tissues of the central nervous system (Williams and Young 1980) and was later classified as a transmissible spongiform encephalopathy (Williams and Young 1992).

Histopathological comparisons of multiple parts of the brain of mule deer affected by CWD to unaffected mule deer included the medulla oblongata, pons, thalamus, cerebellar cortex, mesencephalon, and hypothalamus as well as spinal cord tissue. The neurohistopathologic examination revealed widespread “spongiform transformations” or neuronal vacuolations, particularly within the sectioned gray matter tissue. These transformations found in mule deer affected with CWD were not observed in similar tissues of unaffected mule deer that were of the same age (Williams and Young 1980). Additionally, amyloid plaques have been detected through stained sections of the brain (Williams 2005). Although histopathological analyses of central nervous tissues have identified histopathologic regions associated with CWD, PrP^d can be found in lymphoid tissues when lesions are often absent in tissues (Sigurdson et al. 1999, Miller and Williams 2003, O’Rourke et al. 2004).

In a five year period, CWD affected nearly 80% of mule deer held more than two years in the same facility that it was first characterized in as well as a single black-tailed deer (*O. hemionus columbianus*) (Williams and Young 1980). Mule deer held in this facility that contracted chronic wasting disease were either sired from does that were captured in the wild and brought into captivity, orphaned fawns (wild), or fawns of captive facility does. While held in the facility, other wild and domesticated ruminants including cattle, sheep and goats likely had times of discretionary contact with these captive cervids (Williams and Young 1980). Affected mule deer showed clinical body and behavioral symptoms of being abnormally thin (atrophy), likely

from a lack of eating (Williams et al. 2002), but also exhibited other disease symptoms including hair loss (alopecia), pneumonia, dehydration, excessive salivation, vomiting, and overall lack of body control and movement. Additionally, excessive fluid in the rumen as well as sand and gravel was also discovered and reported during necropsy (Williams and Young 1980).

After the first description of CWD (Williams and Young, 1980), an investigation of a captive population of cervids in the Toronto Zoo revealed CWD as the causative agent of mortality of a single mule deer in 1978. Further testing of a total of 105 cervids revealed the CWD prion antigen present in eight individuals (7 mule deer and 1 black-tailed deer). Importation of an infected individual from the Denver Zoo, where orphaned wild mule deer were brought, was determined to be the most plausible explanation of CWD transmission to the Toronto Zoo (Dubé et al. 2006).

The first detection of chronic wasting disease in free-ranging cervids occurred in a population of Rocky Mountain elk (*Cervus canadensis nelsoni*) in Colorado and Wyoming (Williams and Young 1982). Two decades later, the disease was found in wild mule deer herds in west-central Saskatchewan, northwest Nebraska and southwest South Dakota (Miller and Williams 2002) and most recently in southwestern states including New Mexico and Texas. In 2001, the first case of CWD in free ranging white-tailed deer was detected in south-central Wisconsin (Joly et al. 2003) and has since spread to non-continuous wild populations of white-tailed deer throughout mid-west United States including Minnesota, Missouri, Illinois, Iowa, Kansas, and Michigan. Eastern states including Maryland, Virginia, West Virginia, Pennsylvania, and New York, have also detected the transmissible disease in both wild and captive herds (USGS 2017). Diagnosis of CWD in free ranging populations of Shiras's moose (*Alces alces shirasi*) occurred in 2005 (Baeten et al. 2007). Additionally, although CWD in wild

populations of reindeer (*Rangifer tarandus tarandus*), which naturally overlap with CWD-infected populations of elk and white-tailed deer, has not been reported, oral inoculation of abnormal prions was found to be a viable transmission route (Mitchell et al. 2012).

Although CWD was previously only detected in cervid populations in North America, international detection of CWD has only occurred in a deer farm in Chungkov, in the Republic of Korea (Kim et al. 2005). In 2001, the Korean farm reported a death of an individual elk that was imported from Canada. Histopathological examination of central nervous tissues from this individual revealed neuronal vacuolations consistent with CWD. Those individuals that cohabitated with this individual were slaughtered to reduce the possibility of further transmission. Further testing of the additional individuals imported from Canada revealed nine of 144 deer had CWD (KIM et al. 2005). However, although testing of CWD in Europe was previously only limited to Germany (Schettler et al. 2006), CWD in a free-ranging Norwegian reindeer was detected in March 2016, making this the first case of CWD in Europe. However, due to minimal disease testing, prevalence of the disease is unknown (Benestad et al. 2016).

While differences in disease detections have been observed geographically, prevalence differences across demographic categories between cervids have also been observed. An increased detection rate of CWD in male white-tailed deer has been observed in several studies (Greear et al. 2006, Osnas et al. 2009), independent of age class. The increased detection rate is important for the continued spread of CWD as both dispersal rates and distances have been observed to be greater in male white-tailed deer (Hawkins and Klimstra 1970, Nixon et al. 1991, Nelson 1993, Rosenberry et al. 1999). Additionally, while significant detection differences were not observed between male and female mule deer, there was age disparity in males as prevalence

increased with age until its peak in ages 5-6 and then decreased thereafter (Miller and Conner 2005).

CWD Systemic Spread

Although CWD has been previously detected through various sectioned central-nervous tissues (Williams and Young 1980), prolonged incubation periods result in average clinical symptom (physical appearance) detection of 16 months (Williams 2005); however, accumulation of the prions in lymphoid tissue can be detected in 42 days post-oral exposure (Sigurdson et al. 1999). When first detected in the brain, three months post exposure, the prion is widely distributed throughout the lymph tissues and by nine months, the prion can be detected in the myenteric and submucosal plexi in the gastrointestinal tract in the vagus nerve (Fox et al. 2006). Additionally, the prion can be detected in the vagosympathetic trunk, nodose ganglion, adrenal medulla and pancreatic islets (Sigurdson et al. 2001). After 16 months post exposure, the infected prion can be detected throughout the brain, central nervous system (Fox et al. 2006) and even the retina (Spraker et al. 2010).

Detections of PrP^d in lymphoid tissues in elk, white-tailed deer, and mule deer during periods of incubation have been made through immunohistochemistry (IHC) (Spraker et al. 2004) and enzyme-linked immunosorbent assay (ELISA) (Hibler et al. 2003). However, advancements in the sensitivity of CWD prion detection have been made through serial protein misfolded cyclic amplification (sPMCA) (Haley et al. 2012), protein misfolding cyclic amplification (PCMA) (Saá et al. 2006), and real-time (RT)-quaking induced conversion (QuIC) (Haley et al. 2014). Compared to IHC, which is considered the gold standard of prion detection, sPMCA detected the infectious prion significantly sooner (2.78 months) (Haley et al. 2012) and

in lower amounts of CWD prions (Saá et al. 2006). This technique has also been used to detect CWD prions in elk feces (Pulford et al. 2012). Additionally, in a single-blind study, blood samples from 98 free-ranging white-tailed deer as well as the medial retropharyngeal lymph nodes (MRPLN) were used to compare detection levels of CWD in blood by PCMA (ante-mortem) and MRPLN by IHC (post-mortem). While PCMA detected CWD in 100% of clinical individuals, the test only detected 53% of individuals that had early stages of the disease. As importantly, the test had 100% specificity, indicating a potential ante-mortem technique in detecting CWD (Kramm et al. 2017).

Real-time quaking induced conversion (RT-QuIC) has been shown to be used as a non-invasive, ante-mortem technique for detecting CWD prions in both body fluids including saliva (Henderson et al. 2013) as well as urine (John et al. 2013). Furthermore, using both RT-QuIC and tyramide signal amplification immunohistochemistry (TSA-IHC), Hoover et al. (2017) was able to detect prion seeding activity in oropharyngeal lymphoid tissues at 1 month post exposure in mule deer with detections in all lymphoid tissues .

Although the misfolded prion protein isoform is initially detected in the lymphoid tissues and then later found systemically throughout the central nervous system (Sigurdson et al. 1999, Miller and Williams 2002, Spraker et al. 2004), the infected prion has also been detected in other tissues including the pancreas, adrenal gland (Sigurdson et al. 2001, Fox et al. 2006), and both skeletal (Angers et al. 2006) and cardiac muscle. Additionally, the infectious prions have also been detected in adipose tissues, as well as reproductive, secretory and excretory systems (Selariu et al. 2015). In most cases however, the majority of animals (97%) which were detected to have CWD were subclinical (Williams et al. 2002).

Deposition of PrP^d in lymphoid tissues has been absent in elk, but present in brain tissues (Spraker et al. 2004). Additionally, serial protein misfolding cyclic amplification, a newly developed assay, detected CWD associated prions in 15 of 19 Rocky Mountain elk dams where immunohistochemistry only detected CWD in three of 19. In the 15 positive dams, the prion was not detected in either central nervous tissue or lymphatic tissue. Furthermore, the presence of disease associated prions was found in 80% of elk fetuses (Selariu et al. 2015). Additionally, in Reeves' muntjac deer (*Muntiacus reevesi*), CWD prions were detected in fetal tissue from mothers within early and late stages of disease infection (Nalls et al. 2013), further suggesting that CWD can be transmitted vertically.

Transmission

Perpetuation efficiency of diseases is contingent on the variety of host species, as well as ease, environmental conditions, and number of routes that transmission can occur. Horizontal (animal to animal) and maternal (dam to offspring) transmission routes of a captive mule deer herd were compared in a 5-year study period within a high-density, fenced facility (Miller and Williams 2003). While maternal transmission likely contributed little to disease perpetuation, horizontal transmission through direct deer contact resulted in a high incidence of disease (89%) within the herd.

Identification of the medium through which the infectious prion can be contracted was determined through a controlled lab experiment where fawns were exposed to blood, saliva, urine and feces and brain material from mule deer testing positive for the disease (Mathiason et al. 2006). White-tailed deer inoculated with brain, blood and saliva of infected mule deer were detected to have CWD through brain and tonsil biopsies. White-tailed deer inoculated with urine

and feces were not detected to test positive for CWD (Mathiason et al. 2006); however, later studies confirmed that urine and feces excreta can contain CWD prions (Haley et al. 2009, Tamgüney et al. 2009). Shedding of prions through saliva and urine can occur in as early as three months post exposure (Henderson et al. 2015b) and can contain 1-5 LD₅₀ per 10 ml of expelled fluid (Henderson et al. 2015a). Additionally, in a controlled laboratory study, aerosol transmission was shown to be another efficient prion transmission route for cervids (Denkers et al. 2013) and in a study of PrP transgenic mice, those with abrasions in the lingual regions had enhanced susceptibility to CWD (Denkers et al. 2011).

While tissue, blood, feces, and urine have been shown to serve as mediums for harboring CWD prions, the degree of exposure by which transmission of CWD can occur in a natural environment is difficult to identify. However, in a captive facility, decaying CWD-infected deer carcasses lead to infections of more mule deer with CWD in the same facility (Miller et al. 2004). This may be a result of the efficient binding and adherence of prions shed from a decaying carcass to soil particles (Johnson et al. 2006b) and may in fact increase transmission efficiency in clay soils (Johnson et al. 2007, Walter et al. 2011, Saunders et al. 2012). Prions bound to soil particles were found to be resistant to the environments within the rumen (low pH, anaerobic conditions) (Saunders et al. 2012).

Controlled lab experiments by Rasmussen et al. (2014) and Pritzlow et al. (2015) demonstrated the ability of plants to uptake prions bound to soil particles through the roots. However, while Rasmussen et al. (2014) did not detect prions in the stem or leaves of wheat (*Triticum aestivum* L.), Pritzlow et al. (2015) found that prions shed from feces or urine adhered to the leaves of wheat grass and ingestion of these leaves or roots were shown to lead to infection of susceptible hamsters. Therefore, ingestion of these plant materials indicates that plants can

uptake prions. These studies not only reveal an additional transmission pathway, but highlights the high efficiency and evolutionary potential of prion transmission.

While efficient conversion of the normal PrP to CWD-associated PrP is found in cervids, the detection of PrP^d in cattle after inoculation of brain matter from infected mule deer demonstrated the possibility of transmission to another ruminant that is susceptible to prion diseases (BSE). However, the period of inoculation (5 years) and unnatural transmission route suggests that transmission from infected cervids to cattle is very unlikely (Hamir et al. 2005). The reduced efficiency into the pathogenic state in human and bovine PrP demonstrate a potential molecular barrier for CWD transmission (Raymond et al. 2000). These barriers are likely due to sequence differences in the PrP genes that encode steric zippers structures (Apostol et al. 2011).

Although other mammalian species, including predators, have certainly been exposed historically to CWD prions, transmission of CWD in many of these species has not been fully demonstrated. Infection of CWD through oral exposure was not seen in raccoons (*Procyon lotor*) through three years of exposure to the CWD prion (Hamir et al. 2003). However, ferrets (*Mustela putorius furo*), a domesticated member of mustelidae (weasel family; carnivorous mammals), contracted CWD through intracerebral exposure (Bartz et al. 1998, Sigurdson et al. 2008). Additionally, after exposing coyotes (*Canis latrans*) to CWD prions by consumption of CWD positive elk brain homogenate, prions were able to be detected up to 3 days post ingestion in feces (Nichols et al. 2015).

Genetics

Although the prion protein gene *PRNP* is highly conserved among cervid species with only 16 amino acid polymorphisms within the 256 amino acid reading frame (Robinson et al. 2012a), the remaining variability of amino acid sequence within the protein prion gene of cervids has been previously found to have potential impacts on the delayed incubation rate of chronic wasting disease. These changes were first discovered in both captive and free-ranging Rocky Mountain elk (*Cervus elaphus nelsoni*). A single nucleotide polymorphism (SNP) at PrP codon 132 resulted in an amino acid change from Methionine to Leucine (O'Rourke et al. 1999). Chronic wasting disease was found to be more prevalent in individuals that were homozygous at PrP codon 132 compared to individuals without the disease. Using simulation modeling of populations of elk infected by CWD for at least 30 – 50 years, allele frequencies for the Leucine at codon 132 were nearly twice as great than those from uninfected populations (Monello et al. 2017). Additionally, Jewell et al. (2005) surveyed populations of free-ranging mule deer in Colorado and Wyoming for polymorphisms in the *PRNP*. Mule deer with genotypes SF (Serine, Phenylalanine) or FF (Phenylalanine, Phenylalanine) were underrepresented in the number of free-ranging mule deer that were found to have CWD. Those homozygous SS (Serine, Serine) at codon 225 had 30 times greater odds of getting CWD than those that were SF at 225, suggesting that incubation rates of those that are 225SF or 225FF are much longer. However, only 9.3% of the sampled population had the less susceptible genotypes.

In a study of white-tailed deer inhabiting a region with CWD, four polymorphisms were identified in the amino acid sequence at the 95th, 96th, and 138th codons (Johnson et al. 2003). The majority of deer had one copy of the wild type PrP allele QGS (codon 95 (glutamine), codon 96 (glycine), codon 138 (serine)). This particular allele combination was found in 86.5% of white-tailed deer that tested positive for CWD deer and 68% of white-tailed deer that tested

negative for CWD. However, the QSS (Glutamine, Serine, Serine) allele was under-represented in deer testing positive for CWD and was not identified in CWD positive deer when homozygous.

When a larger number of individuals were genotyped in white-tailed deer populations within Wisconsin, additional polymorphisms were discovered within the 95th and 96th codon. Additionally, a new polymorphism in the 226th codon was reported (Johnson et al. 2006a). Changes within the 95th codon from Glutamine to Histidine as well as Glycine to Serine in the 96th codon resulted negative association of CWD detection. However, one individual that was homozygous for the amino acid Serine at the 96th codon did test positive for CWD. Furthermore, the change of the amino acid Glycine to Serine at the 96th codon was also attributed to slower progression of the disease. Polymorphisms in the 95th and 96th codons have also been reported in white-tailed deer populations in other populations in Illinois (Kelly et al. 2008, Wilson et al. 2009). However, Kelly et al. (2008) did not find any positive CWD individuals with the Serine-Serine genotype. Additionally, a change from Alanine to Glycine at the 116th codon resulted in decreased association to CWD in white-tailed deer populations in Alberta, Canada (Wilson et al. 2009) and Nebraska (O'Rourke et al. 2004).

While polymorphisms within *PRNP* sequences have been associated with prolonged incubation rates, contributions from other genetic factors are generally unknown. While studies have shown no association between differences in promoter regions of the *PRNP* (Heaton et al. 2003, White et al. 2010), and C1q (Blanchong et al. 2009) gene and CWD status, the neurofibronmin 1 gene was associated with reduced CWD states in mule deer (Matsumoto et al. 2013).

Strain diversity

While comparisons of CWD prion protein through Western blot glycoform pattern analysis failed to distinguish distinct strains within the disease (Race et al. 2002), studies of transgenic mice have provided evidence for the possibility for strain diversity. Isolates of CWD from mule deer, white-tailed deer, and elk were inoculated into several species of hamsters including Syrian golden, Djungarian, Chinese, Siberian, and Armenian. While species of hamsters had varying susceptibility to CWD, the Syrian golden hamster had short (85 – 89 days) or long (408 – 544 days) incubation periods. Variability in incubation periods was dependent on injection of the mule deer derived CWD (short incubation) or the elk-derived strain (Raymond et al. 2007). Similar patterns of progression time and distribution of CWD prions were also observed in ferrets (*Mustela putorius furo*) (Perrott et al. 2012). Additionally, a study of transgenic mice expressing the *wt* allele (tg33) or S96 allele (tg60) showed that inoculations of CWD prions from four different PRNP genotypes (*wt/wt*, *S96/wt*, *H95/wt*, and *H95/S96*) produced a novel strain in tg60 mice when inoculated with the *H95/wt* and *H95/S96*. Isolates from the novel strain in tg60 into tg33 mice showed a disease presentation that appeared to be a mixture of two strains, suggesting that transmission between individuals with different genotypes could result in novel CWD strains (Velásquez et al. 2015).

Control Strategies

In 2001, the first case of CWD in free-ranging white-tailed deer was detected in south-central Wisconsin (Joly et al. 2003). In response to the discovery that has been labeled as “fire in the Sistine chapel” (Heberlein 2004), the Wisconsin Department of Natural Resources’ (DNR) initial management goal was to eradicate the disease through an objective based strategy that

included defining the geographic distribution of the disease, determining disease origin, and guiding future management decisions through the enhancement and incorporation of available science. Additionally, initial management practices focused on herd reduction through extended hunting seasons, out-of-season shooting permits, government-agency sharpshooters and monetary incentives to remove deer. The purpose of these management strategies was to extirpate all deer in the area (411 mi²) where CWD positive individuals were found as disease prevalence and deer density were found to increase transmission in mathematical models (Storm et al. 2013). This strategy, if fully executed, would eliminate both potential hosts and vectors for disease spread.

After continued detection of CWD positive deer outside the initial region, the controversial CWD eradication program ended. Continued CWD surveillance within Wisconsin identified two core regions with high levels of detection, indicating that the disease continues to spread outwardly. Amid waning public interest in deer reductions, loss of program funding, and the inability of hunters to harvest a sufficient number of deer required to affect the prevalence of the disease, the implementation of the original management strategies has ceased. Present adaptive management strategies from the Wisconsin DNR now include regulations that restrict baiting and feeding as well as carcass transportation within any county that has tested positive for CWD.

One year after the first detection of CWD in white-tailed deer, an additional case of CWD was detected in northern Illinois in 2002. Similar management strategies to that of Wisconsin were adopted including CWD surveillance along with increased hunter harvest permits and sharpshooters. Like Wisconsin, it was discovered that hunters were unable to significantly reduce the number of deer, likely because hunters' willingness to apply and use antlerless permits is

inadequate to reduce population density (Riley et al. 2003). However, where government agency sharpshooting management actions occurred, CWD prevalence did not increase (Manjerovic et al. 2014) and prevalence in young and females significantly decreased (Mateus-Pinilla et al. 2013). Therefore localized culling of cervid herds may serve as an effective tool for reducing spread (Cullingham et al. 2011).

In addition to localized culling strategies, Potapov et al. (2016) evaluated six harvest strategies that have been used to control CWD prevalence in deer through modeling previously developed by Potapov et al. (2013). The modeling accounted for age of males, density dependent effects, intensity and preferences of hunter harvests, as well as temporal differences in infection rates. Following parameterization, intensity of harvest was shown to reduce disease prevalence the greatest as well as a non-age bias in harvesting male white-tailed deer (Potapov et al. 2016).

While management of CWD in wild cervid populations has focused on limiting the number of susceptible hosts through increased culling, recent studies have also considered immunizations as a tool for CWD management. Although the development of some vaccine strategies have failed to protect cervid species from disease infection (Pilon et al. 2013), several vaccines have been able to generate anti-prion activity (Pilon et al. 2007, Jeong et al. 2012, Taschuk et al. 2015). Additionally, a recent vaccine developed though an attenuated *Salmonella* vaccine has shown a prolonged incubation rate in most deer and an individual deer showed no symptoms of CWD. Immunoglobins A in saliva and immunoglobins G found systemically throughout the body were found to react with CWD prions in Western blot tests (Goñi et al. 2015). Additionally, CWD infected transgenic mice treated with 2-aminothiazole IND 24 showed an increase incubation time (twice as long); however CWD prion structures were not changed by IND 24 (Berry et al. 2015).

To reduce further spread of CWD through prion infected environments, clean-up of contaminated areas can become a management option, especially in fenced-in areas. Saunders et al. (2010) found that a topical treatment of the enzyme Prionzyme disrupted and degraded PrP^d prions bound to a wide range of soil particles. Treatments for other prion diseases including scrapie (Xu et al. 2013) and BSE (Dickinson et al. 2009), have also had success decontaminating prion infected areas.

Management of CWD in West Virginia

Chronic wasting disease was first discovered in a road-killed white-tailed deer in 2005 and in 2006 the first hunter-harvested sample was found to test positive for the prion in Hampshire County. Like Wisconsin, both special collections (sharpshooting) and the issuance of increased hunter opportunities were established to reduce white-tailed deer densities as part of the initial management plan. Although sharpshooting events ceased after 2012, increased harvest limits remain as part of the adaptive management plan.

To control mechanical spread of the prion, a CWD containment area has been established by the WVDNR. Previously, the containment area included all of Hampshire County, Hardy County east of state route 259 and north of Corridor H (U.S. Route 48), as well as Morgan County west of Route 522. However, as an adaptive management tool, the area was expanded in 2015 to include Grant, Hardy, Hampshire, Mineral, Morgan, Berkeley, and Jefferson counties. Baiting and feeding of white-tailed deer in Morgan, Hampshire, and Hardy counties are restricted in order to avoid increased deer-to-deer contact. Additionally, white-tailed deer harvested within Hampshire, Hardy, and Morgan Counties are prohibited from being transported outside of its boundary with the exception of de-boned meat, quartered deer without the head and

spine, finished taxidermy mounts, cleaned hides, and cleaned skull plates and antlers without any tissue attached.

Since the initial detection, over 15,000 deer have been tested for chronic wasting disease using immunohistochemistry (IHC) of retropharyngeal lymph nodes. As of June 2015, there have been 189 confirmed cases of CWD in West Virginia from hunter-harvested, road-killed, and special collections (West Virginia Hunting and Trapping). The majority of these have been found in Hampshire County, but three white-tailed deer have tested positive in Hardy County.

Disease effects on society

The short-term effects and long-term implications of a poorly understood disease, coupled with a large diversity of interest groups, make measuring the consequences of a devastating disease likely immeasurable. However, while not fully measurable, the reduction of cervid herds across the United States has already greatly affected a diversity of small and large economies. Businesses that benefit from hunting activities, tourism, and captive cervid farming have adversely been affected due to losses in the number of hunters, deer, and public uncertainty associated with CWD. Shortly after the discovery of CWD in Wisconsin, license sales declined 9.9% as compared to previous years resulting in estimated monetary losses between \$53 million and \$79 million in 2002 and \$45 million and \$72 million in 2003 (Bishop 2004). However, a survey of hunters in 2002 indicated only half of those not participating in the 2002 white-tailed deer hunt were from concerns of CWD (Vaske et al. 2004) and in Alberta, hunting behavior is believed to be unchanged following CWD detection (Zimmer et al. 2012). However in Colorado, where CWD was first discovered, hunter participation decreased 10% to 20%. Annual

economic reductions in the state of Colorado from this disease is likely tens of millions of dollars (Seidl and Koontz 2004).

The decline in hunter participation could have long-term impacts for the sport as recruitment of youth may diminish across the United States. The WVDNR has incorporated special regulations such as special youth seasons and reduced lifetime license fees (purchased before the age of 2) to recruit and retain youth in the sport. This is especially important for a state like West Virginia, where populations of wild game support 350,000 license holders (roughly the equivalent of one hunter per family) and estimates of the economic contribution of hunting activities to the state's economy are \$270 million annually (supporting 5,000 local and state-funded jobs; Southwick Associates 2012) ,

Additionally, while immeasurable, the intrinsic worth of losing a resource or a tradition that involves a resource could be devastating to a larger majority of people that may or may not be benefitting from the economic products. Residents of CWD-affected counties in Wisconsin were concerned with consumption of CWD-affected meat, reducing the pleasure of the hunting experience (Petchenik 2003) Additionally, while the level of trust between hunters and non-hunters of the Wisconsin DNR was equal (Stafford et al. 2007), hunters who did not participate in hunting following the detection of CWD in Wisconsin were found to mistrust the information published by the Wisconsin DNR compared to those who did hunt, therefore eroding the needed relationship between hunter and government agency (Vaske et al. 2004).

Future Trends

Ongoing widespread detection of a transmissible infectious disease within a wide variety of highly-mobile cervid hosts not only presents a challenge for existing natural resource

managers, landowners, and other stakeholders, but current implemented policies must consider incorporating models that account for future biological trends. Bayesian modeling of individuals with genotypes that confer longer incubation times indicated that white-tailed deer had a four times less risk of CWD infection and, because of the prolonged incubation rate, these deer were able to survive 49% longer (8.25 more months). This difference in selection suggests that the less susceptible genotype could become more dominant over a long period of time (Robinson et al. 2012a). Modeling of elk populations also found that sex-specific harvesting (males only) along with the differences in genotypes would stabilize populations, whereas harvesting both sexes and not incorporating a less susceptible genotype resulted in near extinction of the current population (Williams et al. 2014).

Although scientific and human dimension research into chronic wasting disease continues to contribute to the understanding of societal acceptance, disease transmission, species barriers, and management of the disease in wild cervid populations, there are several focal areas of research that need to be addressed in order to further progress understanding and managing the disease. These include, but are not limited to, a live animal test with comparable detection levels to that of ELISA and IHC, ability to identify strains in vitro, development of a fully preventable vaccine and method for dispersal to individuals, and a protocol for clean-up of contaminated environments (Haley and Hoover 2015).

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Chapter 2

Spatio-temporal analysis of genetic diversity associated with extended pre-clinical symptoms of Chronic Wasting Disease

Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) that affects cervids throughout North America and was detected in a free-ranging, white-tailed deer *Odocoileus virginianus* within West Virginia in 2004. While infection with the disease invariably results in a 100% fatality rate, previous studies have indicated that nucleotide polymorphisms in the 285th and 286th position of the prion precursor gene (*PRNP*) have been associated with delayed clinical disease symptoms. To determine the association with CWD positive individuals in a CWD-affected population in West Virginia and determine temporal differences in polymorphism frequency before and after the first detection, 513 CWD negative female white-tailed deer were sampled in Hampshire County, WV. In addition, 146 CWD positive male and female white-tailed deer were sampled from 2006 – 2014. Female white-tailed deer (CWD positive and negative) were also selected to determine any changes associated with *PRNP* polymorphisms and disease status as well as temporal changes before disease detection within a high density area of CWD detections ($>1/\text{km}^2$). While polymorphisms at *PRNP* 285 (Adenine to Cytosine) were not statistically associated with disease detection at either sampling, polymorphisms at *PRNP* 286 (Guanine to Adenine) were statistically associated with the prevalence of disease detection at both samplings ($P=0.000$, $P=0.0478$). Comparisons between observed allele frequencies at *PRNP* 286 following CWD detection and expected allele frequencies if the locus was under selection found a significant decrease in frequency of the beneficial allele in both the high density sampling as well as the Hampshire County sampling,

which is counter to the expected increase in frequency if selection was occurring. These data suggest that while polymorphisms at *PRNP* 286 are associated with the absence of CWD, the frequency of the beneficial allele is instead decreasing, likely due to migration or genetic drift.

Introduction

Chronic wasting disease (CWD) is a naturally occurring transmissible spongiform encephalopathy (TSE) found in free ranging cervids across North America including white-tailed deer (*Odocoileus virginianus*) (Spraker et al. 1997, Joly et al. 2003), mule deer (*O. hemionus*) (Williams and Young 1980), Shiras moose (*Alces alces shirasi*) (Baeten et al. 2007), and Rocky Mountain elk (*Cervus elaphus nelson*) (Spraker et al. 1997, Williams and Young 1982).

Associated with CWD is the presence and accumulation of the misfolded isoform of the prion protein (PrP^d) (Prusiner 1982). The diseased isoform acts as a template for the conversion of the normal PrP host protein (Basler et al. 1986, Caughey et al. 1990, Safar et al. 1993, Horiuchi and Caughey 1999) into the irregular and infectious shape known as a prion (Prusiner 1982).

Affected cervids display clinical symptoms of being abnormally thin, likely from a lack of eating (Williams et al. 2002), but also exhibit other symptoms including hair loss, pneumonia, dehydration, excessive salivation, depression, vomiting, and overall lack of body control and movement (Williams and Young 1980). These symptoms have led to changes in population dynamics of cervids including decreased population growth (λ) as a result of decreased survival and abundance (Miller et al. 2008, Edmunds et al. 2016). While clinical symptom detection averages 16 months post exposure (Williams 2005), the large majority of animals (97%) detected to have CWD are subclinical (Miller and Williams 2002).

Understanding mechanisms for CWD transmission in free ranging populations has become important for initiating measures to control further spread. After the initial discovery of CWD in Colorado, disease detection has occurred in distant patches and diffused outward from those initial areas. Horizontal (animal to animal) transmission is believed to be primarily responsible for disease perpetuation (Miller and Williams 2003) through shedding of prions through bodily excreta (saliva, blood; Mathiason et al. 2006, 2009, Haley et al. 2009), urine (Haley et al. 2009), feces (Tamgüney et al. 2009) and decaying carcasses (Miller et al. 2004). Additionally, because prevalence of CWD is greater in males (Gear et al. 2006, Osnas et al. 2009) as compared to females and prevalence increases with age of individuals of both sexes (Miller and Conner 2005), there is a possibility that diffusion of the disease is associated with male dispersal, especially considering that dispersal rates as well as distances are greater for males compared to females (Hawkins and Klimstra 1970, Nixon et al. 1991, Nelson 1993, Rosenberry et al. 1999).

Genetic variability (single nucleotide polymorphisms, SNPs) within the highly conserved prion protein (PrP) genes, *PRNP*, has been shown to alter disease progression (i.e., prolonged incubation periods) as efficiency of normal protein forms (PrP^c) conversion to diseased protein conformers (PrP^d) is delayed (Caughey 2003). These changes were first discovered in both captive and free-ranging Rocky Mountain elk where CWD was found to be more prevalent in individuals that were homozygous at *PRNP* 394 (PrP codon 132) compared to individuals without the disease (O'Rourke et al. 1999). In white-tailed deer, multiple studies have noted that polymorphisms within the *PRNP* at nucleotide base 285 (Adenine to Cytosine; 285 A/C) and 286 (Guanine to Adenine; 286 G/A), which results in an amino acid change from glutamine to histidine and glycine to serine, respectively, have been associated with reduced susceptibility and

a prolonged incubation period (Raymond et al. 2000, Johnson et al. 2003, C. Johnson et al. 2006, Kelly et al. 2008, Blanchong et al. 2009, Wilson et al. 2009, Robinson et al. 2012). However, while Kelly et al. (2008) and Wilson et al. (2009) did not find any positive CWD individuals with the 286 A/A genotype, Blanchong et al. (2009) reported a single CWD positive individual homozygous for Adenine at nucleotide 286. Thus, while there may be a statistically significant association of *PRNP* polymorphisms and disease detection, individuals with polymorphisms are not resistant to CWD. In a controlled laboratory environment, Johnson et al. (2011) determined that white-tailed deer homozygous (*wt/wt*) at both *PRNP* positions survived on average 693 days past inoculation (DPI) of CWD, and individuals 286 G/A survived 956 days past incubation when exposed. Additionally, individuals with 285 A/C succumbed to disease symptoms 1,508 DPI (Johnson et al. 2011).

While polymorphisms within the *PRNP* gene have been associated with a prolonged incubation rate, yet invariably fatal to white-tailed deer, the persistence of the proteinaceous prion in the environment creates the opportunity for localized areas of high prion density. In addition to surviving in highly acidic internal conditions (e.g., rumen; (Saunders et al. 2012)), prions shed through decaying carcasses (Miller et al. 2004) and other bodily fluids (Mathiason et al. 2006, 2009, Henderson et al. 2013, 2015*b*) present an opportunity to accumulate and further contaminate variably depending on soil types (Johnson et al. 2006*b*, Saunders et al. 2011, Walter et al. 2011). Additionally, long-term persistence is further aided by prion uptake by plant material (Rasmussen et al. 2014, Pritzkow et al. 2015). The persistence and further accumulation of diseased prions over a period of time along with rapid shedding of the infectious prion (i.e., three months post exposure; Henderson et al. 2015) provides the opportunity for possible genetic selection of individuals with polymorphisms within the *PRNP*. However, population

admixture, fueled by the mobility of white-tailed deer and ecology, may negate any possible selection (Blanchong et al. 2009), especially as clinical detection may be delayed by more than a year (Williams 2005). However, the reduced likelihood of female white-tailed deer dispersal (Porter et al. 1991, Miller et al. 2010) as compared to males may provide the opportunity for adaptive population divergence.

Although state agencies continue to monitor and survey the prevalence of CWD across the landscape, there has been no study examining temporal variation across an infected area. In West Virginia, the abnormally shaped prion associated with CWD was first detected in a road-killed white-tailed deer in 2004. In 2006, five hunter-harvested samples were found to test positive in Hampshire County. Since then, over 15,000 deer have been tested for chronic wasting disease across West Virginia using enzyme-linked immunosorbent assay (ELISA) of retropharyngeal lymph nodes and confirmed through immunohistochemistry. As of June 2015, there have been 183 confirmed cases of CWD in West Virginia. Therefore, the objective of this study was to assess the relationship between genetic diversity at *PRNP* positions 285, 286 and CWD prevalence at both a county-wide scale and a high-density CWD area (>1 positive individual/km²). Additionally, temporal variations in the presence of polymorphisms were also assessed in Hampshire County, but also within an area of CWD detection >1 / km² to determine if allele frequency changes conformed to predicted allele frequencies after selection.

Methods

Sample Collection and Selection

Starting in 2006, in conjunction with West Virginia Division of Natural Resources (WVDNR) monitoring of CWD in hunter-harvested white-tailed deer, tissue samples consisting

of ear notches or abdominal skin were collected, individually labeled, and stored in 95% ethanol for DNA analysis (Figures 1 and 2). Each individual deer was aged through tooth wear and replacement (Severinghaus 1949) and the hunter identified on a map the 1-km² grid cell from which the white-tailed deer was harvested. The centroid of the cell became the geographic location of the sample. Retropharyngeal lymph nodes were collected and tested for CWD using enzyme-linked immunosorbent assay (ELISA). Samples testing positive through ELISA testing were confirmed through immunohistochemistry (IHC). Collection of hunter harvested white-tailed deer samples occurred at WVDNR biological check stations in areas including and surrounding Hampshire County during a special 2-day antlerless deer season in October from 2006 through 2011 and annually during the first three days of firearms season (late November) from years 2006 through 2014. In addition to hunter-harvest samples, special collections consisting of sniper-selected deer were also collected from 2006 – 2012 to reduce deer densities in areas with a CWD positive individual within Hampshire County.

A subsample consisting of female white-tailed deer was selected as dispersal rates and distances of females are considered to be less than males (Hawkins and Klimstra 1970, Tierson et al. 1985, Porter et al. 1991, Aycrigg and Porter 1997), especially those ≥ 1.5 years of age (Miller et al. 2010). To avoid misassigning the spatial location of an individual where a grid cell encompasses both sides of a potential barrier to movement (Blanchong et al. 2008, Long et al. 2010, Robinson et al. 2012b), a 1-km buffer was placed around major highways and major rivers. Samples within the buffer were excluded from sample selection. Finally, females testing positive for CWD were removed from the initial stratified sampling (geographic) to avoid any variation in movement from disease symptoms. From the remaining samples, a minimum of one female from each grid cell was selected. In locations where grid cells have multiple samples, a

single individual was randomly selected. Based on these criteria, a total of 513 female white-tailed deer tissue samples were selected in Hampshire County, WV. In addition to these samples, all male (N= 104) and female (N= 42) white-tailed deer that tested positive for the prion associated with chronic wasting disease (i.e. hunter harvest, special collection) were included in the sample.

To examine potential genetic selection in an area with a high density of CWD positive individuals, the spatial location of each CWD positive white-tailed deer from 2006 – 2014 was mapped in ArcGIS 10.2.2 (ESRI 2014) followed by a point density analysis. Areas with densities > 1 CWD positive individual per square kilometer were mapped. Through this analysis, a 97 - km² high-density area was selected (Figure 2). All females harvested through hunter selection or special collections from this area were selected for analysis. To examine potential temporal shifts in *PRNP* polymorphisms of nucleotides 285 and 286, the year of parturition was calculated using the age data of each individual. Male white-tailed deer were removed from the analysis as males have been found to be more likely to disperse from outside of the study area than females (Hawkins et al. 1971, McCoy et al. 2005, Nixon et al. 2007, Long et al. 2010).

DNA extraction and amplification

Genomic DNA was extracted using the Wizard® SV-96 DNA purification system (Promega, Madison, WI) according to manufacturer's protocol. DNA was quantified using a spectrophotometer (NanoDrop, Wilmington, DE) and standardized to a concentration of 10 ng/μL. Amplification of the coding region of the mature prion gene occurred through the published primer set (Johnson et al. 2003): forward primer 223(5'-ACA CCT CTT TAT TTT GCAG-3') and reverse primer 224 (5'-AGA AGA TAA TGA AAA CAG GAAG-3') by creating

20 μ L reactions consisting of 0.4 mM dNTP, 2.0X Buffer, 1.25 mM MgCl₂, 2.0 U Taq Polymerase, 0.2 mM F Primer, 0.2 mM R Primer and 40 ng of template DNA. Extracts were amplified using an initial heating of 95°C for 5 minutes followed by 30 cycles at 95°C (30 sec), 54°C (30 sec), 72°C (60 sec), with a final hold of 72°C for 7 minutes. Resultant fragments were purified using Promega Wizard[®] SV 96 PCR Clean-up system following manufacturer's protocol. The 860-basepair fragment was sequenced using a BigDye Terminator Sequencing kit and an Applied Biosystems[™] 3130 XL genetic analyzer. Resultant chromatograms of sequencing were evaluated using the software BioEdit version 7.2.5 (Hall 1999). Sequences were aligned using the program CLUSTALW (Thompson et al. 1994) to identify SNPs at *PRNP* positions 285 (95th codon) and 286 (96th codon).

Statistical Analysis

To determine the significance of differences between observed and expected frequencies at *PRNP* positions 285 and 286 in CWD positive and CWD negative individuals, a Fisher exact test (Fisher 1935) was performed in the program R (R Core Team 2015) using an α level of 0.05. Selection coefficients for each genotype and sampling strategy were calculated based on relative fitness of the three possible genotypes for *PRNP* 286. *PRNP* 285 was not included in this analysis because it was not correlated to CWD detection (see Results). To determine if selection was occurring in the population as a result of CWD, allele frequencies at *PRNP* 285 and 286 were calculated before (1998 – 2003) and after (2005 – 2012) CWD detection (2004) using the natal year of each individual. The expected allele frequency under selection for each nucleotide polymorphism and sampling was calculated based on allele frequency predictions under directional selection ($p_1 = p / (1-sq^2)$) and compared to the observed post-CWD allele frequencies

to determine if selection was occurring. A Fisher exact test (Fisher 1935) was performed in the program R (R Core Team 2015) using an α level of 0.05 to determine statistical significance.

Results

A total of 659 white-tailed deer tissues samples were analyzed, consisting of female white-tailed deer in Hampshire County, WV (N=513) as well as all positive individuals (N=146). Based on the total sample set from Hampshire County, 1.4% of individuals carried the cytosine allele at *PRNP* 285 (*wt* = Adenine), whereas 16.5% of individuals had the Adenine allele at *PRNP* 286 (*wt* = Guanine), Table 1). Although a total of 10 individuals were 286 A/A, no individuals were 285 C/C (Figure 2). Additionally, no positive individuals that had a nucleotide substitution at *PRNP* 285 or homozygous A/A at *PRNP* 286, five 286 A/C individuals (3.4%) tested positive for CWD. Although polymorphisms at *PRNP* position 285 were not significantly associated with CWD detection ($P = 0.219$; Table 1), a statistically significant association between CWD negative individuals with SNPs at position 286 was detected ($P = 0.000$). In addition to a non-statistically significant association between polymorphisms at *PRNP* 285, the selection coefficient for homozygous *wt* individuals was lower at both samplings compared at *PRNP* 286 (Table 1). However, heterozygous individuals to *PRNP* 286 within the Hampshire County sampling had a lower selection coefficient ($s = 0.051$) compared to those in the density selected sampling ($s = 0.114$).

For individuals selected in the density analysis (N=316, Figure 3), similar patterns of polymorphic loci were present with 2.8% of individuals carrying the allele for the delayed incubation at *PRNP* 285 (95th codon) and 23.3% at *PRNP* 286 (Table 1). Similar to the Hampshire County sampling, polymorphisms at *PRNP* 285 were not significantly associated

with CWD presence ($P = 0.118$); however, a statistically significant association was detected between individuals with SNPs at position 286 (96th codon) ($P = 0.0476$); Table 1). When comparing observed post-CWD detection allele frequencies to expected allele frequencies if the locus was experiencing directional selection, a significant difference was detected at *PRNP* 286, with the frequency of the beneficial allele decreasing post-CWD in both Hampshire County ($P = 0.003$) and high density sampling ($P = 0.000$, Table 2).

Discussion

Polymorphisms in the *PRNP* gene have been identified in many cervid species including Rocky Mountain elk (O'Rourke et al. 1999), mule deer (Jewell et al. 2005, Wilson et al. 2009), Alaskan moose (Huson and Happ 2006) and white-tailed deer (Johnson et al. 2003, 2011, Kelly et al. 2008, Blanchong et al. 2009, Wilson et al. 2009, Robinson et al. 2012a). In social animals like white-tailed deer, where disease transmission is influenced likely by the exchange of bodily fluids that harbor the diseased prion (Mathiason et al. 2006, Haley et al. 2009, 2016, Henderson et al. 2013, 2015b), factors that influence the conversion efficiency of the normal protein, resulting in a prolonged incubation rate of the disease, are especially important for understanding CWD spatial epidemiology and population growth rates (Johnson et al. 2011, Robinson et al. 2012a). This study provides a further understanding of *PRNP* diversity and chronic wasting disease association as well as any selective differences that would influence the temporal frequency of *PRNP* genotypes when individuals are sampled using two different strategies.

The first objective of this study was to determine if significant associations between CWD and polymorphisms in *PRNP* 285 and 286 could be detected in a population of white-tailed deer in West Virginia. The results of this study indicate that in both a county-wide

sampling as well as a high density CWD area, polymorphisms at *PRNP* 286 were significantly associated with the detection of CWD prions. While no homozygous mutant individuals at the *PRNP* 286 (N=15) tested positive for CWD at either sampling strategy, five heterozygous individuals tested positive for CWD in the Hampshire County sampling (3.5%) as well as five in the high-density area (5.0%). This result contradicts a laboratory study of transgenic mice that suggested complete disease resistance when polymorphic alleles were present at the 96th codon, (Meade-White et al. 2007), but concurs with other wild cervid studies that detected similar patterns of CWD detection in heterozygous and homozygous individuals (Kelly et al. 2008, Wilson et al. 2009). While a statistically significant relationship was not found when individuals were polymorphic at the 95th codon in either sampling, several studies including this one have reported the absence (Blanchong et al. 2009, Wilson et al. 2009), or low percentage of CWD positive individuals (3.9%, Kelly et al. 2008) with polymorphisms at *PRNP* 285. However, only 1.4% of individuals in the Hampshire County sampling and 3.0% of individuals in the high density sampling had these changes.

The second objective of this study was to determine whether temporal variations at *PRNP* 286 correspond to the expectations of directional selection. By estimating the natal year of each individual through tooth wear and replacement (Severinghaus 1949), the results of this study indicated significant deviations from the expectations of directional selection within the Hampshire County sampling as well as the high-density sampling. Although it has been suggested that beneficial *PRNP* alleles would increase in frequency due to selective pressure (Robinson et al. 2012), we instead observed a decrease in frequency of the beneficial allele after CWD detection. Our sampling period represents approximately 12 generations and our calculated expected allele frequency change was after a single generation of selection, so

sufficient time should have passed to permit the detection of a selection signature. This result indicates that high levels of gene flow may be reducing any selective pressure against the wildtype allele within the population by swamping out any local adaptation. This phenomenon was previously observed by Foster et al. (2007) where Hawaii amakihi (*Hemignathus virens*), a sedentary honey creeper species, had localized levels of malaria resistance compared to two dispersing honey creeper species, iiwi (*Vestiaria coccinea*) and apapane (*Himatione sanguinea*) which have much higher rates of dispersal for food. Additionally, continuous migration of non-native rainbow trout (*Oncorhynchus mykiss*) was the primary cause of rapid hybridization with native cutthroat trout (*O. clarkii*) despite strong selection against individuals with genetic admixture (Kovach et al. 2015).

While multiple cervid studies have noted a significant association of genetic diversity with disease detection, there is no known function additional to that of coding for amino acids within the highly conserved *PRNP*. A decrease in the percentage of individuals with alleles that code for slower disease progression may suggest a possible fitness advantage for *wt* individuals. Within humans, polymorphisms in the *PRNP* have been associated with clinical disorders not associated with prion diseases (Ma 2010). Additionally, the observed decrease in frequency of the beneficial allele is counter to a previous study by Robinson et al. (2012a), where a 1% selective advantage for individuals carrying alleles at *PRNP* 286 was predicted to rapidly increase the frequency of polymorphic individuals within the population due to an increased selective advantage when infection rates are higher (Robinson et al. 2012a). Differences between the results may be due to the sampling methodology. While Robinson et al. (2012a) utilized a stratified age sampling within a CWD core area to sample individuals, we focused our sampling in a small area (97 km²) defined by the density (>1 km²) of CWD positive individuals detected.

The absence of strong selection caused by CWD may likely result from a combination of multiple uncertain factors including hunting pressure, white-tailed deer ecology, and extent of prion exposure. Although CWD rate of infection increases in both sexes with age (Gear et al. 2006), hunting pressure is much stronger for males than females (Wasserberg et al. 2009), which has likely led to a skewed male age structure (Ginsberg and Milner-Gulland 1994). While the age structure is likely skewed, the infection rates of males are almost twice the rate compared to females (Gear et al. 2006) likely due to behavioral differences (Miller and Wild 2004, Miller and Conner 2005, Gear et al. 2006). Specifically, male white-tailed deer have been found to have dispersal rates as high as 80% (Hawkins et al. 1971), which have been attributed to temporal influences including inbreeding avoidance, mate competition (Long et al. 2008) and post-natal care (Hölzenbein and Marchinton 1992).

Additionally, while studies have also indicated that dispersal distances of male white-tailed deer have been affected by the percentage of forest cover (Long et al. 2005), research of female white-tailed deer movements have indicated an absence of dispersal rates and distances in highly forested regions (Porter et al. 1991, Miller et al. 2010). Although Nelson (1993) determined that 20% of females dispersed from their natal ranges between ages 1.0 – 1.5, this study assumed that female white-tailed deer did not make significant dispersal movements from their natal area and thus the harvest location of each individual represented presence of the genetic signal and also localized disease pressure.

Increased infection rate positively correlated to dispersal distances in males may likely be due to increased contacts between white-tailed deer and the CWD prion. However, natural exposure thresholds for CWD detection to occur when *PRNP* polymorphism are present remains unknown. While Sigurdson et al. (1999) detected prions in brain matter from CWD positive

individuals 42 days past inoculation, natural exposure to CWD prions can occur through different routes including blood, saliva (Mathiason et al. 2006), aerosolization (Denkers et al. 2013), urine, feces (Haley et al. 2009, Tamgüney et al. 2009) and ingestion of plant materials (Pritzkow et al. 2015). These biological materials, including differences in exposure efficiency within the environment (e.g., clay soils; (Johnson et al. 2007, Saunders et al. 2011, Walter et al. 2011)), could lead to different prion exchanges following exposure. While Henderson et al. (2015a) quantified the LD₅₀ of saliva and urine within three months post exposure and determined that the formation of amyloid fibrils have been shown to have a linear relationship with the amount of CWD prions (Henderson et al. 2015a), the conversion efficiency of normal conformers to diseased isoforms is greatly reduced in polymorphic *PRNP* (Basler et al. 1986, Caughey et al. 1990, Safar et al. 1993, Horiuchi and Caughey 1999), thus likely obscuring the understanding of the complex relationship between environmental factors, CWD prion detection, and *PRNP* diversity. While the frequency of polymorphic individuals in our study ranged between 12.8 – 16.5% at the *PRNP* 286 codon and 1.4 – 3.0 % at *PRNP* 285 between samplings, these results are different from genetic studies in other parts of the United States that reported higher percentages of polymorphisms at *PRNP* 286. In a study of white-tailed deer in central Wisconsin, Blanchong et al. (2009) reported 26% of individuals having a guanine-adenine genotype, and 3.6% of individuals having an adenine-adenine genotype. Similar to our study, both Wilson et al. (2009), who studied polymorphism occurrence in western Canada, and Blanchong et al. (2009) reported a low frequency of individuals with a polymorphism at *PRNP* 285 (2.2% and >1% of individuals, respectively).

Management implications

Managing a wild population of cervids to increase the frequency of a genetic variant that may not be under strong selection may be difficult, if not impossible, as natural characteristics of the organism as well as properties of the disease and migration may reduce selective pressure in a wild population. Although other cervid populations (e.g., Rocky Mountain elk) exhibit seasonal range fidelity (VanDyke et al. 1998), white-tailed deer are a highly mobile organism that exhibits sex-biased dispersal qualities (Greenwood 1980).

In addition to the ecology of white-tailed deer removing genetic selection in a CWD endemic area, the epidemiology of CWD may reduce the likelihood of genetic selection. While CWD is unconditionally terminal, the conversion of normal conformers to diseased isoforms (Basler et al. 1986, Caughey et al. 1990, Safar et al. 1993, Horiuchi and Caughey 1999) results in a slow progression of physical symptoms (Williams 2005) and mortality following exposure (Johnson et al. 2011). CWD positive females carrying wild type alleles at either position will still be able to produce 1 – 2 fawns depending on the age of the individual (Green et al. 2017). The kin's inherited alleles will therefore dilute any selective characteristics, adding to the likelihood of a signal for genetic selection. Furthermore, harvests through hunting activities may also confound any localized genetic advantages. Female white-tailed deer remaining in their natal home range are more likely to be harvested than transient females (Vercauteren and Hygnstrom 1998), and the influence of male white-tailed deer dispersal likely removes the possibility of strong genetic signals in localized areas.

Although studies investigating the distribution of *PRNP* polymorphisms and their association with the detection of CWD prion in free-ranging and captive cervids provide an opportunity to assess genetic diversity within the gene, the absence of a complete barrier to the disease necessitates a complex characterization regarding the importance of *PRNP*

polymorphisms. While the umbrella term “susceptibility” has been used to describe a variable predisposition to CWD, the extended pre-clinical period should not be confused with the ability to obtain diseased prions. At this moment, all individuals regardless of polymorphisms within the *PRNP* are susceptible to CWD; however, the delayed rate of degrading physical symptoms allows for increased opportunities for different mortality events to occur (e.g., harvesting, vehicle collision, predators). Therefore, further management research of the disease in wild herds should focus on practices that reduce the spread of the infectious disease, modeling population dynamics including growth rate, and monitoring continued spread.

Table 1. Number, percentage of genotypes, and selection coefficients for *PRNP* positions 285 and 286 which are associated with a delayed incubation rate of CWD. The presence of CWD was determined through enzyme-linked immunosorbent assay (ELISA) and confirmed by immunohistochemistry (IHC). Asterisks indicate a statistically significant association between genotype and CWD detection ($P \leq 0.05$)

Location	Locus	Genotype	Number of Individuals	Percentage of Sample	Number of CWD Positive Individuals	Percentage of CWD Positive Individuals	Selection Coefficient
Hampshire County	285	AA (<i>wt</i>)	650	98.6	146	100	0.225
	285	AC	9	1.4	0	0	0.000
	285	CC	0	0	0	0	0.000
	286	GG (<i>wt</i>)	550	83.5	141	96.5	0.256
	286	GA*	99	15.0	5	3.5	0.051
	286	AA*	10	1.5	0	0	0.000
Density Selected	285	AA (<i>wt</i>)	307	97.0	80.0	100	0.261
	285	AC	9	3.0	0	0	0.000
	285	CC	0	0	0	0	0.000
	286	GG (<i>wt</i>)	276	87.2	76	95.0	0.275
	286	GA*	35	11.1	4	5.0	0.114
	286	AA*	5	1.7	0	0	0.000

Table 2. Allele frequency for *PRNP* 286 within the CWD Density Sampling, with the wildtype allele denoted by *wt*. Chi-square comparison between observed allele frequency following CWD and expected allele frequency under directional selection was used to test for significance.

Location	Allele	Observed Frequency Pre-CWD	Observed Frequency Post-CWD	Expected Frequency Post-CWD	Chi-Sq	P-Value
Hampshire County	G (<i>wt</i>)	0.914	0.943	0.879	9.029	0.003
	A	0.086	0.057	0.121		
High Density	G (<i>wt</i>)	0.914	0.943	0.886	12.419	0.000
	A	0.086	0.057	0.114		

Figure 1. Map of female white-tailed deer sampled (N=513) in Hampshire County, West Virginia.

Figure 2. Map of CWD positive white-tailed deer (N=160) in Hampshire and Hardy County, West Virginia and density selected area (in grey).

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Chapter 3

Creating a Genetic Database to Understand the Genetic Structure of White-tailed Deer in West Virginia

Abstract

The illegal taking of game and non-game species (i.e., poaching) is both a domestic and international problem. Within West Virginia, white-tailed deer (*Odocoileus virginianus*) are a large economic and cultural resource, however, they are a common target of illegal wildlife activities. While biological evidence collected from poaching crime scenes or other illegal activities can be used to determine species, probability of identity, and geographic origin, a database of white-tailed deer in West Virginia is currently absent. A total of 635 tissue samples were collected from 22 counties in West Virginia with the spatial location of each individual being georeferenced to a 1-km² grid cell. After amplifying four multiplex suites consisting of a total of 16 microsatellite loci as well as sequencing of the mitochondrial d-loop region, the results of this study indicate broad-scale genetic differentiation of white-tailed deer across West Virginia. Following correction for multiple comparisons using a sequential Bonferroni test, pairwise F_{ST} scores indicated minimal within-region differences, but statistically significant differences between regions. While the Bayesian genetic program STRUCTURE indicated the likelihood of four genetic populations, another genetics program GENELAND indicated 10 distinct groups, with genetic differentiation largely explained by their geographic region. However, only 2% of genetic variation could be explained within the region using an AMOVA. Additionally, after grouping haplotypes into mitochondrial clades, the majority of neighboring sampled counties had similar mitochondrial clade consistencies. Although a county by county

genetic assignment could not be determined, the results of this study indicate that geographic assignment can be used to assign individuals to regions. Additionally, the genetic database has been already used for several forensic cases and provides the opportunity for regional assignment.

Introduction

The intentional (Muth and Bowe 1998) and illegal taking of game species (poaching; Musgrave et al. 1993) is viewed as theft of public resources due to both economic and cultural values. Globally, the poaching of fauna and flora market is estimated between hundreds of millions of dollars (World Wildlife Fund 2017) to \$20 billion USD per year (Interpol 2018) with products being largely exported from the Philippines, Hong Kong, India, and Thailand (Kurland and Pires 2017). Although crimes against wildlife and the environment are often viewed as “victimless” as they do not immediately affect individuals or groups of humans, the aesthetic value and ethnic importance they bring to a local area, region, or country is important. In 2012, hunting and related activities alone contributed over \$86 billion dollars to the United States economy (Southwick Associates 2012) with deer hunting alone contributing nearly \$40 billion USD. However, poaching of deer or other associated crimes against deer are not uncommon. Haines et al. (2012) documented a total of 67 poaching cases of white-tailed deer (*Odocoileus virginianus*) within a single county in Iowa (Fayette) from 2000 – 2009. Additionally, Kindall et al. (2011) documented a total of eight poaching events of reintroduced elk in Tennessee, which accounted for nearly 12.9% of mortalities. Within West Virginia, hunting activities accounted for a total economic contribution of \$552 million dollars with white-tailed deer hunting contributing to the large majority of the total (\$400 million USD). However, in 2013 – 2014 there were a total of 67 cases of illegal killing of deer, turkey or boar, 374 cases of illegal possession of wildlife or

parts, and 64 cases of hunting white-tailed deer after sunset within West Virginia (Lt. Col. Trader, West Virginia Natural Resources Police, Personal Communication).

Molecular techniques can provide an applicable tool for combatting crimes against wildlife. Using either mitochondrial or nuclear genetic markers, applications of genetic tools include species identity, regional, population and individual identity, as well as information on parentage (see review; Alacs et al. 2010). However, determining if samples have identical genetic profiles limits the applicability that the samples are not identical by chance without context of the specific markers utilized at the sampled region (i.e., genetic database of species). Using a genetic database of 10 microsatellite loci for mule deer (*Odocoileus hemionus*), Jobin et al. (2008) were able to determine a probability of identity for two mule deer samples of 1 in 1.7 billion in Alberta, Canada. Additionally, the genetic data from their study were able to determine the number of populations ($K=1$) as well as the genetic diversity across the province, thus serving as both a tool for forensic scientists as well as biologist to understand the genetic diversity of the population.

While genetic markers can generate individual genetic profiles that can be used to calculate probability of identity, a comprehensive spatial genetic database that represents the species' inhabited areas can also be utilized to genetically assign individuals to geographic regions. Lorenzini et al. (2011) utilized a microsatellite genetic database of Sardinian mouflon (*Ovis aries musimon*) along with Bayesian assignment testing to genetically identify a poached individual and also confirm its geographic location. However, to assign an individual to its likely natal origin, the genetic structure of the species needs to be identified across the studied landscape for robust genetic assignment to occur (Berry et al. 2004). While species with limited dispersal have a genetic structure that is restricted spatially by their movement ability (Peakall et

al. 2003, Coster and Kovach 2012), highly mobile species such as white-tailed deer, present a greater challenge for delineating spatio-genetic populations (Pelletier et al. 2012). Both high dispersal rates (>80%; Aycrigg and Porter 1997) and distances (38 km; Nixon et al. 1994) of white-tailed deer have been documented across the species' range, likely resulting in a genetically admixed population. However, larger rivers (Blanchong et al. 2008, Robinson et al. 2012) and larger highways (Robinson et al. 2012) have been identified as landscape barriers to gene flow in white-tailed deer and could influence the genetic structure of white-tailed deer.

In addition to landscape features, dispersal or philopatric behaviors exhibited by either sex can impact the spatial genetic structure of species (Sugg et al. 1996, Storz 1999). The identification of local genetic clusters in wild boar (*Sus scrofa*) was attributed to the philopatric behavior of female wild boars where sub-populations corresponded to closely related kin individuals (Podgórski et al. 2014). A similar pattern of female structuring has been found in populations of white-tailed deer. First- and second-order kin had a higher spatial overlap than unrelated deer (Magle et al. 2013), thereby making it possible to delineate fine scale, spatial-genetic structure within a continuous habitat. Additionally, the genetic structure of a high density white-tailed population within the central Appalachian Mountains was observed at a very fine (<1 km) spatial genetic structure using microsatellites (Miller et al. 2010).

In cases where dispersal is male dominated, a geographic genetic population structure can be delineated at a scale determined by the female's range using maternally-inherited mitochondrial DNA (mtDNA) (Avice 1995) and may provide finer resolution of sub-population structuring compared to nuclear inherited genetic markers like microsatellites (Karl et al. 1992). As a haploid genetic marker, mtDNA has a significantly reduced effective population size compared to diploid, bi-parentally inherited microsatellites, making it more susceptible to

genetic drift (Birky et al. 1989). Analyses of a continuous population of American black bear (*Ursus americanus*) in Ontario, Canada using mitochondrial DNA (mtDNA) revealed three populations. The identification of two populations was attributed to an isolation by distance gradient and the third to anthropogenic impacts. However, population clusters identified through microsatellite analysis were less distinct when compared to prior mtDNA analysis, which is likely attributed to dispersal of the males (Pelletier et al. 2012). Populations of white-tailed deer have also been delineated with mtDNA analysis. Gear et al. (2010) indicated that spatial-autocorrelation of relatedness was 50-fold higher than compared to a suite of twelve microsatellite loci. Additionally, high haplotype diversity was observed in 20 white-tailed deer populations (336 individuals) in the southern United States. Populations had an average of six mtDNA haplotypes (range 1 – 10) and a mean haplotype diversity of 0.67 (Sumners et al. 2015). Additionally, genetic clustering identified through spatial autocorrelation was stronger for mtDNA (Lang and Blanchong 2012).

The objectives of this study were to (1) create a white-tailed deer genetic database for West Virginia to be used for probability of identity and to (2) determine spatial genetic structure in West Virginia using both microsatellites and mtDNA for use in genetic assignment.

Methods

Sample collection

Approximately 30 tissue samples consisting of either an ear notch or abdominal skin were collected from hunter-harvested white-tailed deer in 2014 at 17 WVDNR Biological Check Stations and in Hampshire, Hardy, Mineral, Morgan, and Grant counties from 2006 – 2013

(Figure 1). Tissues were immediately stored in 95% ethanol for DNA preservation and were geo-referenced to a 1-km² grid cell by identification of the harvest location by the hunter. The center of the grid cell became the location of the harvested white-tailed deer.

Laboratory Procedures

Genomic DNA was extracted by using the Wizard® SV-96 DNA purification system (Promega, Madison, WI) according to manufacturer's protocol. DNA was quantified using a spectrophotometer (NanoDrop, Wilmington, DE) and standardized to a concentration of 10 ng/μL. A suite of 16 highly polymorphic loci was used to genotype individual white-tailed deer. Microsatellite loci include K, N, P, D, Q, O (Jones et al. 2000), RT-5, RT-7, RT13 (Wilson et al. 1997), BM 6506, BM 6438, BL42, BL25 (Bishop et al. 1994), Cervid 1 (DeWoody et al. 1995), OarFCB193 (Buchanan and Crawford 1993) and INRA011 (Vaiman et al. 1992). The sixteen loci were grouped into four different multiplex PCR primer suites with Suite 1 consisting of loci OarFCB193, Cervid 1, INRA011, BL42; Suite 2 consisting of D, BM 6506, RT-7, N; Suite 3 O, Q, F, BM 6438; and Suite 4 BL-25, K, P, RT-13. Primer concentrations for each primer are listed in supplemental table 1.

Amplification of each suite was completed with the Qiagen® multiplex PCR kit by creating reactions of 5 μL QIAGEN Multiplex PCR Master Mix, 1 μL Primer Mix, 2 μL dH₂O and 2 μL of template DNA. Each suite followed the manufacturer's recommended amplification protocol of an initial heating of 15 minutes followed by 35 cycles at 94°C (30 sec), 60°C (90 sec), 72°C (90 sec), with a final hold of 72°C for 10 minutes using either a MJ Research PTC-200 or BioRad C1000. Resultant fragments were genotyped using GenomeLab™ GeXP genetic analysis system (Beckman Coulter, Brea, California). Each of the 22 sampling sites (counties) was treated as an individual population. Detection of possible genotyping errors or null alleles

was through MICRO-CHECKER (version 2.2.3; Van Oosterhout et al. 2004). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested in GENEPOP (Raymond and Rousset 1995) using 10,000 iterations.

The control region of the mtDNA (D-Loop) was amplified using the primers developed by Miyamoto et al. (1990) (accession ODOMTFVLA) which include F1 (5'-TCT CCC TAA GAC TCA AGG AAG-3') and R1 (5'-GTC ATT AGT CCA TCG AGA TGT C-3') using an initial heating of 94°C for 2 minutes followed by 41 cycles at 94°C (45 sec), 53°C (90 sec), 72°C (60 sec), with a final hold of 72°C for 5 minutes. The 699-bp fragment was sequenced using an Applied Biosystems™ 3130 XL genetic analyzer. Sequences were trimmed in BioEdit (Hall 1999) and aligned using ClustalW (Thompson et al. 1994) using 1000 bootstrap repetitions.

Genetic Analysis

To determine spatio-genetic differences between sampled counties, genetic differentiation between all sampling locations, measured as F_{ST} (Wright 1951), was calculated using the software FSTAT (version 2.9.3; Goudet 2001) with significance based on 1000 permutations and significance of each F_{ST} value corrected for multiple comparisons using a sequential Bonferroni correction (Rice 1989). In addition, a Mantel test was executed in R (Core 2015) to determine if genetic relatedness was significantly correlated to geographic distance (Euclidean distance) between sampling sites. Pairwise relatedness was calculated using the Queller and Goodnight (1989) statistic in the program SPAGeDi (Hardy and Vekemans 2002).

To determine the most likely number of populations K , the Bayesian software STRUCTURE (Pritchard et al. 2000) was used on the microsatellite data to determine likelihoods of possible values for $K = 1 - 24$ (Number of Sampled Counties +2) using ten iterations of possible values (100,000 Burn-ins and 100,000 Monte-Carlo Markov Chain

repetitions) with an alpha value of 0.2. The results were imported into STRUCTURE HARVESTER (Earl and vonHoldt 2012) to visually determine the most likely value of K by examining the $\ln$ likelihood of the number of populations and ΔK (Evanno et al. 2005) and the graphical results created through the program STRUCTURE PLOT (Version 2: Ramasamy et al. 2014). An iterative approach was taken to further define the population structure of each cluster at a finer scale (Vähä et al. 2007). Each grouping of K from the original determination was re-run separately through STRUCTURE (Pritchard et al. 2000) using the same parameters until $K = 1$. Furthermore, genetic clusters were identified through Discriminate Analyses of Principle Components (DAPC; Jombart et al. 2010); a Bayesian multivariate program that detects the number of clusters by accounting between group and within group genetic variation. The spatio-genetics software program GENELAND (Guillot et al. 2005) was used to determine the locations of genetic clusters. The most likely number of populations was determined through the highest log posterior probability and was visualized spatially using the coordinates of each individual and its respective cluster. Genetic differentiation due the regional sampling was accounted for by apportioning populations to their respective clusters determined through STRUCTURE (Pritchard et al. 2000) by conducting an AMOVA in Arlequin (Version 3.5.1.2; Excoffier and Lischer 2010). Significance was determined through 10,000 permutations.

Haplotypes of all individuals were calculated using TCS v 1.21 (Clement et al. 2000); however, haplotypes only occurring once were excluded from further analysis (Sumners et al. 2015). Pairwise F_{ST} scores for each sampled county were calculated in Arlequin (Version 3.5.1.2; Excoffier and Lischer 2010) with significance based on 10,000 permutations and significance of each F_{ST} value corrected for multiple comparisons using a sequential Bonferroni correction (Rice 1989). Mitochondrial clades were identified in the program NETWORK v.5.0.0.3 (fluxus-

engineering.com; Bandelt et al. 1999) by connecting haplotypes into networks through an iterative approach that maximizes the number of haplotypes included. Haplotypes that were separated by 5 or fewer mutational steps were grouped into clades (Summers et al. 2015).

Results

A total of 635 white-tailed deer tissues samples were collected in 22 counties (Figure 1). Although MICROCHECKER did not indicate the presence of null alleles, a homozygote excess was found, indicating the likelihood of unsampled populations. Additionally, the majority of loci significantly deviated from expectations of HWE after correcting for multiple comparisons using a sequential Bonferroni correction test, likely due to the presence of movement within our studied region (Supplemental Table 1). The majority of pairwise F_{ST} scores indicated significant differentiation across a broad landscape with the majority of clustered counties (Southeastern, Eastern, Western) being significantly different (Table 1). However, Summers County, located in the southeastern portion of West Virginia was not significantly different compared to any other county. Using the Queller and Goodnight (1989) r statistic, genetic relatedness between individuals significantly decreased with increasing distance ($P < 0.000$) indicating that relatives appear to be staying near related individuals (Figure 2).

After using a hierarchical approach to determine the number of populations in STRUCTURE, four populations ($K=4$) were determined to be the most likely number as a K of 4 had the highest log likelihood (Figure 3). Populations were largely concordant with the pairwise F_{ST} scores as genetic clusters were identified based on their geographic region location (Figure 4). Similar to the pairwise F_{ST} , Pocahontas County was significantly differentiated compared to all other counties with the exception of Summers County (Table 1). Similar to the STRUCTURE

results, DAPC identified a total of five populations (Figure 5) with the majority of population overlapping, indicating a small level of genetic admixture between populations (Figure 6). Spatial genetic analysis using the program GENELAND revealed a total of 10 populations with strong genetic distinction across a broad landscape scale (Figure 7) with all 13 samplings in the northwestern section grouping together as well as the majority of sampled counties in the southeastern and northeastern sections. Sample locations that differentiated from their regional grouping were on the outside edge of the sampling (Raleigh County, southeastern region; Hardy County, northeastern region), indicating the potential for isolation by distance. Areas on the map that are colored, but do not have samples in them are an artifact of the Poisson-Voronoi tessellation model by which the membership is assigned through a probability distribution that is both distributed uniformly and approximately centered between known polygons. However, only 1.9% ($P < 0.000$) of genetic variation within samples could be explained by region using an AMOVA with the remaining variation explained within sampled counties (Table 2).

A total of 88 mitochondrial haplotypes were identified. Haplotypes that only occurred once ($N=41$) were removed from further analysis. Mitochondrial pairwise F_{ST} scores were similar to the microsatellite data with the large majority of significant differentiation occurring between regions sampled as well as comparisons within regions (Table 3). However, Pocahontas County was not significantly different than any other county, likely due to the presence of a single haplotype. Additionally, the remaining 47 haplotypes were grouped into four clades. Similar the microsatellite analysis (i.e. pairwise F_{ST} , STRUCTURE, GENELAND), frequencies of clades were largely associated with the geographic region, with individuals in Pocahontas county having only one clade (Figure 8).

Discussion

The results of this study indicate a broad-scale level of genetic differentiation of white-tailed deer in West Virginia, which was largely explained between geographic regions. Similar genetic studies of white-tailed deer (DeYoung et al. 2003*a, b*, Sumners et al. 2015) found an absence of fine-scale spatial genetic structuring, but the presence of broad-scale genetic differentiation. The absence of fine-scale, nuclear genetic structure within a geographic range is not unusual as bi-parental inheritance provides a high level of contemporary variability especially considering the dispersal ecology of white-tailed deer. Dispersal rates of juvenile males ranged from 44% in Texas (McCoy et al. 2005) to > 80% in Illinois (Hawkins et al. 1971) and have been attributed to temporal influences including inbreeding avoidance, mate competition (Long et al. 2008) and orphaning (Hölzenbein and Marchinton 1992*a*). Additionally, juvenile male dispersal distances of white-tailed deer averaged between 3 km (western Virginia; Hölzenbein and Marchinton 1992*b*) and 38 km (eastern Illinois; Nixon et al. 1994). In contrast, low levels of natal dispersal ($\leq 3\%$) have been observed in female white-tailed deer (Hawkins et al. 1971, Nelson and Mech 1984, Aycrigg and Porter 1997), some studies have noted high rates of movement of individual females in both agricultural (Brinkman et al. 2005, Nixon et al. 2007), forested (Lutz et al. 2015), and mixed habitat (Oyer et al. 2007), which have been attributed to high deer density (Nixon et al. 2007, Lutz et al. 2015). The movement of both sexes in West Virginia (Wood, unpublished data) as opposed to a distinct sex-biased dispersal behavior (Greenwood 1980) likely explains the indistinguishable genetic structure within the large majority of geographic regions.

Similar to previous studies of white-tailed deer that found rivers to be barriers to white-tailed deer movement (Blanchong et al. 2008, Lang and Blanchong 2012), this study found an

absence of distinct dispersal barriers. Samples from Wood County were collected from Blannerhassett Island, located between West Virginia and Ohio within the Ohio River, during herd health collections. Although the 2.1 km² island is approximately 150 meters from the shoreline of Parkersburg, WV (30,600 residents, U.S. Census Bureau) and Belpre, Ohio (~6,000 residents, U.S. Census Bureau), previous observations of white-tailed deer indicated their strong swimming ability (Severinghaus and Cheatum 1956). Although rivers may serve as possible semi-permeable barriers for disease (Blanchong et al. 2008), individuals collected from Blannerhassett Island (Wood County) were genetically similar to those in the corresponding region. A single effective migrant per generation has been described as the minimal number of breeders needed to facilitate gene flow to amounts needed to homogenize the genetic structure (Wright 1931).

Although rivers were not dispersal barriers to movement between Blannerhasset Island (Wood County) and other counties, both the microsatellite analyses (STRUCTURE, GENELAND, F_{ST}) and mitochondrial analysis (single clade) indicated that samples taken from Pocahontas County were genetically distinct when compared to all other counties. Individuals in this county were sampled near the National Radio Astronomy Observatory (NRAO) in Greenbank, West Virginia. This location is 66 km from the next nearest sampling location (Braxton County) and 90 km and 95 km from the two closest sampling locations in two other sampling regions identified (Grant and Greenbrier counties respectively). Although white-tailed deer may make large dispersal movements in agricultural landscapes, dispersal movements are significantly less in forested regions (Hölzenbein and Marchinton 1992b, Long et al. 2005). Land cover is approximately 71% forested (coniferous, deciduous, mixed coniferous) in Pocahontas

County and thus the combination of high forest land cover and geographic distance may be contributing to genetic differentiation.

While STRUCTURE (Pritchard et al. 2000) identified four genetic clusters, the spatio-genetic program GENELAND (Guillot et al. 2005) identified 10 spatio-genetic clusters within the three broad geographic regions identified. Unlike GENELAND, which is based on a hidden partition model that divides the extent of the landscape sampled into the number of polygons, STRUCTURE identifies groups of individuals by maximizing Hardy-Weinberg Equilibrium and minimizing linkage disequilibrium. Although assigning individuals using STRUCTURE provides a powerful tool for genetic assignment (Berry et al. 2004), these methods are not as appropriate for discriminating either continuous and panmictic populations (Manel et al. 2005). Conversely, GENELAND has been documented to overestimate the number of distinct clusters (Chen et al. 2007). In our study, GENELAND identified individuals on the outside of geographic regions as being most likely as a separate cluster (Figure 5), which may be explained by non-random mating outside of the genetic neighborhood causing geographic distance (i.e., isolation by distance; Wright 1943).

The mitochondrial results are similar to Sumners et al. (2015) who observed geographic variation in the number of mitochondrial clades across southeastern states (Texas, Mississippi, Louisiana). Similar to the microsatellite results, the presence and percentage of clades were largely explained by geographic region (Figure 6). Similar to many regions, white-tailed deer in West Virginia (N = 2,307) were stocked from various and unknown sources between 1921 – 1977 (Allen and Cromer 1977) with both quantities and stocking years varying between all 55 counties. Remnant herds of white-tailed deer only remained in the eastern portions of West

Virginia, primarily within isolated pockets of Pocahontas, Greenbrier, Webster, Pendleton, Hardy, Hampshire, and Mineral Counties (Comer 1977).

Differences in the percentage of clades between regions sampled were similar to historic morphometric differences. Gill (1956) detected differences in hind foot length, weight following dressing (removal of intestines, organs, etc.) for both males and females in the age 0.5 and age 1.5 age class. Additional differences between regions were detected in antler beam diameter (males), as well as number of Corpora lutea for females of age 1.5 and 2.5. White-tailed deer within the western counties, which in our study encompassed 13 counties, had the largest hind foot length, heaviest dressed weight, largest antler beam diameter (males), and greatest average number of Corpora lutea. These morphometric differences may be the result of source herds, which likely explains differences in clade percentage between regions found in this study.

While we would expect to observe fine-scale differences within the regions studied due to their inheritance of mitochondrial DNA and female matrilineal relationships (Tiersen et al. 1985, Miller et al. 2010), hunted regions like those in this study may show different patterns of population structure. In a hunted region within the southeastern United States (Savannah River Site, South Carolina), Comer et al. (2005) observed a weak correlation between genetic and geographic distances, indicating an absence of spatial structure within females for both measures. Over time, dispersal behavior of female white-tailed deer, likely decayed the genetic patterns previously hypothesized.

While genetic differentiation could only be achieved across a broad-scale, a county-by-county genetic database was achieved for 40% of the counties (22 out of the 55 counties) in West Virginia. Unlike a database of mule deer (*Odocoileus hemionus*) within Alberta created by Jobin et al. (2008), in which animals were sampled from geographically distinct areas across Alberta,

Canada ($>661,000 \text{ km}^2$), our database is within an area only 10% of the size ($62,258 \text{ km}^2$), which allowed for greater confidence in distinguishing spatio-genetic regions. Additionally, the samples collected within our sample were geographically referenced to a 1-km^2 grid cell and not within a 100-km radius described by Jobin et al. (2008) and accurately represents a county by county database for calculation of probability of identity. This has already been used for several wildlife forensic cases for individuals purportedly harvested in West Virginia and resulted in a conservative estimated probability of identity for each case of 1 in 1 trillion.

Although the current database represents 22 of the 55 counties in West Virginia, the remaining 33 counties need to be sampled. From May 2018 until June 2018, the West Virginia Natural Resource Police officers have been collecting tissues from white-tailed deer involved in a vehicle collision within counties not previously sampled. The addition of these samples will not only provide an increase in the number of counties with a database, but will also provide an understanding of the spatial-genetic structure within the non-sampled area of the state. These data will be important for understanding the potential for parasite (e.g., deer ticks) or disease spread (i.e., chronic wasting disease). Additionally, while we observed genetic differentiation at a broad-scale, making the assumption that white-tailed deer in neighboring unsampled counties have similar allele frequencies reduces the robustness when calculating probability of identity. If allele frequencies are averaged across neighboring counties, this will reduce the frequency of local alleles and decrease the probability of identity, therefore creating a stronger, but false case against the defendant. However, the genetic admixture in our study reduces the likelihood that this phenomenon could occur given the assumption that a barrier is not found on the landscape.

Table 1. Microsatellite pairwise F_{ST} scores for each sampling location calculated by FSTAT version 2.9.3 (Goudet 2001). Pairwise F_{ST} values are above the diagonal and p-values are below the diagonal. Significant values are indicated by bold font. Significant values ≤ 0.00043 following sequential Bonferroni correction (Rice 1989). Sample numbers for each county are in parenthesis located within the top line of the table.

	Hampshire (N=60)	Grant (N=24)	Mineral (N=24)	Morgan (N=36)	Gilmer (N=28)	Doddridge (N=20)	Braxton (N=30)	Wood (N=16)	Marion (N=29)	Mason (N=28)	Putnam (N=29)	Upshur (N=30)	Harrison (N=30)	Tyler (N=30)	Wetzel (N=30)	Jackson (N=30)	Wirt (N=29)	Raleigh (N=27)	Pocohontas (N=16)	Monroe (N=30)	Summers (N=29)	Greenbrier (N=29)
Hampshire	0	0.0057	0.0098	0.0085	0.0186	0.0191	0.0194	0.0321	0.0205	0.018	0.0234	0.0199	0.0272	0.0271	0.0255	0.0242	0.0243	0.0186	0.0388	0.0266	0.0219	0.0269
Grant	0.02619	0	0.0019	0.0142	0.0181	0.0122	0.0107	0.0228	0.0123	0.0103	0.0176	0.0133	0.018	0.0198	0.0248	0.0203	0.0159	0.0177	0.0427	0.0228	0.0206	0.0238
Mineral	0.02121	0.63593	0	0.0146	0.0217	0.0099	0.0206	0.0233	0.0235	0.0186	0.0263	0.016	0.0198	0.0235	0.0327	0.0255	0.0181	0.0165	0.0291	0.0221	0.0238	0.0283
Morgan	0.00022	0.00043	0.00065	0	0.0132	0.0159	0.0109	0.0343	0.017	0.0206	0.0206	0.0157	0.0216	0.0212	0.0235	0.0181	0.0206	0.0179	0.0376	0.0371	0.0339	0.0382
Gilmer	0.00022	0.00022	0.00022	0.00022	0	0.0061	0.0013	0.0162	0.0099	0.0096	0.0021	0.0071	0.0107	0.0084	0.0131	0.0058	0.0093	0.0143	0.0375	0.0283	0.0288	0.0305
Doddridge	0.00022	0.03225	0.00433	0.00022	0.1829	0	0.007	0.0137	0.0032	0.0098	0.0048	0.0045	0.0032	0	0.0073	0.0104	0.0018	0.0141	0.0351	0.0321	0.0272	0.0433
Braxton	0.00022	0.01039	0.00022	0.00022	0.57229	0.21732	0	0.0189	0.0052	0.0179	0.0054	0.0035	0.0056	0.0103	0.0149	0.011	0.0088	0.0126	0.0346	0.0307	0.0225	0.0298
Wood	0.00022	0.00065	0.00043	0.00022	0.00043	0.01039	0.00022	0	0.024	0.0182	0.0175	0.0189	0.0178	0.0128	0.0231	0.0163	0.0157	0.0179	0.0489	0.0336	0.035	0.0434
Marion	0.00022	0.00043	0.00022	0.00022	0.00411	0.24675	0.04091	0.00022	0	0.0092	0.0054	0.0048	0.006	0.0077	0.0079	0.0113	0.0099	0.0199	0.0401	0.0413	0.0384	0.046
Mason	0.00022	0.00087	0.00022	0.00022	0.00455	0.0039	0.00022	0.00022	0.00303	0	0.0088	0.0111	0.0151	0.0084	0.0115	0.0133	0.0157	0.0237	0.0495	0.0339	0.0332	0.0346
Putnam	0.00022	0.00216	0.00022	0.00022	0.16126	0.19762	0.02706	0.00173	0.02641	0.05584	0	0.0025	0.0053	0.007	0.0068	0.0033	0.0088	0.0209	0.0431	0.0339	0.0373	0.044
Upshur	0.00022	0.00022	0.00022	0.00022	0.00108	0.01537	0.03831	0.00022	0.00022	0.00022	0.0461	0	0.0006	0.0015	0.0056	0.0038	0.0036	0.016	0.035	0.0257	0.0298	0.036
Harrison	0.00022	0.00022	0.00022	0.00022	0.00022	0.05736	0.05173	0.00022	0.00411	0.00022	0.02273	0.04242	0	0.0012	0.0088	0.0115	0.0039	0.017	0.0347	0.0322	0.0335	0.0389
Tyler	0.00022	0.00022	0.00022	0.00022	0.00043	0.27576	0.00498	0.00108	0.00411	0.00043	0.00519	0.01017	0.09416	0	0.0044	0.0062	0.0004	0.0202	0.0357	0.0351	0.0345	0.0379
Wetzel	0.00022	0.00022	0.00022	0.00022	0.00022	0.00693	0.00022	0.00022	0.00108	0.00022	0.00476	0.00043	0.00022	0.0132	0	0.0086	0.0091	0.024	0.0518	0.0405	0.0434	0.0551
Jackson	0.00022	0.00022	0.00022	0.00022	0.00065	0.00303	0.00022	0.00022	0.00043	0.00065	0.07446	0.00065	0.00022	0.00043	0.00022	0	0.0035	0.0179	0.0423	0.0317	0.0351	0.0398
Wirt	0.00022	0.00043	0.00022	0.00022	0.00519	0.27879	0.00216	0.00065	0.00303	0.00065	0.00433	0.00606	0.02316	0.13139	0.00043	0.1461	0	0.0126	0.0336	0.0318	0.031	0.0405
Summers	0.00022	0.00022	0.00022	0.00022	0.00022	0.00043	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0	0.0252	0.0137	0.012	0.0165
Pocohontas	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0	0.0524	0.0324	0.0489
Monroe	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00065	0.00022	0	0.0343	0.0089
Raleigh	0.02013	0.04481	0.11277	0.00368	0.06126	0.07316	0.02229	0.01861	0.00411	0.00952	0.01775	0.02727	0.01991	0.00887	0.00195	0.00823	0.05238	0.28333	0.01255	0.05238	0	0.0117
Greenbrier	0.00022	0.00065	0.00022	0.00022	0.00022	0.00022	0.00022	0.00043	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.00022	0.01126	0.00022	0.09091	0.47879	0

Table 2. Analysis of molecular variance partitioning white-tailed deer populations by groupings determined in STRUCTURE (Pritchard et al. 2000).

Source of variation	Sum of Squares	% of variance (total)	P-value
Among Regional Populations	59.78	1.94	0.0000
Within Populations	4218.96	98.06	
Total	4278.74	100.00	

Significance was determined through 10,000 permutations in ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010).

Table 3. Mitochondrial pairwise F_{ST} scores for each sampling location calculated by ARLEQUIN version 3.5.1.3 (Excoffier and Lischer 2010). Pairwise F_{ST} values are above the diagonal and p-values are below the diagonal. Significant values are indicated by bold font. Significant values ≤ 0.0004 following sequential Bonferroni correction (Rice 1989). Sample numbers for each county are in parenthesis located within the top line of the table.

	Hampshire	Grant	Mineral	Morgan	Gilmer	Doddridge	Braxton	Wood	Marion	Mason	Putnam	Upshur	Harrison	Tyler	Wetzel	Jackson	Wirt	Raleigh	Pocohontas	Monroe	Summers	Greenbrier
Hampshire		0.0197	0.0711	0.0722	0.1651	0.1581	0.1487	0.1287	0.1135	0.1257	0.1459	0.0987	0.1761	0.1260	0.1383	0.1264	0.1150	0.1035	0.1351	0.1776	0.1748	0.1293
Grant	0.1286		0.0812	0.1029	0.1851	0.1611	0.1510	0.1478	0.1394	0.1024	0.1105	0.1384	0.1366	0.1406	0.1514	0.1191	0.1375	0.1173	0.1667	0.1986	0.1954	0.1475
Mineral	0.0037	0.0212		0.1032	0.1234	0.1432	0.1331	0.0904	0.0836	0.0560	0.0555	0.0924	0.0736	0.1184	0.1298	0.0679	0.1129	0.0907	0.1250	0.1789	0.1756	0.1225
Morgan	0.0017	0.0006	0.0004		0.1633	0.1460	0.1356	0.0132	0.0930	0.1135	0.1331	0.0428	0.1672	0.1000	0.1201	0.0922	0.0621	0.0906	0.1053	0.1681	0.1649	0.1156
Gilmer	0.0000	0.0000	0.0030	0.0000		0.1687	0.1599	0.2303	0.0783	0.1245	0.1537	0.1104	0.1816	0.1493	0.1769	0.0957	0.1818	0.1370	0.2418	0.2339	0.2128	0.1843
Doddridge	0.0000	0.0002	0.0002	0.0000	0.0000		0.0000	0.2223	0.1001	0.1263	0.1388	0.1532	0.1698	0.0789	0.0890	0.1675	0.1562	0.0677	0.1985	0.2134	0.0436	0.1540
Braxton	0.0000	0.0000	0.0001	0.0000	0.0005	0.9999		0.2088	0.0929	0.1179	0.1311	0.1420	0.1598	0.0729	0.0828	0.1579	0.1467	0.0606	0.1765	0.2028	0.0417	0.1440
Wood	0.0263	0.0527	0.1557	0.3386	0.0106	0.0148	0.0128		0.1573	0.0873	0.0499	0.0626	0.0569	0.1772	0.2002	0.0218	0.0760	0.1652	0.3333	0.2606	0.2570	0.1991
Marion	0.0000	0.0000	0.0032	0.0005	0.0099	0.0019	0.0019	0.0231		0.0908	0.1076	0.0378	0.1333	0.0961	0.0442	0.0888	0.0922	0.0720	0.1385	0.1823	0.1522	0.1251
Mason	0.0000	0.0022	0.0303	0.0001	0.0003	0.0003	0.0002	0.1120	0.0005		0.0013	0.0967	0.0525	0.1086	0.1318	0.0559	0.0622	0.1059	0.1467	0.1867	0.1740	0.1289
Putnam	0.0000	0.0022	0.0287	0.0001	0.0002	0.0006	0.0008	0.1760	0.0000	0.3879		0.1165	0.0000	0.1402	0.1517	0.0144	0.0678	0.1195	0.1946	0.2103	0.1804	0.1593
Upshur	0.0000	0.0000	0.0021	0.0434	0.0020	0.0000	0.0000	0.1901	0.0488	0.0000	0.0001		0.1398	0.1164	0.1298	0.0686	0.0583	0.1008	0.1510	0.1885	0.1842	0.1378
Harrison	0.0000	0.0023	0.0225	0.0000	0.0001	0.0004	0.0004	0.1987	0.0001	0.0312	0.7406	0.0001		0.1726	0.1869	0.0197	0.1107	0.1522	0.2681	0.2473	0.2160	0.1998
Tyler	0.0000	0.0000	0.0001	0.0003	0.0000	0.0134	0.0136	0.0108	0.0000	0.0000	0.0000	0.0000	0.0000		0.0448	0.1407	0.1270	0.0940	0.1446	0.1854	0.1426	0.0930
Wetzel	0.0000	0.0000	0.0000	0.0000	0.0000	0.0043	0.0060	0.0040	0.0262	0.0000	0.0000	0.0000	0.0000	0.0274		0.1541	0.1335	0.0848	0.1552	0.1902	0.1559	0.1093
Jackson	0.0000	0.0011	0.0215	0.0022	0.0093	0.0000	0.0000	0.3368	0.0009	0.0200	0.1930	0.0075	0.1629	0.0000	0.0000		0.0327	0.1153	0.1767	0.2017	0.1987	0.1536
Wirt	0.0000	0.0000	0.0003	0.0134	0.0000	0.0000	0.0000	0.1639	0.0000	0.0088	0.0090	0.0134	0.0022	0.0000	0.0000	0.0836		0.1028	0.1508	0.1885	0.1803	0.1314
Raleigh	0.0000	0.0000	0.0000	0.0000	0.0000	0.0071	0.0124	0.0065	0.0005	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000		0.0767	0.0549	0.0763	0.0193
Pocohontas	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999		0.2267	0.2210	0.1299
Monroe	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0028	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0189	0.0804		0.2239	0.0027
Summers	0.0000	0.0000	0.0000	0.0000	0.0000	0.0678	0.0706	0.0016	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0044	0.2440	0.0000		0.1697
Greenbrier	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0065	0.0000	0.0000	0.0000	0.0000	0.0000	0.0012	0.0001	0.0000	0.0000	0.1398	0.2583	0.3667	0.0000	

Figure 1. Map of sampled individuals in 22 sampling locations in West Virginia (N=635).

Figure 2. Correlation of pairwise relatedness between individuals and Euclidean distance ($r = 0.008$, $P < 0.000$) to test for isolation by distance (Wright 1943).

Figure 3. Natural log of likelihood plotted against the number of possible populations K , and ΔK plot. Low variability between iterations provides support for four possible populations.

Figure 4. STRUCTURE plot after 100,000 MCMC using sampling locations as known priors, $K = 4$. Analyses reveal strong discontinuities among spatially distant samples.

Figure 5. Plot of Bayesian Inference Criterion versus the number of populations. The inflection at $K=5$ indicates the likelihood of five populations.

Figure 6. Discriminate Analysis of Principle Components (Jombart et al. 2010) when $K = 5$. Overlapping clusters with the exception of cluster 2 indicates low levels genetic admixture.

Figure 7. Locations of populations following identification of 10 population segments using the program GENELAND (Guillot et al. 2005). Each dot corresponds to an individual sample. Clustering was largely explained by geographic sampling region.

Figure 8. Map of West Virginia with percentage of each clade above the sampled county. Size of circle corresponds to the number of samples relative to the other sampled counties.

Supplemental Table 1. Deviations from HWE expectations following corrections for multiple comparisons (Rice 1989). Significant values ($P \leq 0.05$) are indicated by italicized font. Final primer concentrations of each locus and corresponding suite numbers.

Locus	HWE P-Value	Suite	Forward and Reverse Primer Final Concentration (μM)
OarFCB193	0.098	1	0.25
Cervid 1	<i>0.0000</i>	1	3.0
INRA011	0.297	1	0.5
BL42	<i>0.001</i>	1	0.5
N	<i>0.000</i>	2	0.5
RT-7	<i>0.004</i>	2	0.5
D	<i>0.000</i>	2	3.0
BM 6506	<i>0.000</i>	2	1.0
RT-5	0.600	3	1.0
O	<i>0.000</i>	3	2.0
Q	<i>0.000</i>	3	2.0
BM 6438	<i>0.000</i>	3	1.0
K	<i>0.000</i>	4	3.0
BL-25	<i>0.000</i>	4	2.0
P	<i>0.000</i>	4	2.0
RT-13	<i>0.000</i>	4	2.0

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Chapter 4

Using genetic relatedness to measure dispersal distance of white-tailed deer with Chronic Wasting Disease

Abstract

Understanding the dispersal patterns and distance of diseased individuals is critical for establishing management efforts to reduce further infection spread. In 2004, the prion associated with chronic wasting disease (CWD) was detected in a road-killed white-tailed deer in West Virginia and in 2006, CWD positive individuals were detected in hunter-harvested white-tailed deer. To determine the dispersal distance of 40 white-tailed deer (20 males, 20 females) testing positive for CWD, 559 CWD negative, female white-tailed deer (≥ 1.5 YOA) were sampled in Hampshire County, West Virginia from 2006 – 2014. Genotype profiles were generated using a suite of 16 microsatellite loci, and the control region of the mitochondria (D-loop) for each individual was sequenced. Pairwise relatedness was calculated between the 559 females and a focal individual testing positive for CWD and interpolated across the study region (Hampshire County). Areas of high genetic relatedness of the individual were identified and were compared to locations of mitochondrial haplotypes to determine the most likely natal region. Although dispersal distances of females (14.6 km) were not statistically different from males (16.1 km; $P=0.57$), the dispersal pattern of females contrasted that of males. Females dispersed into a management area in which deer density was reduced through “special collections” whereas males dispersed through an area of high CWD density ($>1 \text{ km}^2$), supporting previous studies which indicated that males are the dispersers of CWD prions. Management efforts should continue to reduce deer densities to decrease the likelihood of females dispersing into areas with high CWD density.

Introduction

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) first described in mule deer (*Odocoileus himionus hemionus*) (Williams and Young 1980). CWD has currently been detected in free-ranging populations of white-tailed deer throughout the mid-western United States (Wisconsin, Michigan, Minnesota, Missouri, Illinois, Iowa) and eastern states including Maryland, Virginia, West Virginia, Pennsylvania, and New York (USGS 2018). Once contracted, survival from CWD averages two years, but is nevertheless fatal (Williams et al. 2002, Belay et al. 2004). While infected survivors continue to shed the abnormally shaped prion protein (Prusiner 1982) through blood, saliva, urine and feces (Mathiason et al. 2006, Haley et al. 2009, Tamgüney et al. 2009), prions will adhere to soil particles (Johnson et al. 2006, 2007), especially clay soils (Johnson et al. 2007) and have been demonstrated to be taken up by some plant species (Rasmussen et al. 2014, Pritzkow et al. 2015). While disease prevalence is greater in males (Gear et al. 2006, Osnas et al. 2009) and increases with age (Miller and Conner 2005), the probability of infected females is not influenced by the number of nearby infected males, but rather by close relationships with female kin (Gear et al. 2010). Therefore dispersal of infected males is believed to be responsible for the natural spread of CWD across a broad-scale geographic area.

Dispersal of mammals has been described as the movement of individuals from their natal range to a different area outside of their initial range. For the majority of mammals, these movements are sex-biased (see review; Lawson Handley and Perrin 2007) and typically involve the dispersal of juvenile males (Greenwood 1980). Asymmetric sex dispersal, specifically male-biased dispersal, has been observed in white-tailed deer (*Odocoileus virginianus*) throughout their range. Dispersal rates of juvenile males ranged from 44% in Texas (McCoy et al. 2005) to >

80% in Illinois (Hawkins et al. 1971) and have been attributed to temporal influences including inbreeding avoidance, mate competition (Long et al. 2008) and orphaning (Hölzenbein and Marchinton 1992a). Average movement of individuals has been demonstrated to vary across the range. While Nixon et al. (1994) noted average dispersal distances of 36 km and 38 km in eastern Illinois and northern Illinois, respectively, studies in the eastern United States noted average dispersal distances between 3 km (Western Virginia; Hölzenbein and Marchinton 1992b) and 10 km (Rosenberry et al. 1999) with maximum distances between 7.1 (Rosenberry et al. 1999) and 56 km (Rosenberry et al. 1999) observed. Additionally, Long et al. (2005) observed average dispersal distances of 8 km in Western Pennsylvania (40.6 maximum dispersal distance), but noted that while habitat has not been recognized to influence dispersal rates of male white-tailed deer, a reduction in forest cover increased average and maximum dispersal distances (Long et al. 2005).

In contrast, low levels of natal dispersal ($\leq 3\%$) have been observed in female white-tailed deer (Hawkins et al. 1971, Nelson and Mech 1984, Aycrigg and Porter 1997), which could be attributed to mother-fawn social isolation from four to six weeks post-partum (Ozega et al. 1982). Female white-tailed deer commonly have overlapping home ranges formed by successive generations of females (Hawkins and Klimstra 1970, Tierson et al. 1985, Aycrigg and Porter 1997), creating a social structure analogous to that of a rose petal (Porter et al. 1991). While individual females appear to stay within the same social group (Tierson et al. 1985, Miller et al. 2010), with groups not overlapping with each other (Tierson et al. 1985), overlapping social groups have been observed in areas with high density populations (Miller et al. 2010). However, some studies have noted high rates of movement of individual females in both agricultural (Brinkman et al. 2005, Nixon et al. 2007), forested (Lutz et al. 2015), and mixed

habitat (Oyer et al. 2007) which have been attributed to high deer density (Nixon et al. 2007, Lutz et al. 2015).

Although dispersal rates and distances of both sexes has been documented across the range, understanding the dispersal patterns of white-tailed deer is important to manage for disease spread. Quantifying the dispersal distance of CWD positive white-tailed deer requires two major conditions to be satisfied: locations of both origin and conclusion, as well as a reliable test to detect prions associated with CWD. Previous movement studies of white-tailed deer in the eastern United States have used radio telemetry (Hölzenbein and Marchinton 1992*b*, Rosenberry et al. 1999, Long et al. 2005), with GPS radio telemetry being an additional viable option (Bowman et al. 2000). However, these types of studies are limited to a small number of collared individuals as well as conditions that affect geo-location (i.e., habitat) and battery life of the unit (Jiang et al. 2008). Additionally, these studies also require a reliable ante-mortem test when fitted for a telemetry unit, or post-mortem CWD positive test, therefore requiring that the collared individual was detected for CWD before the collaring or that the individual contracts CWD following collaring. Inherently, this reduces the probability of measuring dispersal distance of CWD individuals, especially when CWD prevalence is low. While progress has been made to detect CWD prions in urine and saliva ante-mortem (Henderson et al. 2013, John et al. 2013), the reliability of these tests is still less than that of accepted post-mortem tests (i.e., immunohistochemistry (IHC)) for CWD detection. Therefore, a balance is needed to assign dispersal distances of white-tailed deer through a broad-scale sampling effort, as well as have a reliable test for prion detection.

Genetic assignment has become a powerful and efficient tool to understand movement of individuals across a landscape (Manel et al. 2005) and to understand disease transmission

(Remais et al. 2011). Genetic assignment estimates the likelihood that an individual originated from the sampled population utilizing the genotypes of the sampled population as well as in the individual of interest. Green et al. (2014) sampled 10 locations of white-tailed deer across Illinois and assigned both male and female CWD positive and negative individuals to their natal locations using two assignment programs. Probability of assignment for individuals ranged between 66% and 100% using the program ONCOR (Kalinowski et al. 2007) with assignments being confirmed through the program GENECLASS2 (Piry et al. 2004). However, the reliability of genetic assignment is limited to populations in which genetic differentiation is high (Berry et al. 2004). Although some studies have indicated the presence of fine-scale population differentiation (Miller et al. 2010), other studies have indicated the absence of strong genetic differentiation of white-tailed deer across a broad scale (Cullingham et al. 2011), reducing the opportunity for robust assignment within a fine-scale.

Measures of genetic relatedness have also been used as assignment alternatives when genetic differentiation between populations is low. Parent-offspring assignment approaches have been used to understand movement in avian species (e.g. Peery et al. 2008). However, the use of sibship or parentage approaches requires a significant sampling effort to have adequate coverage of the large majority of parents. Similarly, kinship-based analyses have also provided a tool for assessing movement within a fine-scale. Using the family groups of individuals, the sibship based approach assesses dispersal through pedigree reconstruction (e.g. brook trout; Kanno et al. 2011, 2014, Whiteley et al. 2014). However, much like the pedigree reconstruction, sibship based analyses are limited to a single age-class and also requires the large majority of the population's genetic diversity to be sampled (Whiteley et al. 2014).

Alternatively, the use of an analogous measure of family structure, genetic relatedness, has been used to characterize population movement. Magle et al. (2013) used measures of pairwise relatedness to identify spatial overlap of white-tailed deer female social groups and found that highly related females overlapped. These periods of overlap and close contacts greatly increase the opportunity for CWD transmission between individuals. Measures of pairwise relatedness have also been used to find significant genetic discontinuities across the landscape. In a study of a continuous population of brown bears (*Ursus arctos*), Norman et al. (2017) were able to delineate significant areas of relatedness and characterize features on the landscape which limit dispersal. By using the landscape relatedness (LandRel) approach, which interpolates quantitative levels of relatedness across the region, areas of high pairwise relatedness of that individual can be identified, making this an appropriate method to assign CWD positive individuals to areas on the landscape with high levels of relatedness.

Methods

Study area

This study was primarily conducted in Hampshire County West Virginia (Figure 1) located in the eastern portion of the state. The majority of land cover for Hampshire County consists of a mixed deciduous forest (74%) with total forest (deciduous, mixed deciduous, coniferous) covering 81.2%. Pasture and cropland cover the remaining majority of the county (14.2%; 2011 National Land Cover Dataset, Homer et al. 2015). Historically (early 1900's) white-tailed deer were nearly extirpated in the state, with a small number of isolated populations believed to occur in both counties. Limited harvests in the 1920's and 1930's as well as stocking (N=23; Hampshire County; N=28, Hardy County) between 1930-1945 and between 1961-1965 (Hampshire County; Allen and Cromer 1977) improved herd numbers, which allowed for liberal

harvest numbers (2014, N=2,545; James Crum, West Virginia Division of Natural Resources, personal communication). However, in 2005, the prion associated with chronic wasting disease (CWD) was detected in a 2004 road-killed white-tailed deer in Hampshire County. In response to disease presence, the West Virginia Division of Natural Resources (WVDNR) instituted several management measures to decrease disease spread including special collections (e.g. sharpshooting) within areas having infected individuals from 2006 – 2011. Additionally, the WVDNR instituted a CWD containment area in Hampshire, Hardy, and Morgan counties that restricts the transportation of white-tailed deer carcasses and body parts as well as feeding and baiting throughout the year (West Virginia Division of Natural Resources 2017).

Sampling methods

Starting in 2006, in conjunction with WVDNR surveillance of CWD in hunter-harvested white-tailed deer, tissue samples consisting of ear notches or abdominal skin were collected, individually labeled, and stored in 95% ethanol for DNA analysis. Each individual deer was aged through tooth wear and replacement (Severinghaus 1949) and the hunter identified on a map the 1-km² grid cell from which the white-tailed deer was harvested. The centroid of the cell became the geographic location of the sample. Retropharyngeal lymph nodes were collected and tested for CWD using enzyme-linked immunosorbent assay (ELISA). Samples testing positive through ELISA testing were confirmed through immunohistochemistry (IHC). Collection of hunter harvested white-tailed deer samples occurred at WVDNR biological check stations in areas including and surrounding Hampshire County during a special 2-day antlerless deer season in October from 2006 through 2011 and annually during the first three days of firearms season (late November) from years 2006 through 2014. In addition to hunter harvest samples, special

collections consisting of sniper selected deer were also collected from 2006 – 2011 to reduce deer densities in areas with a CWD positive individual within Hampshire County.

A subsample consisting of female white-tailed deer was selected as dispersal rates and distances of females is considered to be less than males (Hawkins and Klimstra 1970, Tierson et al. 1985, Porter et al. 1991, Aycrigg and Porter 1997), especially for females ≥ 1.5 years of age (Miller et al. 2010). To avoid misassigning the spatial location of an individual where a grid cell encompasses both sides of a potential barrier to movement (Blanchong et al. 2008, Long et al. 2010, Robinson et al. 2012), a 1-km buffer was placed around major highways and major rivers. Samples within the buffer were excluded from sample selection. Finally, females testing positive for CWD were removed to avoid any variation in movement from disease symptoms. From the remaining samples, a minimum of one female from each grid cell was selected. In locations where grid cells have multiple samples, a single individual was randomly selected. Based on these criteria, a total of 559 female white-tailed deer tissue samples were selected in Hampshire County, WV (Figure 1). In addition to these samples, a random sample of 20 male and 20 female white-tailed deer which tested positive for the prion associated with chronic wasting disease was also included.

Laboratory Methods

Genomic DNA was extracted using the Wizard® SV-96 DNA purification system (Promega, Madison, WI) according to manufacturer's protocol. DNA was quantified using a spectrophotometer (NanoDrop, Wilmington, DE) and standardized to a concentration of 10 ng/ μ L. A suite of 16 highly polymorphic microsatellite loci was used to genotype individual white-tailed deer. Microsatellite loci included K, N, P, D, Q, O (Jones et al. 2000), RT-5, RT-7, RT13 (Wilson et al. 1997), BM 6506, BM 6438, BL42, BL25 (Bishop et al. 1994), Cervid 1

(DeWoody et al. 1995), OarFCB193 (Buchanan and Crawford 1993) and INRA011 (Vaiman et al. 1992). The sixteen loci were grouped into four different multiplex PCR primer suites with Suite 1 consisting of loci OarFCB193, Cervid 1, INRA011, BL42; Suite 2 consisting of D, BM 6506, RT-7, N; Suite 3 O, Q, F, BM 6438; and Suite 4 BL25, K, P, RT-13. The sixteen loci were grouped into four different multiplex PCR primer suites with Suite 1 consisting of loci OarFCB193, Cervid 1, INRA011, BL42; Suite 2 consisting of D, BM 6506, RT-7, N; Suite 3 O, Q, F, BM 6438; and Suite 4 BL-25, K, P, RT-13. Primer concentrations for each primer are listed in supplemental table 1.

Amplification of each suite was completed with the Qiagen[®] multiplex PCR kit by creating reactions of 5 µL QIAGEN Multiplex PCR Master Mix, 1 µL Primer Mix, 2 µL dH₂O and 2 µL of template DNA. Each suite followed the amplification protocol of an initial heating of 15 minutes followed by 35 cycles at 94°C (30 sec), 60°C (90 sec), 72°C (90 sec), with a final hold of 72°C for 10 minutes using either a MJ Research PTC-200 or BioRad C1000. Resultant fragments were genotyped using GenomeLab[™] GeXP genetic analysis system (Beckman Coulter, Brea, California).

The control region of the mtDNA (D-Loop) was amplified using the primers developed by Miyamoto et al. (1990) (accession ODOMTFVLA) which include F1 (5'-TCT CCC TAA GAC TCA AGG AAG-3') and R1 (5'-GTC ATT AGT CCA TCG AGA TGT C-3') using an initial heating of 94°C for 2 minutes followed by 41 cycles at 94°C (45 sec), 53°C (90 sec), 72°C (60 sec), with a final hold of 72°C for 5 minutes. The 699 basepair fragment was sequenced using an Applied Biosystems[™] 3130 XL genetic analyzer. Sequences were trimmed in BioEdit (Hall 1999) and aligned using ClustalW (Thompson et al. 1994) using 1000 bootstrap repetitions.

Genetic Analysis

Estimates of pairwise relatedness were calculated in the program SPAGeDI (Hardy and Vekemans 2002) using the Lynch and Ritland (1999) relatedness coefficient. The Lynch-Ritland coefficient is a regression based estimator that reduces the variances as increased marker loci are used and has been shown to outperform other estimators (e.g. Queller and Goodnight 1989) in several studies (Thomas 2005, Csilléry et al. 2006). The relatedness coefficient, or *r*-value, represents the percentage of shared genetic material that is identical between two individuals. A pair of identical twins would have an *r*-value of 1.0, whereas full-siblings and parent-offspring, should have an *r*-value of approximately 0.50. Additionally, half-siblings and grandparent-grandchild relationships would have an *r*-value of 0.25 and first-cousins a value of 0.125. Lynch-Ritland relatedness values with negative scores would indicate that individuals are less related than related. Interpolations of pairwise relatedness were conducted using integrated nested Laplace approximations (Rue et al. 2009) using the package INLA implemented in R (R Core Team 2013). Similar to Markov Monte Carlo methods used in other Bayesian assignment programs, INLA utilizes latent Gaussian model to approximate posterior margins within structured additive regression models.

Interpolations were carried out on each individual separately. Areas with the highest 1% of relatedness were identified in R (Figure 3), extracted, and the coordinates of each were imported into ArcGIS 10.2.2 (ESRI 2014). The spatial location of mitochondrial haplotypes were mapped in ArcGIS. A 2-km buffer around the relatedness areas was extracted. Haplotypes that matched the focal individual within the 2-km buffer were investigated to determine relatedness. The highest pairwise relatedness between any individual with the same haplotype was recorded for the subsample of 40 CWD positive white-tailed deer (20 males, 20 females).

The dispersal distances were measured by the Euclidean distance from the natal area identified to the harvest location.

To examine the influence of areas with high-CWD detection rates, the spatial location of each CWD positive white-tailed deer from 2006 – 2014 (N=160) was mapped in ArcGIS 10.2.2 (ESRI 2014) followed by a point density analysis. Areas with densities > 1 CWD positive individual per square kilometer were mapped. Through this analysis, a 43-km² high-density area was selected (Figure 2), which consisted of 97 CWD positive individuals. By identifying this area, individuals could be identified as a CWD disperser (carrying prion away from high density area) or newly infected CWD hosts (dispersing into high density CWD area).

Results

Individual genotypes consisting of 16 microsatellite loci and haplotypes from the mtDNA D-loop were generated from 559 female white-tailed deer using the selection criteria previously discussed as well as 40 CWD positive white-tailed deer that were identified through ELISA and IHC testing. Ages of CWD positive deer ranged between 1.5 YOA to 4.5 YOA with average ages of 2.45 and 2.51, for females and males, respectively ($P = 0.834$). Dispersal distances ranged between 4.4 km (female; 1.8 YOA) to 42.4 km (male; 1.5 YOA). Female dispersal distances averaged 14.6 km and males 16.1 km ($P = 0.573$).

The majority of CWD positive individuals had natal regions outside of the CWD high density area, with the majority of individuals dispersing within or adjacent to this area (Table 1, Figure 4). Only 30% of females had natal regions within or closely adjacent to the high density CWD area, with the majority of individuals dispersing into the area. Similarly, four males were assigned to natal areas within or nearly adjacent to the high-density CWD area, but were

harvested outside the area (Figure 5). Of the 40 individuals sampled, similar haplotypes were identified within 2 – km for 78% of the areas identified. The r value for individuals with the same haplotype and within a 2 – km buffer ranged between 0.011 to 0.52 (Table 1).

Discussion

The results of this study indicate that levels of genetic relatedness of a highly mobile species can be detected across a discrete landscape and contemporary time period. While this study used a similar method (LandRel) as Norman et al. (2017) to model levels of relatedness across the study area, our study further used the method to assign an individual to their natal region based on their pairwise relatedness. Although this study used females as a foundation to detect pairwise relatedness with the assumption of the non-dispersing female white-tailed deer (rose-petal hypothesis; Porter et al. 1991), average female dispersal distances of 14.6 km were not significantly different from male dispersal distance (18.08 km). This result is not unlike other studies that found similar movements of females. Nelson and Mech (1984) recorded dispersal distances between 17.6 – 168 km in northeastern Minnesota. Additionally, outside of fawn-mother relationships, Kelly et al. (2010) found positive spatial autocorrelation up to 48 km, indicating that while female kin are likely close, matrilineal relationships can be maintained at far distances. Additionally, Oyer et al. (2007) documented movement of a single female in southern Wisconsin and found dispersal and establishment of ≥ 2 new home ranges 35 km and 41 km beyond that of her natal range. While the distance of females dispersal was not statistically different from that of males, the majority of females that were harvested within the CWD high density area dispersed within or from outside that region. For several years, the density of females within this area was reduced through “special collections” (James Crum, West

Virginia Division of Natural Resources, Personal Communication). While a decrease in forest cover has been an indicator of dispersing distances of white-tailed deer (juvenile males; Long et al. 2005, Skuldt et al. 2008), an increase of deer density has resulted in an increase in the home range size of white-tailed deer (Kilpatrick et al. 2001). Therefore, by reducing the density of white-tailed deer within the region, females likely dispersed into the area as home ranges became available to inhabit.

Although there was a significant effort in sampling white-tailed deer in Hampshire County, WV, this limited the opportunity to assign individuals that could have dispersed from outside of Hampshire County. Although natural and unnatural landscape factors can influence white-tailed deer movement (Lang and Blanchong 2012, Robinson et al. 2012), the absence of a strong dispersal barrier influences the scope of the study beyond that which was sampled and the applicability of this method to understand dispersal distance. However, while it is likely that individuals dispersed from regions outside of Hampshire County (i.e., natal region was outside the county and they moved into the CWD area), this method can be used to exclude the possibility that the natal area of the individual is within or adjacent to the high density CWD area. Understanding dispersal direction helps to understand the contribution of high CWD prevalence to individuals outside of these areas. In this study, females were more likely to be affected by the high density CWD area and were detected within the CWD area with a single female dispersing 16.1 km from her natal area adjacent to the high density CWD region. Although these highly mobile females pose a threat to future spread, it is the males who have been documented to travel distances greater than 100 km (Kelly et al. 2010). Additionally, marking the territory of males (i.e. ground scrapes, tree rubs; Marchinton and Hirth 1984) results in the spreading of biological material that contains prions including urine (Haley et al. 2009,

John et al. 2013, Henderson et al. 2015) and antler velvet (Angers et al. 2009). Because these prions withstand the large majority of environmental conditions with bacterial enzymes only being relatively effective at both high pH (10 – 12) and high temperatures (50 – 60°C), these prions are likely to remain effective after infecting the environment (McLeod et al. 2004, Yoshioka et al. 2007).

Although our study examined dispersal distance and direction of white-tailed deer, this method can be used for other populations to understand dispersal movements. Norman et al. (2017) empirically tested this program to detect population level changes in Scandinavian brown bears (*Ursus arctos*) to identify features that contribute to population differentiation. In addition to the utility for different species that exhibit dispersal, this method also has the advantage of not needing to conform to expectations of Hardy-Weinberg equilibrium (HWE) including the absence of migration. As previously discussed and found in this study, populations of white-tailed deer violate this Hardy-Weinberg assumptions due to high dispersal of both sexes' (Hawkins et al. 1971, Nelson and Mech 1992, Long et al. 2005, 2008, McCoy et al. 2005, Kelly et al. 2010, Green et al. 2014). This continuous genetic admixture reduces the power and reliability to assign individuals to a particular area (Berry et al. 2004). Additionally, genetic relatedness is less effected to other violators of HWE including homozygous excess caused by marker reliability (e.g., null alleles).

This study was also able to combine the use of a contemporary genetic marker (microsatellites) with a historical marker (mitochondrial DNA). The use of this approach helps in identifying the most likely natal area by identifying areas of relatedness with the same haplotypes, but this approach is limited to maternal lineage. However, when multiple areas of

high relatedness are identified in a study, the use of this tool aids in final selection of a natal region.

Management Implications

The identification of natal dispersers is a key element in describing dispersal patterns of white-tailed deer. The majority of white-tailed deer dispersed from outside a high-density CWD (>1km) area, whereas a large number of individuals dispersed into this area, or a region around it. Prior management actions have used “Special Collections” to remove individuals in areas around detections to limit the number of contacts between infected and non-infected individuals. In a study of where government agency sharpshooting management actions occurred, CWD prevalence did not increase (Manjerovic et al. 2014) and prevalence in young and females significantly decreased (Mateus-Pinilla et al. 2013). However, in our study, females likely dispersed into the area due to the decrease in deer density. Therefore localized culling of cervids herds may serve as an effective tool for reducing spread to other regions (Cullingham et al. 2011), but may provide sinks to infect new migrants. In addition to reducing deer density, hunting activities within this region need to continue to reduce deer density across the county and the surrounding areas to limit dispersal of individuals away from their natal area and into areas with high CWD prevalence. However, other states (e.g., Wisconsin) that have enacted liberal hunting regulations as well as herd reduction strategies in areas of a CWD detection lost the trust of the public, resulting in an ill perception of the management actions limits (Heberlein 2004). In addition to localized culling strategies, Potapov et al. (2016) evaluated six harvest strategies that have been used to control CWD prevalence in deer through modeling previously developed by Potapov et al. (2013). The modeling accounted for age of males, density dependent effects, intensity and preferences of hunter harvests, as well as temporal differences in infection

rates. Following parameterization, intensity of harvest was shown to reduce disease prevalence the greatest as well as a non-age bias in harvesting male white-tailed deer (Potapov et al. 2016). However, Riley et al. (2003) found that hunters were unable to significantly reduce the number of deer, therefore a balance is needed between public perception of management strategies and the effectiveness of those strategies for controlling the spread of infectious prions.

Table 1. Biological data for each individual as well as distance traveled from natal region to harvested location. Highest relatedness value indicates individual with same mitochondrial haplotype within 2 km of region identified. The absence of a relatedness value (N/A) indicates that no mitochondrial haplotype was sampled within a 2 km buffer.

Individual	Age	Sex	Distance Traveled (km)	Highest Relatedness Value
12WV20275414	1.5	Male	36.9	0.52
10WV20271230	2.5	Male	14.2	N/A
14WV20277040	1.5	Male	42.4	0.08
09WV20278636	2.5	Male	24.2	0.12
08WV20276247	2.5	Male	7.9	0.36
12WV20275023	3.5	Male	12.8	0.11
11WV20273546	2.5	Male	9.4	N/A
09WV20279590	2.5	Male	16.4	0.12
10WV30273271	2.8	Male	5.5	0.011
13WV20276743	3.5	Male	11.1	0.16
13WV20276732	2.5	Male	2.3	0.17
13WV20276558	3.5	Male	19.1	0.24
13WV20276557	3.5	Male	15.8	0.20
13WV20276730	1.5	Male	15.1	0.07
10WV20271086	1.5	Male	16.5	0.20
14WV20277739	2.5	Male	13.2	0.17
09WV20278450	3.5	Male	8.5	N/A
08WV20272761	2.8	Male	7.1	0.13
06WV90270408	0.8	Male	24.9	0.10
06WV90270325	2.8	Male	18.5	0.27
10WV20270770	2.5	Female	16.1	0.21
10WV30273252	1.8	Female	17.5	0.08
08WV30272940	2.8	Female	21.3	N/A
08WV20276002	4.5	Female	13.8	0.24
07WV20273755	2.5	Female	13.1	0.21
13WV20276752	3.5	Female	18.9	N/A
13WV20275557	1.5	Female	8.2	0.02
11WV20273647	3.5	Female	6.2	N/A
13WV20275705	2.5	Female	34.8	0.04
10WV20270770	2.5	Female	16.1	0.21
08WV30272740	1.8	Female	4.4	N/A
09WV30273057	0.8	Female	12.6	0.02
10WV30273275	2.8	Female	5.6	0.20
08WV30272744	2.8	Female	14.5	0.03
10WV30273249	2.8	Female	19.5	N/A
11WV20273562	1.5	Female	14.7	0.20
13WV20276699	1.5	Female	15.0	N/A
09WV20279113	2.5	Female	18.5	0.40
10WV30273251	3.8	Female	11.2	0.09
09WV30273037	2.8	Female	13.6	0.36
08WV30272921	0.8	Female	10.7	0.06

Figure 1. Map of female white-tailed deer sampled (N=559) in Hampshire County, West Virginia.

Figure 2. Map of Hampshire County with darkened area indicating >1 CWD positive individual per km². Area selected is approximately 43 km² and includes 97 CWD positive individuals.

Figure 3. Map of relatedness pairwise relatedness values interpolated across Hampshire County using LandRel (Norman et al. 2017) for individual 09WV20278636. Areas of red indicate likely natal region. Black dots indicate highest 1% of relatedness values. Spatial coordinates of these areas were extracted and mapped to determine spatial distance as well as direction of positive individual.

Figure 4. Map of 10 female individuals natal location and harvest location. Individuals have corresponding colors with closed circles indicating natal area identified by relatedness and open circles indicate harvest location.

Figure 5. Map of 10 male individuals natal location and harvest location. Individuals have corresponding colors with closed circles indicating natal area identified by relatedness and open circles indicate harvest location.

Supplemental Table 1. Final primer concentrations of each locus and corresponding suite numbers.

Locus	Suite	Forward and Reverse Primer Final Concentration (μM)
OarFCB193	1	0.25
Cervid 1	1	3.0
INRA011	1	0.5
BL42	1	0.5
N	2	0.5
RT-7	2	0.5
D	2	3.0
BM 6506	2	1.0
RT-5	3	1.0
O	3	2.0
Q	3	2.0
BM 6438	3	1.0
K	4	3.0
BL-25	4	2.0
P	4	2.0
RT-13	4	2.0

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Chapter 5

Environmental and temporal variables that contribute to DNA degradation in white-tailed deer blood

Abstract

Crimes against culturally and economically important wildlife vary both spatially and temporally across a landscape. While officer response times to these crimes and ensuing collection of evidence may also vary spatiotemporally, exposure of evidence to environmental factors including changing temperatures and UV radiation may degrade evidence below qualities and quantities suitable for DNA analysis. Additionally, materials from which evidence is collected may contribute to DNA degradation. Aliquots of blood from white-tailed deer were exposed in a 35°C incubator for 24 and 48 hour time periods to determine the effects of heat on DNA quantity. An additional study was conducted to test for degradation of DNA due to UV radiation. A 40 µL volume of blood (equivalent to 1 drop) was placed on separate pieces of 6.36 mm plastic and 1.6 mm steel and exposed to UV light (8.15 mW/cm^2) for 1- and 2- hour time periods. Genomic DNA from each exposed sample and a non-exposed control was extracted and purified using a Qiagen DNeasy blood and tissue kit. DNA quantity was measured through Real-Time PCR using a white-tailed deer microsatellite primer (OarFCB193). Analysis through a one-way ANOVA indicated that both exposure to high temperatures and substrate significantly affected DNA quantity ($P=0.000$, $P=0.0119$, respectively); however, exposure time had no influence on DNA quantity ($P=0.444$). Samples exposed to UV radiation were not significantly affected by exposure ($P=0.785$). Additionally, although substrate type did not significantly influence DNA quantity ($P=0.258$), an increase in exposure time significantly decreased DNA quantity ($P=0.018$). Results from this study indicate that exposure to the environment can reduce the quantity

of DNA; however, these quantities were large enough to be amplified through qPCR. Continued testing with different environments including high humidity is needed to determine potential interacting effects on biological materials.

Introduction

Poaching, defined as the intentional (Muth and Bowe 1998) and illegal taking of flora and fauna (Musgrave et al. 1993), is both an international and domestic concern for the conservation of species. Although these crimes are considered “victimless” as they do not directly harm the lives of humans, poaching of rhinos, elephants, tigers, and gorillas, which are listed as endangered, are common targets within the international wildlife trade of species due to monetary worth in worldwide markets. Within the United States, millions of dollars are earned annually from poaching (Musgrave et al. 1993); however, between \$10 and \$20 billion USD are earned annually from international trade of wild species (Wylar and Sheikh 2008, World Wildlife Fund 2017). In addition to a financial incentive, additional motivations for poaching include household consumption, recreational satisfactions, trophy poaching, thrill killing, protection of self and property, rebellion, traditional right of use, disagreement with regulations, and gamesmanship (Muth and Bowe 1998). For these many reasons, poaching continues to persist in an advanced, industrial society (Muth 1998), especially for those seeking a trophy individual where larger individuals contribute to a perceived increased social status (Eliason 2012).

Legal protections of wild flora and fauna exist at the local, national (e.g., Lacey Act of 1900, Endangered Species Act of 1973, Marine Mammal Protection Act of 1972), and international level (e.g., Convention on International Trade in Endangered Species of Wild

Fauna and Flora (CITES)) including laws directed towards specific species or groups thereof (e.g., migratory birds) (see review; Wallace and Ross 2012). However, while these policies exist, industrialized countries including the United States, China, Australia, and many of those within the European Union report the highest number of illegal wildlife seizures (United Nations Office on Drugs and Crime 2016). Unlike human crimes in which eyewitness often aid in the identification of those involved, wild species cannot provide an account of the crime(s). However, an exchange of materials between the individuals, location, and items can provide evidence of the crime and those involved (i.e., Locard's exchange principle; Locard 1931).

Scientific methods to investigate crimes against wildlife parallel that of human forensics including the fields of entomology, pathology, hair morphology, toxicology. In addition to these fields, applying genetic tools to wildlife forensic questions (i.e., birds; Burke and Bruford 1987) began shortly after the application to human forensics (Gill et al. 1985). Conservation groups including state agencies, universities, and federal entities (e.g., United States Fish and Wildlife Forensic Laboratory), as well as international non-governmental organizations including TRAFFIC (the wildlife trade monitoring network) and TRACE (Tools and Resources for Applied Conservation and Enforcement) provide tools for the investigation, prosecution, and conviction of individuals violating wildlife law. Additionally, international non-governmental organizations including the Society for Wildlife Forensic Science (SWFS) and the International Veterinary Science Forensic Science Association (IVFSA) have provided ethical guidelines for investigating wildlife crimes.

While authorities may not witness the poaching event, collecting biological evidence including hair, blood, and other tissue that may be produced during the crime can be used to determine the sex (Shaw et al. 2003, Lindsay and Belant 2008), match the identity of an

individual animal (e.g., white-tailed deer; Anderson et al. 2002), and genetically designate individuals to their most likely natal region through genetic assignment (e.g., Sardinian mouflon; Lorenzini et al. 2011). While collection of biological evidence can aid in linking the crime scene to weapons used, vehicles, accomplices, or storage of the animal or its part, environmental variables may degrade biological material and hinder downstream DNA processes. UV radiation can fuse pyrimidine bases adjacent to each other, causing an absence of DNA primer attachment and subsequent amplification of the desired region. Additionally, while DNA is stable at high temperatures as evident by PCR in which DNA is heated to 95°C, covalent bonds in high molecular weight DNA (100 – 200 kb) are more likely to be broken at higher temperatures, especially when exposed for long periods of time (Lahiri and Schnabel 1993).

White-tailed deer (*Odocoileus virginianus*) are both an economically and intrinsically valued species in the United States with nearly 11 million hunters pursuing white-tailed deer annually, resulting in a contribution of \$40 Billion USD to the economy (Southwick Associates 2012). The white-tailed deer's present population in West Virginia serves as a plentiful resource, supporting over 350,000 annual hunting license holders in the state (roughly the equivalent of one hunter per family). Estimates of the contribution of hunting activities to West Virginia's economy are \$290 million annually, creating thousands of local jobs that are crucial to rural communities within the state (Southwick Associates, 2012). However, with the ease of white-tailed deer harvesting and transport, poaching of white-tailed deer, which includes the illegal hunting, harvesting and capture of wild individuals, is a massive problem that is currently being faced by the West Virginia Division of Natural Resources Natural Resource Police (WVNRPP). According to the WVNRPP, as of 1999 there have been 5,368 prosecutions of illegal possession of wildlife and an additional 1,798 cases of illegal killing or taking of white-tailed deer, turkey,

and or boar (Lt. Col. Trader, West Virginia Natural Resource Police). Furthermore, in addition to vacancies within the WVNRP (approximately 20% of counties currently have vacancies), the WVNRP like other state conservation officers, are responsible for civilian police tasks including drug and alcohol related duties (Falcone 2004). Therefore, the response times to wildlife crimes become inherently longer, increasing the likelihood that biological evidence will be degraded. The objectives of this study were to (1) determine if environmental conditions including temperature and UV-light contribute to DNA degradation in white-tailed deer blood and to (2) determine if time of exposure contributes to DNA degradation.

Methods

Whole blood from recently euthanized white-tailed deer was collected and stored in EDTA K2 blood tubes at 4°C. After sterilization with 10% bleach, 40 µL of blood (equivalent to one droplet) was placed on 1.65 mm thick steel plate and 6.36 mm thick polyethylene composite, which are common materials in pickup truck beds. Materials with blood droplets were placed in a 35°C incubator for 24 and 48 hour time intervals. Following exposure, samples were removed with a sterilized razor blade and stored in a 1.5 mL tube at room temperature until extraction. Additionally, a 40 µL sample of blood of each individual not exposed to environmental conditions, was placed in a 1.5 mL tube to serve as a positive control as well as an empty 1.5 mL tube (negative control). Blood droplets from the same individuals were exposed to high temperatures an additional two times for a total of three separate exposure trials. To determine if UV light affected the quantity of DNA, 40 µL of blood was placed on 1.65 mm thick steel plate and 6.36 mm thick polyethylene composite under a UV light (8.15 mW/cm^2) for 1 and 2 hour time periods at room temperature (22.2° C). Following UV-light exposure, samples were removed with a sterilized razor blade and stored in a 1.5 mL tube at room temperature until

extraction. Similar to the temperature tests, a 40 μ L sample of blood not exposed to environmental conditions was placed in a 1.5 mL tube to serve as a positive control as well as an empty 1.5 mL tube (negative control). Blood droplets from the same individuals were exposed to high temperatures an additional two times for a total of three separate exposure trials.

Genomic DNA from each sample was extracted and purified using a Qiagen DNeasy® blood and tissue kit following manufacturer's protocol. Total genomic DNA from all samples was eluted using 200 μ L of elution buffer. DNA was amplified using 10 μ L reactions consisting of 0.3 μ M OarFCB193 forward primer (Buchanan and Crawford 1993), a microsatellite primer designed for ungulate species including those in the cervidae family that has a base pair range of 93-131. Additionally, 5 μ L ThermoFisher Scientific SYBR® Green PCR Master Mix and 2 μ L DNA template were included. DNA quantity was measured using the Bio-Rad CFX Connect™ Real-Time PCR instrument using an amplification protocol with an initial heating of 95° C for 3 minutes and then continued with 36 cycles of 95° C (10 sec) and 57° C (30 sec). Differences in quantity were measured by the quantification cycle (Cq) which is identified by the cycle number in which amplification exceeds baseline instrument fluorescence (i.e. background noise). A one-way ANOVA was used in the program R (Core, 2015) to determine significant effects of exposure groups, materials, and time of exposure on the number of cycles (Cq).

Results

During exposure, all samples dried completely on the material regardless of exposure method or time exposed. Using a one-way ANOVA, the effect of high temperature had a

significant effect ($P < 0.000$) on the number of cycles (Cq) as well as a significant difference between the substrate used ($P = 0.019$) for the temperature trials. However, the amount of time exposed did not have significant differences in DNA quantity ($P = 0.444$). Unlike the temperature trials, exposure to UV-light for 1 and 2 hour time periods did not have significantly affect DNA quantity (Cq; $P = 0.785$). Additionally, while the substrate did not significantly affect DNA quantity ($P = 0.258$), exposure time significantly affected DNA quantity ($P = 0.018$).

Discussion

Following exposure to environmental conditions (high temperature, UV-light) that likely emulate extreme natural conditions compared to those likely be found given the time of day and year that the majority of white-tailed deer poaching events occur (Haines et al. 2012), significant Cq differences were detected between the control group and the majority of exposure groups (80%; Table 1) when exposed to elevated temperatures. Unlike other studies that identified compounds in blood (heme; Akane et al. 1994, hemoglobin; Al-Soud and Rådström 2001) to inhibit PCR amplification, all samples were able to be amplified after exposure. Although samples exposed to high temperatures for periods of 24 and 48 hours had significant differences when compared to the control group, only one sample exposed to UV-light conditions that emulate peak radiation (Sample 1; Table 2) had a significant difference between the control. Previous studies using environmental DNA (eDNA) found that UV light contributed to DNA degradation if stored in a liquid form (Strickler et al. 2015). Similarly, Hall et al. (2014) found that UV-B radiation (wavelength, 280-315 nm) affected bloodstained DNA, likely due to UV energy absorption and transfer to cell structures via the reactive oxygen species (ROS) causing mutagenic affects including lesions (Freidberg et al. 2006). However, microsatellite profiles constructed using dehydrated DNA, similar to the conditions following the blood droplet drying

on the material, were not affected by UV-A or UV-B radiation (Hall et al. 2014), with hydrolytic degradation not occurring until 24 days at 65° C (Marrone and Ballantyne 2010).

While there were significant differences between the majority of samples with the temperature trials, a significant difference between material types was found at the 48 hour time interval for the temperature exposure trials. Amplification differences at 48 hours of samples between the two materials may be due to differences in thermal conductivity between the two materials. However, given that all samples were completely dried, the additional heat conductance of the steel likely would not have affected the sample after drying. In a study of thermal degradation of DNA, Karni et al. (2013) found that complete degradation of DNA in dry conditions did not occur until 190° C. Additionally, although DNA is thermally stable as evidenced by polymerase chain reactions (Mullis et al. 1986), however is less stable in long-term aqueous conditions (Karni et al. 2013). Most commonly, the lesions formed when DNA nucleotides are damaged through various methods causes the absence of PCR primer binding and successful strand replication (Pfeiffer et al. 1999, Hoff-Olsen et al. 2001). Additionally, smaller fragments create false microsatellite genotype profiles during electrophoresis, therefore creating an incorrect profile.

When UV-light was exposed to samples on the two material types (steel, plastic), there was not a significant difference between material types given the two different time periods used. Although UV radiation can fuse pyrimidine bases adjacent to each other, causing an absence of primer attachment and subsequent DNA amplification, rapid drying of DNA can block the progression of DNA degradation (Freidberg et al. 2006), likely due to the absence of oxidative environment that drying out of the blood sample provided as well as the natural anoxic conditions of the nucleus. However, reflection of energy is greater in steel material (grey/silver

color) compared to that of a black polyethylene material that is found in a truck bed liner and thus may be contributing to the increased DNA degradation. The absorption of the energy (heat) rather than the transferring of energy to the blood sample from the plastic material likely reduces the likelihood of damaging the pyrimidine bases in the DNA.

Although this study used a negative control throughout the DNA extraction, purification, and amplification steps, the methods did not utilize a Universal DNA spike to allow for the detection of inhibitors during the extraction and any potential losses of DNA during the experiment. However, the use of multiple samples (individuals) within experiment as well as amplification of all samples regardless of exposure type reduces the possible inaccuracy caused by the absence of this step.

While we emulated two environmental conditions that affect DNA quantity, this experiment did not consider other natural conditions that may or may not affect DNA quality and quantity. Bacteria, which are naturally occurring in the environment (i.e., soil), may contaminate a blood sample due to the presence of exogenous nucleases (Lindahl 1993). In addition, the presence of bacteria may amplify during PCR and thus reduce the quality of the PCR product. However, in our study, each sample was completely dried between 10 minutes and 30 minutes within our normal laboratory conditions (32% relative humidity), which limits the opportunity for bacteria to colonize onto the biological material. Further PCR inhibitors that occur naturally include humic acids that occur in both soil and plant materials (Tsai and Olson 1992, Watson and Blackwell 2000) as well as low pH (Strickler et al. 2015). Although testing of these conditions would be useful in determining further contributors of DNA degradation, these conditions are difficult to replicate in the field due to ever changing weather scenarios.

Although we exposed DNA to conditions outside the normal range (high UV, high temperature) which would be expected for white-tailed deer poaching events (Haines et al. 2012), amplification of DNA templates following exposure is encouraging for future cases involving genetic analysis. Crimes occurring in remote areas may necessitate longer response times for investigation of wildlife crimes (Pendleton 1996), especially considering that the large majority (97%) of the United States is rural (U.S. Census Bureau 2016). In addition to a small number of conservation officers per hunters (1:10,000; Tobias 1998), some agencies are broadening the responsibilities of conservation officers to include responsibilities considered for civilian police (Falcone 2004), resulting in longer response times to wildlife crimes and reducing the opportunity for collecting biological evidence.

Table 1. ANOVA results of C_q, a measurement of detection for real-time PCR, for samples exposed to high temperature (35° C) after three replicates for each sample. * indicates significant difference ($P < 0.05$).

	Sum of Squares	df	F Value	P Value (Pr>F)
Treatment	65.9	1	38.017	0.000*
Substrate	11.1	1	6.431	0.0119*
Time	1.0	1	0.589	0.444

Table 2. ANOVA results of C_q, a measurement of detection for real-time PCR, for samples exposed to UV light (8.15 mW/cm²) after three replicates for each sample. * indicates significant difference ($P < 0.05$).

	Sum of Squares	df	F Value	P Value (Pr>F)
Treatment	0.10	1	0.075	0.785
Substrate	1.64	1	1.288	0.258
Time	7.25	1	5.702	0.018

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