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Genetic variation and leprosy distribution in nine-banded armadillos

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GENETIC VARIATION AND LEPROSY DISTRIBUTION

IN NINE-BANDED ARMADILLOS

By

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Genetic, ecological, and environmental variation within and among populations of hosts and pathogens can influence local susceptibility of hosts and transmission rates of pathogens. These complex interactions can lead to geographic variation in the prevalence of pathogens. The interaction between leprosy and nine-banded armadillos in the southeastern U.S. provides an opportunity to examine the genetic, ecological, and environmental factors contributing to the variation in pathogen prevalence on a large geographic scale. Using genotypic data for loci associated with the immune response to leprosy and for anonymous loci, we have assessed the population structure of armadillo populations across the southern U.S. Additional statistical tests were used to assess any significant differences between functional and anonymous loci that indicate selection. Our results suggest that local adaptation does not influence the geographic distribution of leprosy in armadillo populations and that very little genetic variation can be attributed to differences among host populations.

DEDICATION

I dedicate this work to Fernando Chinchilla.

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INTRODUCTION

Out of over one thousand recognized pathogens that infect humans, 61% are zoonotic highlighting the importance of understanding interspecific transmission and emergence (Taylor et al. 2001). For a pathogen to emerge and cause an outbreak within a new population, ecological and evolutionary factors that genetic diversity in host and pathogen populations must be at work (Morse 1995; Begon 2002; Childs et al. 2007). Infectious disease emergence begins with an accidental infection or "spill over" infection from one species to another (Daszak et al. 2000; Antia et al. 2003). Once an accidental infection has occurred in a susceptible population, pathogen emergence in the new host is not guaranteed.

While infected individuals may transmit the pathogen to more susceptible individuals of the same species, an infectious individual must cause, on average, one or more secondary infections. That is the mean number of secondary infections, called the basic reproduction number (R_0), must be greater than one for the pathogen to persist in that population. There are a number of ecological and evolutionary processes that can influence the basic reproduction number.

For example, adequate contact between hosts must occur for new infections to occur, and infection rate often depends upon host density and the frequency of host contact. Host density, often governed by the carrying capacity of the ecosystem, can dictate the number and duration of contact events between susceptible and infected

individuals in a population (Diekmann et al. 1990; Morse 1995; Daszak et al. 2000; Dobson and Foufopoulos 2001; Taylor et al. 2001). In addition, the frequency of contact between individuals can depend on the behavior of individuals dictated by the need for resources and mates. Environmental conditions such as precipitation and temperature have effects on both carrying capacity and animal behavior and in turn disease distribution (Hagget 1994; Hagenaars et al. 2004; Despommier et al. 2007; Real and Biek 2007). The biodiversity of the ecosystem may also play a role in infectious disease dynamics by governing how often competent hosts are parasitized (Brooks and Zhang 2010). Studies have shown that the affects of an outbreak may be "diluted" with increased biodiversity (Ezenwa et al. 2006). Ecological conditions affecting the diversity of an ecosystem can thus change the probability that an outbreak will occur. When ecological conditions change individual fitness, evolutionary processes, such as adaptation or genetic drift, may be affected leading to an increase or decrease in host susceptibility or in pathogen virulence. Any factor that can alter transmission or recovery time, including genetic variation in host or parasite populations, can influence disease emergence.

Advancements in molecular technology have become valuable to the study of infectious disease by allowing researchers to identify genes involved in immune response and to assess the involvement of host genetic diversity in the epidemiology of outbreaks. Springbett et al. (2003) used an SIR (susceptible, infected, recovered) model to describe the effects of host genetic diversity on R_0. They demonstrated that populations with genetic heterogeneity were much less likely to suffer devastating epidemics than genetically homogeneous ones by varying both disease induced mortality and level of host susceptibility. They also showed that the higher the number of alleles at loci

associated with disease resistance in the host population, the less likely R_0 is to ever reach one and that disease induced mortality decreased with increasing allelic richness. In addition, Zhu et al. (2000) showed that introducing genetic diversity into monoculture rice crops decreased the severity of infection with the fungus *Magnaportha grisea*.

Clearly genetic diversity is important to determining the susceptibility of individuals to disease. It follows that spatial variation in genetic diversity may lead to spatial variation in disease resistance. The geographic mosaic theory of coevolution may explain how geographic distance and heterogeneity can contribute to variation in infection rates (Thompson 1999b). Selection mosaics or interactions between species that affect fitness could explain any differences in immune response in hosts and virulence genes in pathogens between populations in different geographic areas (Gomulkiewicz et al. 2007). The constant reciprocal adaptation between hosts and pathogens can lead to divergence of genetically isolated populations (Kaltz and Shykoff 1998). Gandon (2002) used models based on a metapopulation structure to show that host and parasite migration rates strongly influence local adaptation. When migration rates were low for the pathogen and higher for the host, his model showed that the host population was more resistant. On the other hand, when migration rates were low for the host, high parasite migration was met with lower resistance from the host population. This demonstrates that local host genotypes have adapted to local pathogen genotypes. When migration rates were high for both parasites and hosts, local adaptation was less likely to occur. It is not surprising then that genetic structure in the landscape can alter the epidemiology, and ultimately the emergence, of infectious diseases (Gomulkiewicz et al. 2007).

The ideal system to explore the relative roles of genetics and ecology in the emergence of infectious diseases is one in which the host has broad habitat requirements allowing for comparison of environmental conditions and highly variable population densities across sites allowing for comparison of ecological conditions. Additionally, available genomic data for both host and pathogen and a stable disease distribution are characteristics of an ideal system for studying the roles of genetics and ecology in emergence on a large geographic scale. The association between *Dasypus novemcinctus* (the nine-banded armadillo, referred to as armadillo throughout) and *Mycobacterium leprae* (the causative agent of leprosy) possesses all of these characteristics. Because *M. leprae* thrives optimally in environments that are between 27-33°C, the low basal metabolic rate and therefore low core body temperature (average 34°C) of nine-banded armadillos makes these animals one of the only the ideal hosts for *M. leprae* (McNab 1980; Franzblau and Harris 1988; Boily 2002).

With genomic information, it has been shown that *Mycobacterium leprae* is very reliant upon its host for survival and replication due to its extremely limited genome. About 27% of the genome consists of presumably non-functional pseudogenes and has led to the dependence of *M. leprae* on its host for molecules that provide energy and essential nutrients (Cole et al. 2001). The pathogen's genomic data has also allowed researchers to conclude that worldwide genetic variation of *M. leprae* is very low (Truman et al. 2004; Monot 2005). Because *M. leprae* relies on its host in order to survive and replicate and because the level of genetic variation is so low, we assume that the mechanism of infection is conserved across host species (Britton and Lockwood 2004; Cole et al. 2001). The reliance of *M. leprae* on its host and its temperature

requirements also greatly limits the number of host species that the bacteria can infect (Cole et al. 2001; Britton and Lockwood 2004).

One of the only natural hosts of *M. leprae*, *Dasypus novemcinctus,* has only inhabited the United States since the mid-nineteenth century, and the relatively recent migration of the nine-banded armadillo into the southern United States has been documented in several publications (Talmage and Buchanan 1954; Taulman and Robbins 1996). Taulman and Robbins (1996) cited sources from as early as 1854 that described the migration of the nine-banded armadillo into southern Texas, and Talmage and Buchanan (1954) attribute the armadillo's presence in Florida to a human introduction between the 1920's and 1930's. The range now extends from Texas to Florida and north to Missouri and southern Illinois. Since the discovery of leprosy in armadillos, several researchers have used histopathological examination, ELISA, and polymerase chain reaction based analyses to test hundreds of animals for the disease across much of the species range in the United States (Fox et al. 1977; Kirchheimer 1977; Kirchheimer and Sanchez 1978; Smith et al. 1978; Smith et al. 1983; Walsh et al. 1986; Truman 2005; Loughry et al. 2009).

With the range expansion and distribution of disease in mind, Loughry et al. (2009) hypothesized that a wave of infection is traveling from west to east through armadillo populations as the range of the host species expands. However, some armadillo populations from west to east have been spared infection with *M. leprae*. This suggests that some factor other than just an expanding disease front is playing a role in the observed pattern of infection. Truman (2005) proposes an alternative hypothesis that the presence or absence of leprosy in armadillo populations is governed by environmental factors especially soil composition, resource availability, and moisture. Truman (2005)

has noted differences particularly in the habitats of infected and uninfected populations. He reasons that certain ecological conditions can be conducive to outbreaks, while sites where infection is not seen may not provide such conditions. Differences in soil types, moisture and temperature levels between sites can create variation in resource availability and thus differences in host density and ultimately the host's ability to fight infection.

D. novemcinctus has the largest range of any member of the family Dasypodidae and so inhabits a variety of habitats. Conditions of habitat in the United States alone vary greatly with average temperature varying from 58° F to 70° F and rainfall varying from 28 inches to 57 inches (NOAA Satellite and Information Service). In addition, soil type varies greatly across the range (www.priweb.org). The variation in soil types not only dictates differences in vegetation and food types but also the temperature and moisture levels in burrows. These differences could have an important consequence on the spread of leprosy within armadillo populations. The greatest levels of infection have been noted in the lowlands along the Mississippi River Alluvial Plain where soil type and moisture levels are affected by the Mississippi River (Truman 2005). The areas where leprosy is most prevalent are predominantly of the vertisol soil type, which is clay capable of holding large amounts of water. Viable *M. leprae* has been found in soil samples and could be the source of infections where soil, temperature, and moisture levels are appropriate for the pathogen's survival (Lavania et al. 2008).

In conjunction, the differences between sites in soil type, temperature, and moisture may affect host interaction and disease transmission as well as the overall health of the host. To fully understand the extent of differential ecological conditions contributing to leprosy distribution, we must examine each aspect of this hypothesis including the affect of environment on both the pathogen and host. This includes

examining the environments of each population as well as determining the extent to which gene expression and genetic variation has been changed across different ecological conditions. Identifying ecological factors involved in disease transmission can be very difficult. Therefore, focusing on genetic differences in host populations is the next step in determining the cause of disease distribution. By considering the genetic structure of nine-banded armadillo (*Dasypus novemcinctus*) populations and the prevalence of leprosy in these populations, we can further our understanding of the roles of ecology, population structure, and evolution in dictating the spread of infectious disease.

A new and quickly expanding population, such as the migration of *D. novemcinctus*, can have consequences for genetic diversity, which can affect the spread of disease in the newly established populations. Additionally, the low virulence (pathogen induced mortality of host) of leprosy relative to its low transmissibility provides the perfect opportunity for the pathogen to persist within host populations leading to a stable distribution of infection and the potential for local adaptation (Kale et al. 2002; Blaser and Kirschner 2007; Stearns and Koella 2008). Genotypic data can reveal genetic variation between subpopulations elucidating the structure of armadillo populations. Specifically, the inclusion of genes thought to be involved in the immune response to leprosy can further our understanding of how these genes influence the distribution of leprosy across populations. Taking a quantitative measure of the genetic structure of populations can give us an idea of which of these natural processes is at work and can identify any relationship between leprosy distribution and host genetic diversity in nine-banded armadillo populations.

We hypothesize that local adaptation may have led to differences in allele frequencies between armadillo populations at loci associated with susceptibility or

resistance to leprosy. The differences in allele frequencies at these loci could account for differences in seroprevalence observed at each site. Genetic susceptibility or resistance to *M. leprae* has been noted in human populations leading to the logical assumption that the possibility for such susceptibility or resistance could also occur in the only other natural host in North America, *Dasypus novemcinctus* (Marquet and Schurr 2001; Fitness et al. 2004; Mira 2006; Moraes et al. 2006). Studies in human populations where *M. leprae* is endemic are ongoing and have shed new light on the role of genetic susceptibility to the pathogen. Specific polymorphisms in some immune response genes conferring susceptibility or resistance to leprosy have been identified in human populations (Marquet and Schurr 2001; Fitness et al. 2004; Mira 2006; Moraes et al. 2006). See Table 1.1 for a list including the functions of these genes. Considering the role of the immune response genes in susceptibility to leprosy in humans, seroprevalence in armadillo populations could be patchily distributed due to differences in genotypes between populations at loci associated with genes homologous to human susceptibility genes.

Table 1.1 A list of genes associated with the immune response to leprosy in humans and their functions.

Gene Name	Abbreviation	Function in Humans	Citation
Tumor Necrosis Factor- α	TNFα	Is an inflammatory cytokine; induces apoptosis	(Kindler et al. 1989)
Interferon γ	IFg	Aides in presentation of antigen to macrophages; helps to increase presentation of MHC	(Schroder 2004)
Major Histocompatability Complex	DPA	Involved in expression of antigen on cell surface for recognition by T cells	(Klein 1976)
Vitamin D Receptor	VDR	Helps with the function of cells involved in immune response	(Hayes et al. 2003)
Cytotoxic T-lymphocyte Antigen 4	CTLA4	Involved in destruction of infected cells	(Waterhouse 1995)
Interleukin 10	IL10	Inhibits inflammatory cytokines; Inhibits antigen expression	(Volk et al. 2001; Llorente et al. 1995)
Parkin Co-regulated Gene	PACRG	Involved in ubiquitination	(Shimura 2000)
Mannose binding protein	MBP	Involved in activation of the complement system	(Fraser 1998)

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CHAPTER II

MICROSATELLITE MARKERS IDENTIFIED FOR THE STUDY OF LEPROSY IN NINE-BANDED ARMADILLOS

Seroprevalence of *Mycobacterium leprae*, the causative agent of Hansen's disease or leprosy, in the nine-banded armadillo has what appears to be a stable geographic distribution in the southeastern United States (Truman 2005; Loughry et al. 2009). These circumstances are ideal for studying the effects of environmental variation and host genetic variation on the ecology and evolution of infectious disease. We are particularly interested in determining whether the known pattern of leprosy infection in *Dasypus novemcinctus,* the nine-banded armadillo*,* is correlated with the genetic structure in host populations.

Previous population genetic analyses using mitochrondial and nuclear markers have been conducted and were designed to confirm the presence of polyembryony, to describe the dispersal patterns of identical siblings, and to compare genetic variation between U.S. and South American populations (Prodöhl 1996; Huchon 1999). However, presently available molecular markers are insufficient for an analysis of the distribution of leprosy in this species because none are associated with the immune response to leprosy. Our goal was to develop a set of markers to estimate the genetic structure of *D. novemcinctus* populations in the southeastern United States including microsatellites that are physically linked to genes orthologous to the human genes associated with immune response to leprosy. Genetic susceptibility or resistance to infection with *Mycobacterium*

leprae has been noted in several human populations. Genes found to play a role in this resistance include several HLA (human leukocyte antigen) genes, Interleukin-10 (IL10), Interferon γ (IFg), vitamin D receptor (VDR), and cytotoxic T-lymphocyte antigen-4 (CTLA4) among others (Mira 2006). Because *M. leprae* relies on its host in order to replicate, we began by assuming that the mechanism of infection, and thus of the immune response, is conserved across host species (Britton et al. 2004). Therefore, we were able to employ genes homologous to the human immune response genes to conduct our study.

Using the *D. novemcinctus* data published on Genbank (Project: 12594), we conducted an exhaustive genome wide scan for microsatellites using Tandem Repeats Finder v. 4.03 (Benson 1999; Benson et al. 2008). In addition to anonymous microsatellites, several "functional" loci that are physically linked to the orthologs of the candidate genes involved with immune response to leprosy in humans were identified using Orthomam and Ensembl (Ranwez et al. 2007; Flicek et al. 2008). Primers flanking the chosen microsatellites for both functional and anonymous loci were designed using Primer 3 development software v. 0.4.0 (Rozen and Skaletsky 2000). We altered the forward primer sequences to include the M13 sequence (5'CAC GAC GTT GTA AAA CGA C 3') developed for use in a three-primer PCR protocol (Schuelke 2000). The third primer uses this universal M13 sequence, and it is labeled with fluorophores detectable by standard DNA sequencing equipment. This serves as a cost-cutting technique because individual primer pairs need not be labeled. Initially, 21 loci were identified, and primer sets were designed for each locus.

Dasypus novemcinctus ear tissue samples were collected from St. Catherine's Creek National Wildlife Refuge in Mississippi and Stimpson State Game Refuge and Riverside Access Area in Alabama. Animals from two of the populations (Stimpson and

Riverside) have previously tested positive for antibodies to *Mycobacterium leprae* (Loughry et al. 2009). DNA was extracted from 5-10 mg of *D. novemcinctus* ear tissue using an ABI prism 6100 Nucleic Acid Prep Station and proprietary chemistry (ABI, Foster City, CA). Tissue was macerated using a Retsch MM200 ball mill (Retsch Incorporated, Newtown, PA) and digested overnight in 100µL of NucPrep Digestion Buffer (ABI, Foster City, CA) and 50µL Proteinase K (20µg/mL) at 65°C. We conducted 10 µL PCR reactions with concentrations of 0.2 µM of reverse primer and M13 primer marked with one of two fluorophores (HEX or FAM). Optimal concentrations of forward primer are listed in Table 2.1 and were either 0.08 µM or 0.12 µM. Final concentrations in the PCR cocktails were 2mM $MgCl_2$, 30mM Tricine (pH 8.4- KOH), 50mM KCl, and 100µM of each dNTP. The 10 µL reactions included ~10ng of template DNA and 0.4 U of *Taq* DNA polymerase. The amplification process started with a temperature of 95°C for one minute for denaturation followed by 35 cycles of 15 seconds at 95°C, 15 seconds at the optimal annealing temperature (see Table 2.1) and 30 seconds at 72°C. After the 35th cycle, a terminal elongation phase of 72°C was held for 7 minutes followed by cooling.

Table 2.1 Repeat motifs, product size, and optimal forward primer concentrations are listed along with optimal annealing temperatures for each primer set. The first two loci are associated with functional genes which are mentioned in the text. All others are anonymous.

Locus name	Repeat motif/Size	Primer sequences (Forward/Reverse)	Accession # /Location	Forward Primer	T_a(°C)
DnovCTLA4	$(t)_{1/65}$	5'cac gac gtt gta aaa cga cgg cct ttt tag cca ttc tcc3'	AC156764	0.08	52
	152	5'ggg ttg aat ggg acc tca c3'	28297-28361		
DnovVDR	$(gt)_{2/16}$	5'cac gac gtt gta aaa cga ctt tgg tca ctc tgt gcc ttg3'	AC145507	0.08	52
	220	5'tgg cca agt tag tga aaa gga3'	35955-35988		
Dnov2092	$(gt)_{2/15}$	5'cac gac gtt gta aaa cga cgc aca ttc aga gga ggg aaa3'	CH512092	0.08	52
	212	5'ttc cct ggg gtg tat cag ag3'	14476-14505		
Dnov2179	$(cta)_{4/19.8}$	5'cac gac gtt gta aaa cga cag cca gta ttt gag cca agg3'	CH512179	0.08	52
	241	5'ggt gat ggt agc att cca ttg3'	41788-41863		
Dnov2426	$(at)_{2/16.5}$	5'cac gac gtt gta aaa cga ctt ttg aaa agt gtt tgt ttt tgg a3'	CH482426	0.12	52
	176	5'aga agg gga caa tga aca cg3'	142505-142537		
Dnov2433	$(ac)_{2/12.5}$	5'cac gac gtt gta aaa cga ctg ttg ttt cag ccc atc tga3'	CH482433	0.08	52
	244	5'aaa tta cca cct ggc ttt gg3'	369380-369404		
Dnov3824	$(ag)_{2/18}$	5'cac gac gtt gta aaa cga ctg ctg agg gtc cta agg aag3'	CH483824	0.12	52
	229	5'gct ttt ccc acc act gag ag3'	108474-108509		
Dnov4035	$(ca)_{2/21}$	5'cac gac gtt gta aaa cga ctt ttt ctt ccc aaa agg cta ctt3'	CH484724	0.08	52
	202	5'agt cat cag tcc cca tgg aa3'	55293-55334		
Dnov4724	$(act)_{3/13.7}$	5'cac gac gtt gta aaa cga ctg ttg cag gac agg aaa gtg3'	CH484035	0.08	52
	208	5'aag cca tct tca ggc aca at3'	30557-30598		

Fragment analysis for genotyping microsatellites was conducted on ABI 3730 capillary sequencers (ABI Foster City, CA) at Arizona State University's DNA lab. Chromatograms were scored at Mississippi State University using Peak Scanner software v. 1.0 (ABI, Foster City, CA). We recorded genotypes for forty individuals from each site for a total of 120 individuals per primer set, and we used Micro-Checker to detect any genotyping errors including potential null alleles (vanOosterhout et al. 2004). The genotypic data was used to calculate observed and expected heterozygosities (see Table 2.2) with FSTAT v. 2.9.3 (Goudet, 2001). We used the R statistical package v. 2.6.1 to conduct an exact test for significant departures from Hardy-Weinberg equilibrium in the three populations at each locus (www.R-Project.org). No locus deviated from Hardy-Weinberg equilibrium suggesting that loci are appropriate for use in empirical studies of *D. novemcinctus* population genetics. Of the 21 markers originally developed, 16 loci amplified reliably via PCR. Of these 16, seven anonymous markers and two functional markers were identified as polymorphic

Table 2.2 Observed and expected heterozygosities for each subpopulation are presented. (RS=Riverside Access Area, SC=St. Catherine's Creek National Wildlife Refuge, ST=Stimpson State Game Refuge) The p-values for an exact test of Hardy Weinberg equilibrium for each population and locus are also included. Level of significance for these tests is 0.05.

Locus	$H_{O(RS)}$	$H_{E(RS)}$	p-value	$H_{O(SC)}$	$H_{E(SC)}$	p-value	$H_{O(ST)}$	$H_{E(ST)}$	p-value	# of Alleles
DnovCTLA4	0.3636	0.487	0.6836	0.4595	0.4935	1	0.3056	0.52	0.2385	3
DnovVDR	0.6154	0.6037	0.4741	0.4865	0.5198	1	0.2	0.2582	0.8395	5
Dnov2092	0.4103	0.3783	0.795	0.5	0.4434	1	0.3684	0.3723	1	4
Dnov2179	0.2222	0.2727	0.6142	0.5143	0.4767	1	0.375	0.313	0.904	5
Dnov2426	0.4737	0.5583	0.9494	0.6154	0.7203	0.9491	0.7	0.5921	0.9526	5
Dnov2433	0.425	0.4845	0.8453	0.6923	0.625	0.9433	0.641	0.6324	1	3
Dnov3824	0.6	0.7092	0.2892	0.641	0.6713	0.1394	0.5641	0.6993	0.1333	4
Dnov4035	0.4359	0.5541	0.2689	0.4103	0.4582	0.8434	0.641	0.5275	0.7674	4
Dnov4724	0.4	0.4104	1	0.4	0.3946	0.953	0.325	0.3668	0.8797	3

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CHAPTER III

POPULATION STRUCTURE AND LEPROSY DISTRIBUTION IN NINE-BANDED ARMADILLO POPULATIONS

The relationship between *Mycobacterium leprae* and its host *Dasypus novemcinctus,* the nine-banded armadillo, provides a unique opportunity to investigate the role of genetic structure in the spread of pathogens in wild host populations. Recent outbreaks of diseases such as SARS, West Nile virus, and avian influenza have led to increased interest in the mechanisms that lead to the emergence of zoonotic pathogens in human populations (Daszak et al. 2000; Jones et al. 2008; Woolhouse 2002). Disease emergence requires that a change in exposure, infectivity, or transmissibility occurs within or among populations (Diekmann et al. 1990; Morse 1995; Daszak et al. 2000; Taylor et al. 2001). Differential migration over large spatial scales may induce spatial variation in exposure (Woolhouse 2002 and 2006). Additionally, environmental heterogeneity can alter exposure and transmissibility by affecting host density, host immune response, and pathogen virulence and survival (Hagget 1994; Hagenaars et al. 2004; Despommier et al. 2007; Real and Biek 2007). Ecological heterogeneity that changes host communities can significantly alter the epidemiology of zoonotic pathogens and as a result, the likelihood of emergence (Dobson and Foufopoulos 2001; Brooks and Zhang 2010).

The importance of these ecological factors in disease emergence is ultimately mediated by genetic structure and gene expression patterns for both the host and the

pathogen. For pathogens that induce a significant fitness cost, spatial variation in epidemiology can result in the development of selection mosaics that reinforce geographic heterogeneity in exposure (Thompson 1999b). The consequence is that the risk of emergence for any zoonotic pathogen varies spatially and temporally. With the growing availability of annotated genomes for a variety of hosts and associated pathogens, it is now increasingly possible to assess the role of functional genetic structure as well as ecological variation in the epidemiology of wildlife diseases.

Mycobacterium leprae, the etiological agent of leprosy, provides an opportunity to study the role of environment and genetics in disease emergence. Since the discovery that *M. leprae* can infect *Dasypus novemcinctus* (the nine-banded armadillo, hereafter referred to as armadillo), hundreds of animals across the species recently inhabited range in the United States have been tested. Histopathological examinations, ELISAs, and PCR based assays have been used to establish spatial patterns of exposure and infection (Smith et al. 1983; Smith et al. 1978; Kirchheimer 1977; Kirchheimer and Sanchez 1978; Walsh et al. 1986; Fox et al. 1977; Truman 2005; Loughry et al. 2009). Nine-banded armadillos are very recent members of the small mammal fauna in North America, with only a few animals establishing populations in southern Texas in the mid-nineteenth century (Audubon and Bachman 1854; Taulman and Robbins 1996). A comparison of *D. novemcinctus* range surveys from 1954 and 1972 suggest that migration into the southern United States occurred rapidly (Talmage and Buchanan, 1954; Humphrey, 1974), Tauman and Robbins (1996) show that migration has continued into the present range, from Texas to Florida and as far north as Nebraska, and is still under way. A study published by Huchon et al. (1999) confirms a founder effect during the recent migration of *D. novemcinctus* into the United States indicating recent common ancestry.

Over the time scales for which studies have been conducted, the geographic distribution of armadillo infection with *M. leprae* (Figure 3.1) appears to have remained consistent in spite of continuing migration (Truman 2005). The low virulence and slow progression of *Mycobacterium leprae* in host populations allows the pathogen to persist without causing massive die offs, thus leading to a slowly changing distribution of infection (Stearns and Koella 2008; Blaser and Kirschner 2007; Kale et al. 2002). As a result, this system provides an ideal opportunity to examine the roles of migration, environmental variation, and genetic variation on the persistence of a novel pathogen.

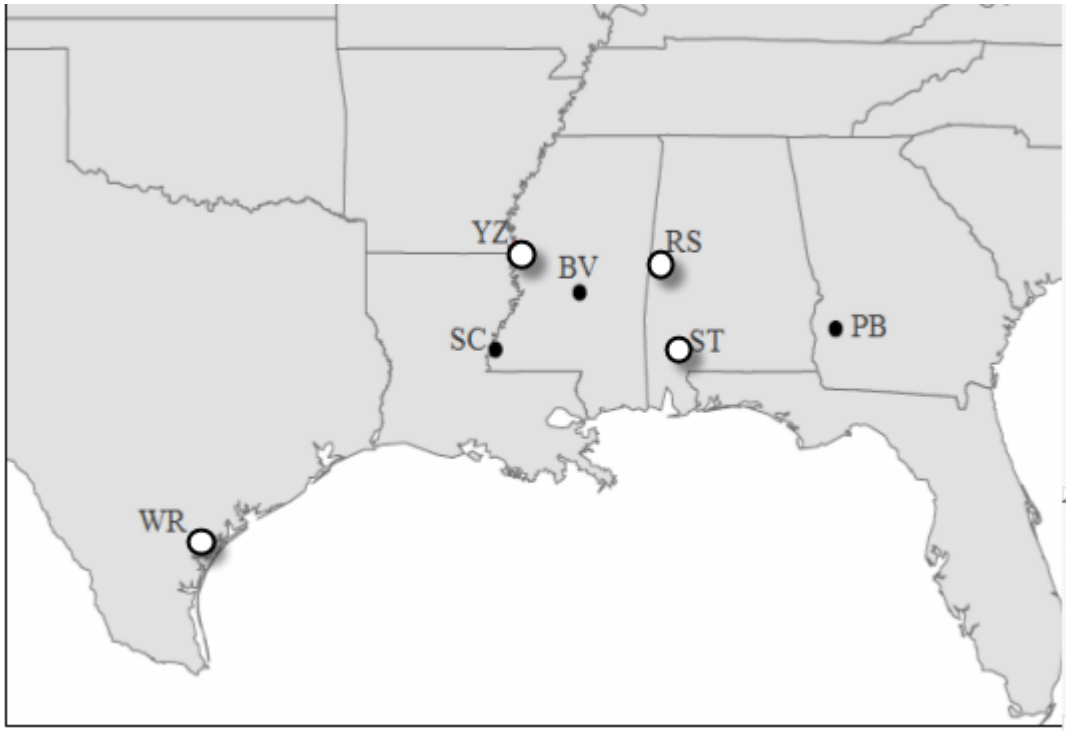

Figure 3.1 This is a map of sites where nine-banded armadillo samples were taken and tested for antibodies to PGL-1, an antigen specific to *M. leprae*. White represents sites with individuals seropositive for leprosy, and black represents sites with individuals only seronegative for leprosy.

Several authors have suggested hypotheses addressing how ecological and environmental variation affects the distribution of *M. leprae*. Loughry et al. (2009) hypothesized that exposure to *M. leprae* has moved from west to east as the range of *D. novemcinctus* expands. Truman (2005) noted that soil composition and moisture levels differ in the environments of infected and uninfected populations and hypothesized that the spatial pattern of infection and exposure may be associated with these ecological conditions.

An alternative, but not mutually exclusive, hypothesis is that there is functional genetic variation between populations of the host or pathogen that has resulted in the observed geographic distribution of disease. To test this hypothesis, we have considered the population structure of both the pathogen and the host. Studies have revealed that worldwide genetic variation of *M. leprae* is very low and that the strains of *M. leprae* infecting both armadillos and humans in the United States are more closely related than to other strains (Monot et al. 2005; Truman et al. 2004). The lack of genetic variability in the pathogen suggests that at least some of the genetic mechanisms of virulence are conserved across host species (Britton and Lockwood 2004; Cole et al. 2001) and that any influence of genetic structure on the geographic distribution of infection is due to the host alone.

Previously published studies suggest that there is little neutral genetic variation across *D. novemcinctus* populations in North America, but all of these previous studies have been limited in either their geographic scope or in the number of markers used. Populations near Carville, LA were found to be monomorphic at more than 30 allozyme loci (Moncrief 1988), while Ramsey and Grigsby (1985) found similar results for 27 allozyme loci over a broader geographic range. Huchon et al. (1999) examined a single

mitochondrial marker and a single nuclear microsatellite marker. The mitochondrial marker yielded only two haplotypes, and there was also very little variation in the microsatellite marker. Prodöhl et al. (1996; 1998) used 9 microsatellite loci to confirm polyembryony in *D. novemcinctus.* Seven of the nine markers were found to be polymorphic, but population structure with regard to functional genes was not established. The evidence provided by these studies suggests that armadillo populations in the southeastern United States have very little genetic structure at neutral loci, which is consistent with the recent common ancestry of these populations. This is also beneficial for the present study because detection of loci associated with locally adaptive gene complexes is more likely when background genetic structure is weak (Beaumont and Balding 2004).

Specific polymorphisms in several immune response genes, given in Table 3.1, conferring innate susceptibility or resistance to leprosy have already been identified in human populations (Mira 2006; Marquet and Schurr 2001; Fitness et al. 2004; Moraes et al. 2006). The genomes of both hosts have been published and are available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The availability of this genomic data allows us to test the hypothesis that local adaptation has occurred leading to differences in the frequency of alleles associated with susceptibility or resistance to leprosy explaining the observed distribution of seroprevalence in the southern United States. We approach this hypothesis by comparing genetic structure in infected and uninfected populations and by comparing the genetic structure observed at anonymous microsatellite markers to that of microsatellite markers that are physically linked to innate immune response genes. If genetic structure correlates with the distribution of *M. leprae* exposure in the host, then a genetic component to the observed

geographic pattern of exposure would be supported. Strong genetic structure in functional versus anonymous markers would suggest strong selection operating on the innate immune response. We also assess differences between the distributions of allele frequencies of anonymous and functional loci. Significant differences in these distributions would suggest that selection has occurred.

Table 3.2 These genes are associated with the immune response to leprosy in humans and their functions.

Gene Name	Abbreviation	Function in Humans	Citation
Tumor Necrosis Factor- α	TNFα	Is an inflammatory cytokine; induces apoptosis	(Kindler et al. 1989)
Interferon γ	IFg	Aides in presentation of antigen to macrophages; helps to increase presentation of MHC	(Schroder 2004)
Major Histocompatability Complex	DPA	Involved in expression of antigen on cell surface for recognition by T cells	(Klein 1976)
Vitamin D Receptor	VDR	Helps with the function of cells involved in immune response	(Hayes et al. 2003)
Cytotoxic T-lymphocyte Antigen 4	CTLA4	Involved in destruction of infected cells	(Waterhouse 1995)
Interleukin 10	IL10	Inhibits inflammatory cytokines; Inhibits antigen expression	(Volk et al. 2001; Llorente et al. 1995)
Parkin Co-regulated Gene	PACRG	Involved in ubiquitination	(Shimura 2000)
Mannose binding protein	MBP	Involved in activation of the complement system	(Fraser 1998)

Materials and Methods

Sampling

Tissue samples were collected from Bienville National Forest (BV) and Yazoo National Wildlife Refuge (YZ) between May and August of 2009 (Figure 3.1). Searches were conducted an average of four nights per week and corresponded to peak activity of *D. novemcinctus* beginning at 5:00 p.m. and ending at 1:30 a.m. (McDonough and Loughry 1997). Once located, armadillos were captured using large dip nets. Ear tissue was collected from each captured animal using an ear notcher and placed in 99% ethyl alcohol. Blood samples were taken from notched ears or clipped toenails when necessary using Nobuto strips, thick absorbent paper strips made of cellulose fibers (Cole-Parmer, Vernon Hills, IL). A topical coagulant was applied to the wound site before animals were released. Nobuto strips were allowed to air dry before being placed into labeled envelopes. Coordinates of all sampling locales and capture sites were recorded using a GPS (global positioning system) unit.

In addition to the samples collected at BV and YZ, we also obtained tissues previously collected by William Loughry (Valdosta State University). These samples were collected from five sites. In Mississippi, fifty-four samples were collected from St. Catherine's Creek National Wildlife Refuge (SC). Two sites in Alabama are included: Stimpson State Game Refuge (ST) (55 samples) and Riverside Access Area in Alabama (RS) (60 samples). Twenty-six samples were collected from Pine Bloom Plantation in Georgia (PB). A total of 74 samples were collected from Welder Wildlife Reserve (WR) in Texas. Thirty-five of those samples were collected by Dr. Richard Truman (1991), and 39 samples were collected by Dr. William Loughry (unpublished data).

Seroprevalence Analysis

Blood samples collected via Nobuto strips were processed at the National Hansen's Disease program labs in Baton Rouge, LA for analysis. In order to assess the exposure of each captured animal to *M. leprae*, ELISA was performed on blood samples to assess antibody titer to phenolic glycolipid-1 (PGL-1), an antigen specific to *M. leprae* (Cho et al. 1983; Truman et al. 1986b). The 39 samples collected at WR by Dr. William Loughry were not tested for antibodies to PGL-1. An individual was considered to be positive for leprosy when absorbance was greater than 0.72 (Truman 1986b). In order to quantify the likelihood that seropositive individuals were present in some of the populations for which none were detected, we assumed that seroprevalence was governed by a binomial distribution. We defined the probability of success as the proportion of seropositive individuals divided by the number of sampled individuals across all populations in which positive serology was detected, reflecting the assumption that seroprevalence across populations was drawn from the same distribution. We then calculated the probability of finding zero seropositive individuals out of the number of animals sampled in each population. This represents our confidence that a population was free of infection. All calculations were conducted using the R statistical package v. 2.6.1 (www.R-Project.org).

DNA Extraction

A Retsch MM200 ball mill (Retsch Incorporated, Newtown, PA) was used to macerate between five and ten milligrams of the collected ear tissue. The softened tissue was then placed in a solution containing 100µL of NucPrep Digestion Buffer (ABI, Foster City, CA) and 50µL Proteinase K (20mg/mL) and was digested overnight in a 65°C water bath. The extraction process was carried out using an ABI prism 6100

Nucleic Acid Prep Station according to the manufacturer's protocol (ABI, Foster City, CA).

Marker Development

In prior work (Molecular Ecology Resources Primer Development Consortium et al. 2010), a genome wide scan for anonymous microsatellites was carried out using Tandem Repeats Finder (Benson 1999). Primers for these microsatellites were designed with Primer 3 v. 0.4.0 (Rozen and Skaletsky 2000). Then, a thorough literature search was conducted to identify genes involved in human immune response to leprosy. The Orthomam and Ensembl databases were then used to identify armadillo genes believed to be orthologous to those associated with leprosy susceptibility or resistance in humans (Flicek et al. 2008). These sequences were then located within the *D. novemcinctus* genome found in GenBank by using BLAST (Altschul et al. 1990). Tandem Repeats Finder software was used again to locate microsatellites within each scaffold in which a homologous sequence was found, and Primer 3 v. 0.4.0 was used to develop primers flanking the sequences (Rozen and Skaletsky 2000; Benson 1999). These microsatellite markers are assumed to be inherited in the same pattern as the associated gene because we allowed no more than 3000 base pairs between the gene and the marker (Kruglyak 1999).

Microsatellite Genotyping

Microsatellites were amplified by means of three-primer PCR in 10 μL reactions. This technique is used as a cost cutting measure. The third primer is the universal M13 sequence (5'CAC GAC GTT GTA AAA CGA C 3'), which is fluorescently labeled. The forward primer is augmented on the 5' end with the M13 extension (Schuelke 2000). A

concentration of 0.2 μM of M13 primer labeled with either HEX or FAM fluorophores, 0.2 μM of reverse primer, ~10ng template DNA, and 0.4 U of *Taq* DNA polymerase were included in the PCR cocktail. Optimal forward primer concentrations were found to be either 0.08 μM or 0.12 μM (Table 3.2). The cocktail had final concentrations of 2mM $MgCl_2$, 30mM Tricine (pH 8.4- KOH), 50mM KCl, and 100μM of each dNTP. PCR for the primer sets with reliable amplification started with a denaturation temperature of 95°C for one minute. Thirty-five cycles of 15 seconds at 95°C, 15 seconds at the proper annealing temperature (Table 3.2) and 30 seconds at 72°C followed. A final elongation step at 72°C followed the 35^{th} cycle. Individuals were genotyped via fragment analysis on an Applied Biosystems 3730 sequencer (ABI, Foster City, CA) at Arizona State University. Twenty individuals each from WR and PB and forty individuals each from YZ, SC, RS, BV, and ST were genotyped based on the detected fragment size using Peak Scanner software (ABI, Foster City, CA). Individuals found to have antibodies to *M. leprae* were deliberately included, but all other individuals were randomly chosen from a pool of 465 individuals with high quality DNA after extraction.

Table 3.3 The primer sequences, forward primer concentrations, and accession numbers for an anonymous microsatellite locus (Dnov3435) and two microsatellites in tight physical linkage with innate immune response genes are listed, as is T_a (C°), the optimal annealing temperature for amplification during PCR. Information for 9 additional loci used in this study were previously published (Molecular Ecology Resources 2010).

Locus name	**Repeat motif/Product Size**	**Primer sequences (Forward/Reverse)**	**Accession # /Location**	**T_a(°C)**	**Forward Primer Conc.**
DnovIFg	$(ag)_{4/22.5}$	5'cac gac gtt gta aaa cga cga aac cag ttt tgc agc tca t3'	AC183482	53	0.08
	201	5'ttt gcc atc aag tca ttg aga3'	47561-47650		
DnovIL10	$(ac)_{4/8}$	5'cac gac gtt gta aaa cga cgt tct ggg cac ctt tgt ctt3'	AC157510	50	0.08
	243	5'ttg gca cta act cgc tga tg3'	1647-1677		
Dnov3435	$(gat)_{3/12}$	5'cac gac gtt gta aaa cga ctt tcc ctc acc tac aat atg aag a3'	CH483435	52	0.12
	223	5'tgg ggt aag tgt tgg gga ta3'	76148-76183		

Data Quality Assessment

Analyses were conducted to determine whether genotypes within all populations and loci were in Hardy-Weinberg equilibrium. Failure to detect genotyping errors leading to deviations from Hardy-Weinberg equilibrium can result in biased estimates of allele frequencies, and ultimately misinterpretation of genetic structure (Shaw et al. 1999). We screened for errors such as null alleles and allelic dropout using Micro-Checker software (van Oosterhout et al. 2004; Chakraborty et al. 1992; Brookfield 1996; Weir 1996). To detect genotyping errors resulting in deviation from HWE, Micro-Checker randomizes genotypes for each locus and then compares randomized genotypes to the observed distribution of genotypes (Weir 1996; van Oosterhout et al. 2004). Micro-Checker provides an estimate of corrected allele frequencies for each locus and population if genotyping errors are found.

Quantification of Genetic Structure

To detect the presence of population structure we conducted two analyses of molecular variance (AMOVAs), which includes a calculation of F_{ST}, using Arlequin v. 3.11 (Excoffier et al. 2005; Weir and Cockerham 1984). First, an AMOVA was run to compare all populations with seropositive individuals to all populations with no evidence of exposure to *M. leprae*. For the second AMOVA, populations with a probability greater than 0.05 of harboring undetected seropositive individuals were omitted and populations seropositive for leprosy were again compared to the remaining seronegative populations.

Comparing Functional and Anonymous Loci

The hypothesis that there is differential selection on functional and anonymous loci was tested using BayesFST (Beaumont and Balding 2004) and Detsel v. 1.0 (Vitalis et al. 2001; Vitalis et al. 2003) with the corrected allele frequencies from Micro-Checker. BayesFST relies on a Bayesian approach in which a multinomial-Dirichlet likelihood is modeled. This approach deals explicitly with allele counts at each locus and in each population. Each parameter associated with migration of alleles in the model is estimated using a Markov chain Monte Carlo (MCMC) approach, and the resulting distributions are used to identify loci under selection (Beaumont & Balding 2004).

We used Detsel v. 1.0 to confirm and clarify the results from BayesFST. Detsel v. 1.0 produces confidence intervals (95%) from a probability density function based on the relationship between F_{ST} and heterozygosity and then uses a pairwise population approach to determine whether loci fall within the expected distribution. High F_{ST} values leading to outliers indicate selection against migrants, whereas low F_{ST} values leading to outliers indicate balancing selection. The expected distributions are determined by a series of coalescent simulations. Nuisance parameters are chosen to give the user control over mutation rate, time of divergence and ancestral population sizes. The mutation rate was set at 0.005, 0.001, or 0.0001. Time since divergence was set as 50, 100, or 200 generations with the time of divergence set at either 50 or 100 generations. Lastly effective ancestral population size was set at 500, 1000, or 10,000 with the population size before divergence at 50 or 500. Such parameters can vary depending on the system of study and must be adapted to fit the life history of the organism. Documentation for the software recommends implementing a series of simulations with different

combinations of the nuisance parameters and accepting that selection has occurred only if all combinations of parameters reveal the locus as an outlier.

Since allelic diversity can be affected by selection, we used calculations from Microsatellite Analyser (MSA) v. 4.05 to determine whether the distributions of allelic diversities of functional and anonymous loci are different (Dieringer and Schlötterer 2003). The allelic diversity data was transformed and plotted for functional and anonymous loci separately for comparison. The distributions of the allelic diversities for both groups were then compared via a Kolmogorov-Smirnov test (Schlötterer and Dieringer 2005). If the distributions are significantly different, variances are compared between the two groups with an F-test, and the means are compared with a Welch's t-test (Schlötterer and Dieringer 2005).

Results

A total of 570 animals were sampled from the seven geographic sites. Twenty-four animals or 4.4% of all animals collected tested positive for PGL-1 antibodies. All seropositive animals came from four populations: WR, YZ, RS, and ST (Figure 3.1). Welder Wildlife Reserve (WR) had the highest percentage of infected animals at 17.1% (6/35) with Yazoo National Wildlife Refuge (YZ) at 6.9% (15/217). Seropositive individuals were identified at both sites in Alabama with 3.3% prevalence in RS (2/60) and 1.8% prevalence in ST (1/55). Based on the results of binomial testing, small sample size in one population, PB, may be too low to reliably establish the absence of *M. leprae*.

Out of 21 primer sets initially developed, 16 amplified reliably via PCR. Functional genes that were identified but that failed to amplify properly included MBP, PACRG, and TNFα. Three anonymous loci and the MHC class II locus (DPA) were

found to be monomorphic and were not used for further analyses. Four functional loci (IL10, IFγ, VDR, CTLA4) and eight anonymous loci were found to be polymorphic and adequate for use in analyses of population structure.

No significant genotyping errors were detected by Micro-Checker, and allelic diversity ranged from two to five alleles per locus. All populations are fixed for one allele at the IFg locus except for the SC population, and all populations are fixed for one allele at the IL10 locus except for the ST population. One of the five alleles at the VDR associated locus was identified in only two populations, RS and BV. The results for observed and expected heterozygosity as well as the number of alleles for each are shown in Table 3.3.

Table 3.4 This table shows allele counts and expected heterozygosity for all populations not included in the previously published work and for each locus.

Population	Locus	# of Alleles	H_o	H_e
PB	CTLA4	2	0.529412	0.507
	Ifg	1	0	0
	IL10	1	0	0
	VDR	4	0.35	0.354
	Anon2092	3	0.473684	0.462
	Anon2179	4	0.214286	0.206
	Anon2426	3	0.631579	0.516
	Anon2433	3	0.7	0.522
	Anon3435	2	0.3	0.387
	Anon3824	4	0.65	0.716
	Anon4035	3	0.8	0.664
	Anon4724	3	0.15	0.145
WR	CTLA4	2	0.052632	0.474
	Ifg	1	0	0
	IL10	1	0	0
	VDR	4	0.473684	0.468
	Anon2092	3	0.55	0.445
	Anon2179	3	0.166667	0.381
	Anon2426	4	0.7	0.643
	Anon2433	3	0.6	0.643
	Anon3435	2	0.05	0.05
	Anon3824	4	0.263158	0.629
	Anon4035	3	0.277778	0.458
	Anon4724	2	0.4	0.384
YZ	CTLA4	3	0.173913	0.462
	Ifg	1	0	0
	IL10	1	0	0
	VDR	4	0.685714	0.625
	Anon2092	3	0.4375	0.389
	Anon2179	4	0.521739	0.643
	Anon2426	4	0.820513	0.614
	Anon2433	3	0.666667	0.63
	Anon3435	3	0.102564	0.253
	Anon3824	4	0.394737	0.535
	Anon4035	4	0.473684	0.535
	Anon4724	3	0.475	0.483
BV	CTLA4	3	0.236842	0.449
	Ifg	1	0	0
	IL10	1	0	0
	VDR	4	0.459459	0.489
	Anon2092	3	0.27027	0.289
	Anon2179	4	0.405405	0.39
	Anon2426	5	0.684211	0.725
	Anon2433	3	0.684211	0.612
	Anon3435	2	0.05	0.328
	Anon3824	4	0.621622	0.672

	Anon4035	3	0.648649	0.547
	Anon4724	3	0.162162	0.224

There was very little genetic variation among populations (F_{ST}=0.0502), and no structure was attributed to anonymous and functionally linked markers. Comparison of populations known to harbor leprosy with those in which we did not detect the pathogen also failed to reveal structure by group (Table 3.4). This is true regardless of whether populations possibly harboring undetected positive individuals are excluded.

Table 3.5 A) This table shows AMOVA results for all populations with individuals seropositive for leprosy as one group and all populations with all individuals seronegative for leprosy as the other group. B) This table shows AMOVA results for all populations with individuals seropositive for leprosy as one group and only populations with less than 5% probability of harboring seropositive individuals as the other group.

A)

Source of Variation	S.S.	V.C.	% Variation	p-value
Among Groups	10.182	0.019	1.23	0.0860
Among Populations	27.152	0.060	3.91	>0.001
Within Populations	685.262	1.449	94.86	>0.001
Total	722.596	1.527	100	

B)

Source of Variation	S.S.	V.C.	% Variation	p-value
Among Groups	8.868	0.019	1.28	0.140
Among Populations	18.577	0.045	2.96	>0.001
Within Populations	628.587	1.448	95.76	>0.001
Total	656.032	1.512	100	

There was little evidence of local adaptation at any locus. Analyses using BayesFST indicated that only one anonymous locus falls beyond the threshold p-value and is likely under selection (Figure 3.2). A confirmatory analysis revealed no evidence that any loci evaluated are under significantly different selective pressures for any pairwise combination of populations and across a wide range and number of

combinations for the nuisance parameters. The distributions of allelic diversities (Figure 3.3) for the functional loci and anonymous loci were significantly different ($p<0.00001$), with both variance differing significantly ($p<0.01$) and means differing significantly ($p<0.00001$).

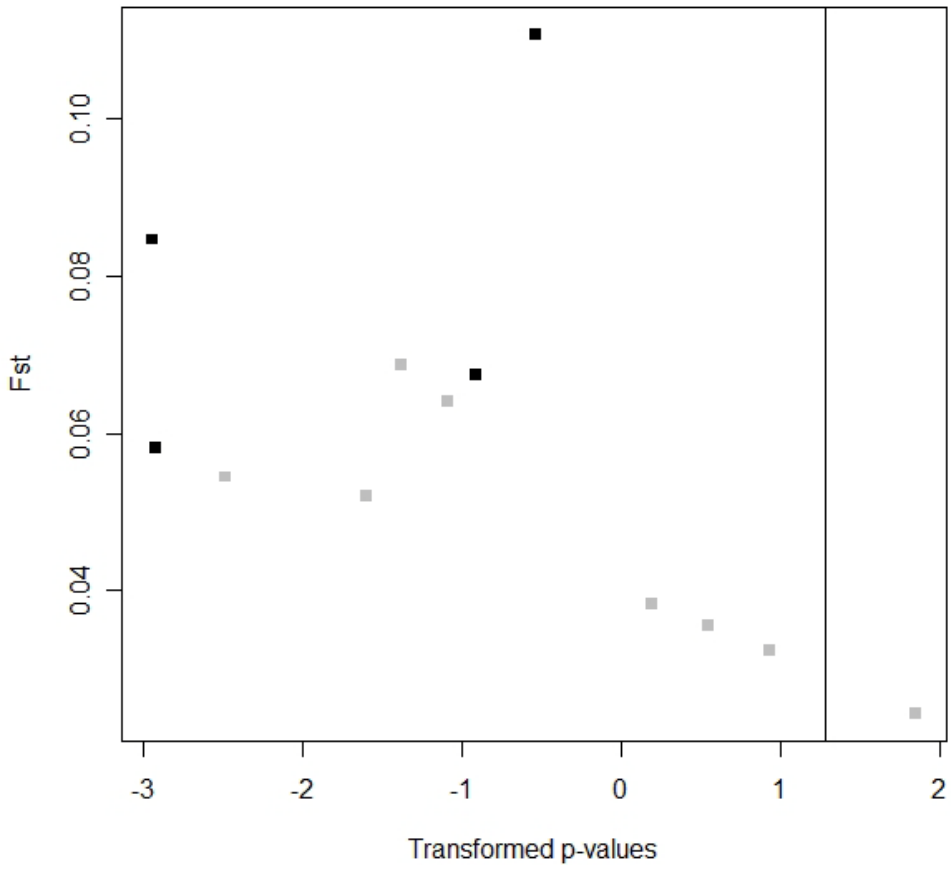

Figure 3.2 This figure represents the output from BayesFST. Black represents functional loci, and gray represents anonymous loci. The black line represents the threshold p-value beyond which loci are considered to be under selection. Only one anonymous locus appears to be under selection based on this analysis.

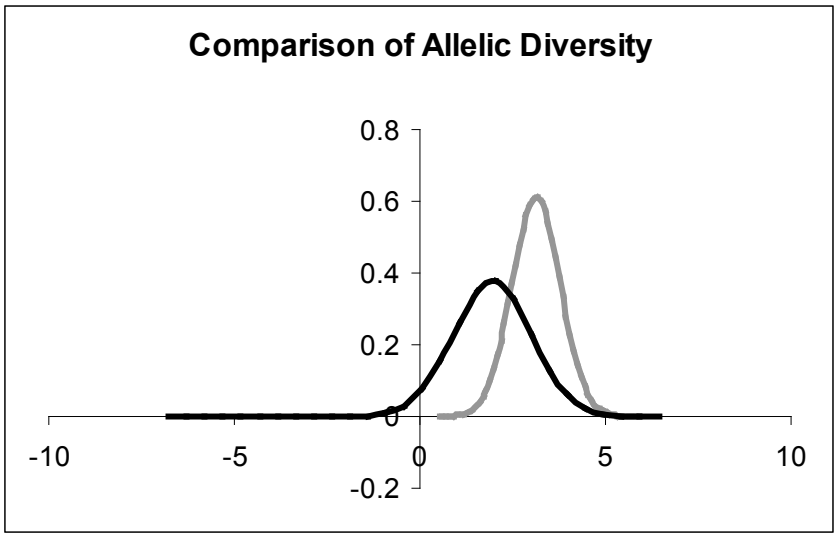

Figure 3.3 The allelic diversity of functional loci is compared to the allelic diversity of anonymous loci. The black curve represents functional loci, and the gray curve represents anonymous loci. It is clear that the mean allelic diversity is much lower and the variance is much higher for functional loci than for anonymous loci indicating selective pressure on functional loci.

Discussion

We hypothesized that differences in the allele frequencies at immune response loci between populations of *D. novemcinctus* have contributed to a patchy distribution of *M. leprae* through these populations. Our results do not support this hypothesis because there is very little genetic structure among populations for anonymous and functionally linked microsatellite markers. Thus, susceptibility or resistance to leprosy is independent of genetic structure among populations at the loci included in this study. The lack of population structure and the lack of detected localized selection at functional loci suggest that leprosy either does not induce a high fitness cost or that rapid range expansion and the resulting homogenization of allele frequencies has counteracted any selective effects. Our results agree with previous assessments of neutral variation in U.S. armadillo

populations (Ramsey and Grigsby 1985; Moncrief 1988; Prodöhl et al. 1996 and 1998; Huchon et al. 1999).

Despite the lack of population structure, we found that allelic diversity for functional and anonymous loci differs significantly. Barring possible sampling bias introduced by our choice of functional markers and their low variability, this finding is consistent with a role for selection acting on the generalized immune response genes of *D. novemcinctus*. The lack of significant differences between infected and uninfected groups largely eliminates *M. leprae* as the causative selective pressure. Other pathogens and parasitic organisms, such as *Trypansoma cruzi* and *Sarcocystis neurona,* have been isolated from nine-banded armadillos and could be exerting selective pressure on the immune response genes (Cheadle et al. 2001; Paige, Scholl and Truman 2002; Vizcai'no and Loughry 2008). However, compared to populations in South America, infection loads in U.S. populations are low, and the observed signature of selection in U.S. populations could be carried over from ancestral populations (Vizcaino and Loughry 2008).

Since there is little genetic variation across the range of *D. novemcinctus* in the U.S., the epidemiology of *M. leprae* in armadillo populations appears to be largely environmentally dependent. Environmental variation may induce changes in host or pathogen ecology and gene expression, in turn causing a change in exposure, infectivity or transmissibility (Hagget 1994; Hagenaars et al. 2004; Despommier et al. 2007; Real and Biek 2007). A recent study supporting the environmental variation argument suggests that viable *M. leprae* can be obtained from soil samples near the homes of infected people and may point to differences in suitable locations for environmental reservoirs of *M. leprae* (Lavania et al. 2008). Additionally, the distribution of infection

in human populations closely mirrors the pattern observed in armadillo populations in the United States (Truman 2005). Many infected people living in these areas have had no known exposure to the *M. leprae* indicating that infection was acquired from an environmental source or from an animal host. It is possible that soil and moisture levels in the endemic areas have provided a suitable climate for *M. leprae* to remain viable for long periods creating more opportunity for exposure and infection. Ecological differences in infected and uninfected host communities, such as increased density or poor host health, may have caused a difference in the number of opportunities for exposure or transmission (Dobson and Foufopoulos 2001; Brooks and Zhang 2010).

The relationship between *M. leprae* and *D. novemcinctus* has provided the opportunity to explore selective processes involved in the spread of zoonotic disease, and our approach to understanding the role of host genetics in epidemiology can have implications for many other host/parasite interactions. Similar studies have been published regarding the effects that local adaptation have on host and pathogen populations, but many of these studies have only employed microsatellites as a means to establish neutral gene flow and population structure (Cousyn et al. 2001; Dionne et al. 2007). However, microsatellite markers linked to genes have become increasingly popular alternatives because of the increase in available genomic data. Luikart et al. (2008) published a study in which variation in microsatellites linked to candidate genes was found to be correlated with the risk of parasitism in bighorn sheep. One genomic region associated with susceptibility or resistance to leprosy was even identified using microsatellite loci for linkage analysis (Mira 2003). Even more interesting is the result of a much earlier study in which a variant of a TNF associated microsatellite was directly related to the rate of AIDS progression (Khoo et al. 1997). Our study along with these

studies has illustrated the importance of exploring the role of genetic variation in disease dynamics, and they have shown that the increase in genomic information available to researchers has made this work possible.

Most certainly, the roles of genetic and environmental variability in the spread of pathogens will become an important part of understanding emergence. Changes in the transmissibility and infectivity of infectious diseases can often be a direct result of genetic variation in host or pathogen populations (Schrag and Wiener 1995; Biek 2010). In turn, genetic variation and phenotypic plasticity in these populations is often dictated by ecological conditions. Considering these factors and implementing safe guards to maintain stable ecological conditions must become part of the protocol for monitoring possible emerging pathogens in wild animal populations. Ultimately, understanding the dynamics of emerging zoonotic pathogens, such as SARS and AIDS, is important to global human health.

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