POPULATION GENETICS OF CANINE HEARTWORM (*DIROFILARIA IMMITIS*)

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2011

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This manuscript has been read and accepted for the
Graduate Faculty in Biology in satisfaction of the
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Abstract

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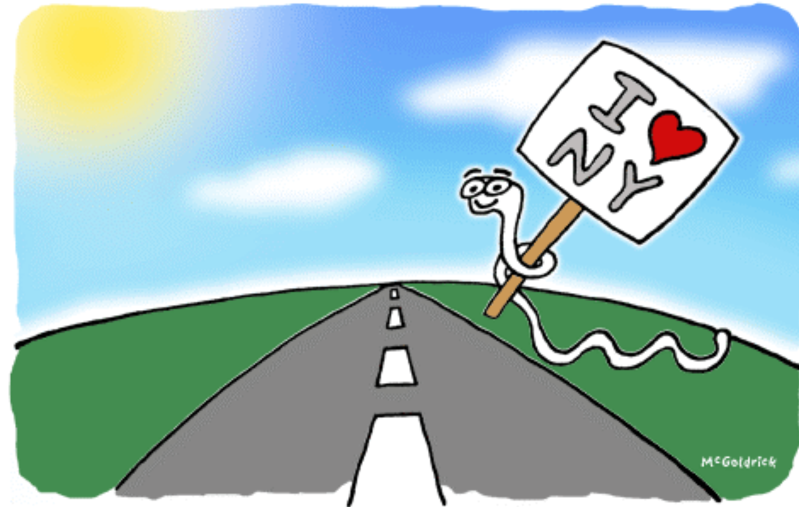
Dirofilaria immitis, canine heartworm, is a filarial nematode that may have genetic features that favor the development of drug resistance, including rapid rates of mutation, large population sizes, and high levels of gene flow. This parasite is currently treated with macrocyclic lactone anthelminthics, and while it has not yet shown evidence for evolving resistance to these chemotherapeutic compounds, resistance has evolved in related filarial nematodes infecting ruminants and humans. Heartworm samples from domestic dogs and coyotes were obtained via donations from veterinarians and researchers across the United States. I isolated and characterized 11 microsatellite loci for canine heartworm. Using the observed distribution of alleles, I determined the amount of genetic variability and quantified the partitioning of genetic variance. In conjunction with microsatellite data, specific mitochondrial (*cox1*) and *Wolbachia* (*wsp* and *ftsZ*) loci were used to genotype a subset of host taxa. Results indicate a lack of mitochondrial diversity and maximum likelihood trees show no discernable geographic patterning on a continental scale. This is not unexpected in a *Wolbachia*-infected organism like *D. immitis* as this bacterium has been shown to purge mitochondrial diversity in numerous model systems. After establishing baseline genetic parameters, a model of population dynamics was created to answer questions about the potential spread of drug resistance

alleles. In the absence of selection, gene flow between subpopulations drives the dispersal of drug resistance alleles. Fixation time is directly proportional to selection pressure. When resistance alleles arise in a source population they spread more rapidly than if they arise in a sequestered population.

Acknowledgements



I would like to thank my advisors Robert Rockwell and Susan Perkins for supporting this work and keeping me on the straight and narrow, my committee members Mark Siddall, Rob DeSalle, Jason Munshi-South, and Charles Criscione for their guidance, and the staff at the American Museum of Natural History for answering all of my silly questions. Additionally, I thank researchers, veterinarians, and wildlife biologists far and wide for their selfless sample collection. Without their help this research could not have been completed. I would also like to offer special thanks to my husband for his fabulous cartoon contribution as well as his ability to make a fine pot of tea.

Dedication

This dissertation is dedicated to my parents, Robert and Donna Belanger, and to my husband Jack McGoldrick, all of who have asked the eternal question:

"Are you ever going to finish?"

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Chapter 1: Introduction

Obligate parasites rely on their hosts for reproduction, transmission, and persistence. They exert selective pressures on their ecological communities and affect the biodiversity of ecosystems (Irvine 2006, Poulin 2007). Due to the dynamic complexity of the parasite-host relationship, the mechanisms underlying the evolution and dispersal of these organisms are poorly understood (Woolhouse *et al.* 2001, Criscione *et al.* 2005). The majority of theoretical and empirical studies focus on parasites prevalent in human populations while the interplay of many animal hosts and their parasites are unexplored. Predicting changes in host range and prevalence relies on identifying the processes that govern genetic variation in natural populations. This information underpins many research areas and is particularly important for understanding the development and spread of drug resistance (Gilleard 2006).

Dirofilaria immitis* and *Wolbachia

Heartworm disease is caused by a parasitic infection of *Dirofilaria immitis* (Leady 1856) nematodes in temperate and tropical animal populations worldwide. Heartworm infects members of the genus *Canis*, its definitive host, as well as cats, foxes, ferrets, and other mammals including humans (Gortazar *et al.* 1994, Foster *et al*. 2003, Nelson *et al* 2003, Pence *et al*. 2003, Riley *et al.* 2004, Sacks *et al.* 2004). Adult worms live in the pulmonary arteries of the host, where mature females release microfilariae into the blood stream. Microfilariae are ingested by arthropod vectors during the course of a blood meal, and develop into third stage larvae within the malpighian tubules (Anderson 2000).

The larvae migrate to the salivary glands in preparation for transmission, and are inoculated into the dermis of the final host with subsequent bites. Larvae reach the pulmonary arteries after several months of migration and maturation, eventually compromising the entire right side of the heart and its associated vessels. Several species of mosquito are competent heartworm vectors (Lee *et al.* 2007, Simon *et al*. 2007).

In North America, numerous canid species harbor heartworm infections, including the coyote (*Canis latrans*), red fox (*Vulpes vulpes*), and domestic dog (*Canis familiaris*) (Sacks and Caswell-Chen 2003). Outbreaks are particularly common in the southern United States where mosquito vectors are present year round. In cooler climes, heartworm infections have a distinct seasonal cycle. Several distinct mechanisms impact host-pathogen interactions including: seasonal changes in host social behavior and contact rate, variation in encounters with infective stages in the environment, annual pulses of host births and deaths, and changes in host immune defenses (Altizer *et al.* 2006). Vector-borne parasites are among those most likely to co-vary with environmental conditions, and heartworm is no exception.

For closely linked parasites, the ability to alter host fitness and behavior is required for a successful relationship. Several parasite systems demonstrate synchronicity between parasites and their vectors (Hurd 2003). For example, *D. immitis* numbers increase in peripheral circulation during the summer to coincide with an increase in mosquito numbers (Otto 1969). As with most ecological relationships, vector transport is a fitness tradeoff. A high hyper-parasite load affects mosquito dispersal. Moore and Gotelli (1990) and Sousa and Grosholz (1991) demonstrated that *Anopheles* and *Aedes* mosquitoes, infected with *Plasmodium* and heartworm respectively, travel

shorter distances than uninfected mosquitoes. Thus, it is the dispersal capabilities of the mammalian host coupled with those of the vector that spread the disease.

Many parasitic nematodes, including perhaps heartworm, possess genetic features that favor the development of drug resistance (Kaplan 2004). Rapid rates of nucleotide substitution combined with large effective population sizes translate into high levels of genetic diversity (Maizels and Kurniawan-Atmadja 2002, Schwenkenbecher *et al.* 2006, Grillo *et al.* 2007). Additionally, many studies of nematodes have shown a population structure with high levels of gene flow (Blouin *et al.* 1995, Johnson *et al.* 2006). These organisms not only possess the genetic potential to respond successfully to chemical attacks, but also the means to assure the dissemination of resistance alleles (Kaplan 2004). Anthelminthic resistance has been documented in small ruminants since the 1950s and appears to be irreversible (Drudge 1957, Leathwick 2001, Mortensen *et al.* 2003, Kaplan 2004, McKeller and Jackson 2004, Wolstenholme *et al.* 2004, Prichard 2005, Geary 2005, Garretson *et al.* 2009, Traversa *et al.* 2009).

D. immitis is capable of establishing two alternate associations with its host: a chronic infection within canids, or a dead end infection within all other mammals. In dogs, microfilariae migrate to the heart, mature, and reproduce. The pulmonary vasculature is affected and inflammatory reactions are critical to the development of the disease process. Adult worms release microfilariae into the host's blood stream to be ingested by the mosquito vector during a blood meal. Immature stages must molt in the digestive system of the vector in order to become infective. In felids, the parasite takes longer to migrate from the infection site and mature, and most infections are amicrofilaraemic (Simon *et al.* 2007). Dead end hosts are not able to harbor viable

heartworm infections. The adult worms either migrate to the wrong place (e.g. lungs or muscle tissue) or are unable to produce microfilariae (Solaini *et al.* 2008). The immunopathology of the disease is complex, and clinical manifestations depend on the immune response stimulated by the parasite. The interpretation of heartworm pathogenesis has changed recently with the rediscovery of their bacterial symbionts. Similar to other filarids, *D. immitis* possesses a *Wolbachia* bacterial endosymbiont (Sironi *et al.* 1995).

Wolbachia is a matrilineally inherited obligate intracellular α2 proteobacterium (order *Rickettsiales*) that infects numerous invertebrate species. In arthropods, it acts as a reproductive parasite and has been shown to purge mitochondrial diversity in these systems (Pfarr and Hoerauf 2006). These “sweeps” can establish a single mitochondrial lineage within an invertebrate population (Galtier *et al.* 2009). Its effect on mitochondrial diversity has yet to be examined in a nematode system.

Wolbachia’s relationship with nematodes is characterized by stable infection, cospeciation, and a relatively long coevolutionary history (Werren *et al.* 1995, Bandi *et al.* 2001, Werren *et al.* 2008). *Wolbachia* are essential for larval development in the mammalian host and for long-term survival of adult worms (Hise 2004, McGarry *et al.* 2004). Found in the lateral chords of both sexes and in the female reproductive apparatus, the bacteria retain intact metabolic pathways for the production of flavins and nucleotides (Foster *et al.* 2005). The genus *Wolbachia* is currently divided into eight taxonomic super-groups based primarily on 16S, *wsp,* and *ftsZ* gene phylogenies (Werren *et al.* 1995, Bandi *et al.* 2001, Casiraghi *et al.* 2001, Casiraghi *et al.* 2004, Baldo *et al.* 2005, Fenn *et al.* 2006). Vertical transmission implies that these bacteria experience little

recombination or are subject to gene transfer; however, there is some discordance within the phylogenies of the *Wolbachia* surface protein (*wsp)* (Jiggins *et al.* 2001, Jiggins 2002, Reuter and Keller 2003). They also contribute to the spectrum of pathogenic effects of filarial infections (Bazzocchi *et al.* 2000, Brattig *et al.* 2004, Simon *et al.* 2008). Antigenicity of *Wolbachia* has been implicated in filarial inflammatory responses indicating molecules released from the worm prompt an immune reaction from the vertebrate host (Bazzocchi *et al.* 2000, Brattig *et al.* 2004). In amicrofilaraemic infections, detection of circulating antigens is a method of diagnosis (Casiraghi *et al.* 2006).

An investigation of heartworm evolution and population dynamics

The spread and persistence of zoonotic macroparasites among multiple host species is poorly understood and relatively unexplored. It has been shown in many instances that patterns of evolution are influenced by human activities. Canine heartworm offers a unique opportunity to assess the genetic variability and population structure of a prevalent parasite in an effort to establish baseline genetic parameters that can be used to model the spread of resistance alleles.

The approach this research takes in the investigation of canine heartworm evolutionary dynamics is as follows:

1: Examine mitochondrial diversity in a *Wolbachia*-infected nematode

2: Determine the level of genetic variability of heartworm populations in the United States using microsatellite loci

3: Model the dispersal of resistance alleles in heartworm populations.

Chapter Two looks at the mitochondrial diversity of *Wolbachia*-infected filarial parasite. Chapter Three describes the genetic variability and population structure of canine heartworm across North America. Chapter Four utilizes the population parameters estimated from the previous chapters to create a model of population dynamics that will help illuminate the possible spread of resistance alleles. I discuss the implications of this research and future possibilities in Chapter Five.

Chapter 2: *Wolbachia* infection and mitochondrial diversity in the canine heartworm (*Dirofilaria immitis*)

Abstract

Many species of filarial nematodes are infected with *Wolbachia pipientis*, a maternally inherited endosymbiont. In addition to manipulating host reproduction, these bacteria also affect the evolution of the mitochondrial DNA with which they are transmitted. Selective sweeps can establish a single mitochondrial lineage within a *Wolbachia*-infected population and purge genetic diversity. While this phenomenon has been studied in insect model systems, it has not been thoroughly examined in a filarial nematode. Patterns of mitochondrial diversity were examined in *Dirofilaria immitis*, a *Wolbachia*-infected species. The levels of genetic diversity observed in canine heartworm were much lower than those in related species not known to be hosts of *Wolbachia*. Results suggest that a maternally inherited endosymbiont can depress mitochondrial diversity in a filarial host.

Belanger DH, Perkins SL. 2010. *Wolbachia* infection and mitochondrial diversity in the canine heartworm (*Dirofilaria immitis*). Mitochondrial DNA 21(6): 227-233

Introduction

Wolbachia pipientis is a matrilineally inherited obligate intracellular α2 proteobacterium (order Rickettsiales) that infects numerous invertebrate species including many arthropods and nematodes. The genus is currently subdivided into eight supergroups based primarily on sequence information from the cell division protein gene *ftsZ*, *Wolbachia surface protein* (*wsp*), and 16S rRNA (Werren et al. 1995, Bandi et al.1998, Bandi et al. 2001, Casiraghi et al. 2001, 2004, Fenn et al. 2006). *Wolbachia* infections are associated with a variety of phenotypic effects that, while adaptive for the bacteria, have far-reaching ramifications for the population biology of the hosts (Werren et al. 2008, Nice et al. 2009).

Arthropod-associated *Wolbachia* are typically reproductive parasites that enhance their own transmission by altering host reproduction. The bacteria may cause cytoplasmic incompatibility (Tram and Sullivan 2002), induce parthenogenesis (Weeks and Breeuwer 2001), feminize males (Negri et al. 2006, Negri et al. 2008), or kill male offspring (Jiggins 2003). Each of these manipulations serves to increase the number of infected females in a population (Werren et al. 2008). *Wolbachia* strains are initially associated with a single mtDNA haplotype and as the bacterial infection spreads throughout a population so do the associated mitochondria (Galtier et al. 2009). This situation can cause severe bottlenecks in mitochondrial haplotypes due to indirect selection of matrilineally inherited genes associated with bacterial endosymbionts (Hurst and Jiggins 2005, Werren et al. 2008). Endosymbiont-driven selective sweeps are well documented in insect systems (Turelli and Hoffmann 1991, Jiggins 2003, Shoemaker et

al. 2004, Hurst and Jiggins 2005, Raychoudhury et al. 2010), but they have never been thoroughly examined in a filarial nematode species.

Wolbachia's relationship with filarial nematodes is characterized by stable infection, cospeciation, and a relatively long coevolutionary history (Werren et al. 1995, Bandi et al. 1998, Bandi et al. 2001, Casiraghi et al. 2001, 2004, Fenn and Blaxter 2006). There is a general concordance between the phylogeny of the bacteria and the phylogeny of its hosts (Casiraghi et al. 2004, Werren et al. 2008). A *Wolbachia* infection is essential for larval development in the mammalian host and for long-term survival of adult worms (Hise et al. 2004, McGarry et al. 2004). The bacterium also contributes to the spectrum of pathogenic effects of filarial infections (Bazzocchi et al. 2000, Brattig et al. 2004, Kozek 2005, Simon et al. 2008). Antigenicity of *Wolbachia* has been implicated in filarial inflammatory responses indicating that molecules released from the worm prompt an immune reaction from the vertebrate host (Bazzocchi et al. 2000, Brattig et al. 2004). Only a few species of filarids lack *Wolbachia* including: *Setaria equina* (Chirgwin et al. 2002), *Oncocherca flexuosa* (Plenge-Bonig et al. 1995), *Loa loa* (McGarry et al. 2003), and *Acanthocheilonema vitae* (Casiraghi et al. 2004).

Dirofilaria immitis, canine heartworm, is a filarial nematode (Nematoda; Onchocercidae) that infects wild and domestic species worldwide. Like most filarids and many arthropods, this parasite harbors a *Wolbachia* endosymbiont, which is found in the lateral chords of both sexes and in the female reproductive system (Sironi et al. 1995, Foster et al. 2005). Phylogenetic analyses of this nematode and its relatives and their *Wolbachia* symbionts have been done (Casiraghi et al. 2004); however, little is known about mitochondrial DNA diversity in *D. immitis* or how a *Wolbachia* infection is

influencing that diversity. To that end, several fundamental questions about *D. immitis* must be answered: What pattern of mtDNA diversity is observed in canine heartworm? Does it differ from *Wolbachia*-free species? If there is a reduction in genetic diversity, is it the result of an endosymbiont-driven selective sweep? Is a sweep currently occurring? We will address these questions using both mitochondrial and *Wolbachia* loci.

Materials and Methods

Samples and DNA extraction

We obtained *D. immitis* specimens through donation from veterinarians, researchers, trappers, and wildlife officials (Figure 1). Nematodes were opportunistically collected in high prevalence areas from domestic dogs (*Canis familiaris*), domestic cats (*Felis catus)*, coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), white-nosed coati (*Nasua narica*), and black-footed ferrets (*Mustela nigripes*). Heartworms were fixed in 70-95% ethanol or 20% dimethylsulfoxide (DMSO) saturated with sodium chloride and they were stored in 100% ethanol at -20°C until the DNA extraction. To extract genomic DNA, we subsampled a segment of each nematode (~15 mg) and then followed the manufacturer's protocols for tissue samples for the DNeasy Tissue kit (Qiagen). Extracted DNA was stored at -20°C.

DNA sequencing

We surveyed the genetic variation in *Wolbachia* by sequencing a portion of the *wsp* (328 bp) and *ftsZ* (661 bp) genes and assessed the mitochondrial variation of *D. immitis* by sequencing 1644 bp of the cytochrome c oxidase subunit I (*cox1*) gene from

37 individuals. A shorter fragment of *cox1* (451 bp) was amplified and sequenced from 210 additional samples. Six additional pairs of *Wolbachia wsp* and *ftsZ* sequences were included to increase geographic representation.

We performed polymerase chain reactions (PCR) in 25 μL volumes containing 1 μL extracted DNA, 1 μL of each 10mM forward and reverse primer, 2.5 μL 25mM $MgCl_2$, 2 μL PCR buffer A (Thermo Fisher Scientific), 1 μL 8mM dNTP mixture, 1 μL 10x BSA, 0.2 μL *Taq* polymerase (Thermo Fisher Scientific) and 15.3 μL H_2O. All primers are listed in Table 1. After an initial denaturation at 94°C for 2 min, the reactions were subjected to 35 cycles of amplification as follows: *cox1* (94°C for 20 s, 52°C for 20 s, 70°C for 60 s), *wsp* (94°C for 45 s, 52°C for 45 s, 72°C for 1.5 min), or *ftsZ* (94°C for 15 s, 55°C for 60 s, 72°C for 3 min). Each thermal profile concluded with a 10 min extension at 72°C.

Phylogenetic analysis

Gene sequences (*cox1, wsp,* and *ftsZ*) were edited using Sequencher v4.2 (Gene Codes Corp.) and, due to the extremely low level of sequence variation in these datasets, could simply be aligned in the same program. Phylogenetic relationships among sequences were investigated using maximum likelihood in RAxML v7.0.4 (Stamatakis 2006) on the CIPRES Portal v2.1 (www.phylo.org). Clade support was evaluated with 1000 rapid bootstrap replicates (Stamatakis et al. 2008). An *Onchocerca volvulus* sequence (GenBank accession number AM749285) was used as the outgroup in the evolutionary analysis, as it is a closely related filarial nematode (Hu et al. 2003, Hu and Gasser 2006).

Patterns of diversity

We used TCS v1.21 (Clement et al. 2000) to construct a statistical parsimony network with a connection threshold of 95% for *D. immitis* mitochondrial haplotypes. Levels of polymorphism were estimated with DnaSP v5.10.01 (Librado and Rozas 2009) and statistical significance was assessed with 1000 coalescent simulations. The number of haplotypes, haplotype diversity (H_d), and nucleotide diversity (π; Nei 1987) were calculated for all *a priori* populations as well as across the sample range. Additionally, the number of segregating sites (θ_W; Watterson 1975) was estimated for the entire dataset. In the case of haploid maternally inherited mtDNA, both θ_W and π are unbiased estimators of $N_{ef}\mu$ under the assumption of neutrality. N_{ef} is the effective population size of females and μ is the locus mutation rate. F_{ST}, based on observed haplotypes, was calculated to assess population differentiation (Hudson et al. 1992). Intraspecific neutrality tests (Tajima's *D* and Fu and Li's *D**) were conducted to determine if the number of polymorphisms observed in the data differed from predictions of neutral evolution. At a neutral equilibrium, both statistics should be zero. Tajima's *D* considers the difference between θ_W and π, and measures neutrality as reflected in low and intermediate frequency alleles. A negative result indicates an excess of low frequency polymorphisms that could be attributed to population expansion or a recent selective sweep (Tajima 1989). A positive result reflects an excess of intermediate frequency alleles resulting from a decrease in population size, population structure, or balancing selection. While Tajima's *D* can detect genetic bottlenecks, population substructure, and selective sweeps, it cannot distinguish between them (Nielsen 2001). Fu and Li's *D**,

which is based on coalescent theory, compares the mutations of a recent past with those of a remote past in order to detect selection (Fu and Li 1993). It is based on the proportion of singleton polymorphisms as compared to the total number of segregating sites. Similar to Tajima's *D*, negative values of Fu and Li's *D** reflect an excess of low frequency haplotypes that can be attributed to population expansion or selection. Genetic hitchhiking was assessed using Fay and Wu's *H* (H_{FW}). This statistic quantifies the frequency of derived mutations by using an outgroup to deduce the ancestral state. It measures departures from neutrality based on the difference between intermediate and high frequency haplotypes. A negative value of H_{FW} indicates an excess of high frequency derived mutations. This is typical for linked loci under selection (Fay and Wu 2000). An *O. volvulus* sequence (GenBank accession number AM749285) was used as the outgroup.

Results

Analysis of 247 *cox1* sequences (451 bp) from *D. immitis* from North America revealed 14 unique haplotypes. Nucleotide variation was reflected in 17 substitutions at 16 polymorphic sites. For the *a priori* populations (Figure 1), the number of haplotypes, haplotype diversity, and nucleotide diversity were calculated (Table 2). Haplotype diversity ranged from 0.4 to 0.76 and nucleotide diversity ranged from 0.001 to 0.004. The Georgia population contained the highest number of haplotypes (h = 7) while the California samples were the most diverse (H_d = 0.76). In general, the level of genetic diversity was consistent throughout the sampling area. F_{ST} revealed no statistically significant population structure.

The maximum likelihood analysis of phylogenetic relationships for the larger fragment of *cox1* (1644 bp; N = 37) showed no geographic patterning and no subdivision based on host taxon or domesticity status (Figure 2). The bootstrap values of all nodes were under 50% and this is likely due to the great similarity between sequences. Figure 3 presents a more extensive picture of mitochondrial diversity by utilizing sequence data from an additional 210 samples.

The *cox1* haplotype network reveals a relationship that displays no readily apparent correlation between haplotype and geographic origin (Figure 3). This complexity could be due to genetic exchanges between heartworm populations via host or vector dispersal – dispersal that could be natural or human-mediated. The predominant genotype (WI18) was found in all sampled populations. Overall, a pattern of low mitochondrial diversity was revealed with relatively few alternative connections occurring between haplotypes within the network.

The average nucleotide diversity (π) for *cox1* in *D. immitis* across the sampling area was 0.0017. This value was similar to those for *Nasonia vitripennis* and *Drosophila recens*, both *Wolbachia*-infected, and degree of magnitude lower than the values for *Necator americanus, Mecistocirrus digitatus,* and *Drosophila subquinaria* which are not infected (Table 3). The level of diversity in heartworm based on the number of segregating sites ($\theta_W = 0.0058$) was three times higher than the level of diversity established by π. In contrast, θ_W and π values for the *Wolbachia*-free species were approximately equal. Both Tajima's *D* and Fu and Li's *D** revealed statistically significant negative values for *D. immitis*; therefore, the hypothesis of neutrality is

rejected (Table 3). The *cox1* sequences of the other species did not deviate from expectations of neutral evolution.

All 43 isolates of *Wolbachia* from *D. immitis* had identical *wsp* sequences and all but three individuals had identical *ftsZ* sequences. The three samples, all from the Wisconsin population, had one nonsynonymous transition at position 452 altering the amino acid sequence from glycine to arginine.

Discussion

Wolbachia pipientis was first identified in the 1920s and current meta-analyses estimate that 65% of insect species harbor this bacterial endosymbiont (Hilgenboecker et al. 2008, Werren et al. 2008). Fitness experiments show that the bacterium's interspecific interactions run the gamut from parasitic to mutualistic (Turelli and Hoffman 1991, Dean 2006). While its reproductive manipulations have been well established in arthropod systems, little is known about the genetic influence it exerts on filarial nematodes. Due to coevolutionary processes, selection favors *Wolbachia* as a fit phenotype in these species. Without this endosymbiont, adult filarial nematodes suffer a decline in survivability and reproductive output. As a *Wolbachia* infection is driven to fixation, the associated mitochondria hitchhike into each successive generation causing a decline in mitochondrial diversity (Turelli and Hoffmann 1991, Jiggins 2003, Shoemaker et al. 2004, Galtier et al. 2009).

As a working hypothesis, we suggest that the level of genetic diversity in *D. immitis* has been depressed by its *Wolbachia* infection. We calculated two summary statistics of nucleotide variation (θ_W, π) and from these we calculated several tests of the

standard neutral model including Tajima's *D*, Fu and Li's *D**, and Fay and Wu's *H*. We also constructed a phylogenetic tree for *cox1*.

Despite identifying 14 unique haplotypes within the sampling area, phylogenetic analysis of *cox1* sequences revealed no geographic patterning and no patterning based on host taxon or domesticity. The *Wolbachia* loci, *wsp* and *ftsZ*, were completely conserved. This suggests that all *Wolbachia* within canine heartworm populations in North America are descended from a single infected female nematode or that multiple infections lost their genetic diversity over time. The mutation rate of mitochondrial DNA is higher than that of *wsp*; therefore, a difference in the number of observed polymorphisms at these two loci is not unexpected (Schulenburg et al. 2000, Raychoudhury et al. 2009). Previous studies show that there is little genetic variation in *Wolbachia* strains and that the bacterium has an estimated mutation rate 120-fold lower than the host's mitochondrial DNA (Raychoudhary et al. 2009, 2010).

A comparison between *N. americanus*, *M. digitatus*, and *D. immitis* showed significant mitochondrial diversity in the former nematode species and a profound reduction in diversity in the latter (Table 3). A similar pattern can be seen when heartworm is compared with the *Wolbachia*-free insect *D. subquinaria* (Shoemaker et al. 2004). When the number of segregating sites and nucleotide diversity were assessed, the values for *D. immitis* were a degree of magnitude lower than those of the uninfected insect. A conclusion of disproportionately low mitochondrial diversity is supported by a comparison between *D. immitis* and other invertebrate species. Demographic fluctuations or an endosymbiont-driven selective sweep could explain this lack of genetic diversity. Neutrality statistics were used to determine which mechanism likely caused the effect.

Tajima's *D*, which measures the difference between nucleotide diversity and the number of segregating sites, is sensitive to population expansion and contraction because the number of segregating sites responds more rapidly to changes in population size than the nucleotide diversity. Fu and Li's *D** measures the difference between high and medium frequency polymorphisms. Table 3 shows that both of these statistics were significantly less than zero for *cox1* in *D. immitis*. The concordance between neutrality tests indicates an excess of low frequency polymorphisms and suggests that a selective force may be influencing *cox1* variation in *D. immitis*. Additionally, the population may also be experiencing a demographic effect. The mitochondrial sequences from the uninfected species did not deviate from neutral expectations.

Fay and Wu's *H* tests for genetic hitchhiking by examining the frequency of derived mutations and measuring departure from neutrality based on the difference between intermediate frequency and high frequency haplotypes. Unlike Tajima's *D*, H_{FW} is not sensitive to population expansion. In the case of canine heartworm, H_{FW} = -0.613 (P>0.1) was not significantly different than zero. This suggests that *D. immitis* is not currently experiencing a mitochondrial sweep. It appears that heartworm populations are in a recovery phase between sweeps and could be recovering diversity for millions of generations (Avise 2000).

In canine heartworm, results suggest that decreased diversity and departure from neutral expectations are most likely due to mtDNA hitchhiking with a *Wolbachia* infection. The H_{FW} statistic becomes more conservative with the pooling of subpopulation data; thus, non-significance is not an artifact of sample size (Fay and Wu 2000). These results are consistent with patterns of diversity observed in other

Wolbachia-infected organisms (Jiggins 2003, Shoemaker et al. 2004, Raychoudhury et al. 2010). They also demonstrate that mitochondrial markers, while popular for population genetics research, are inappropriate in these species (Galtier et al. 2009). Symbiont-driven selective sweeps confound the inference of an organism's evolutionary history from mtDNA (Hurst and Jiggins 2005).

Conclusions

This research provides a unique opportunity to expand our knowledge of the *Wolbachia* – filarial nematode interaction. The data presented here suggest that *Wolbachia* affects the mtDNA diversity of filarial nematodes in a manner similar to that found in insect systems. Additional comparative studies, focused on *Wolbachia*-free species, would more comprehensively support the conclusion of low mitochondrial diversity in canine heartworm and present a clearer picture of the relationship between filarial nematodes and their endosymbionts.

Acknowledgements

Numerous veterinarians, researchers, wildlife officials and trappers across the country enthusiastically provided us with heartworm specimens. We would like to thank E. Greiner (University of Florida), T. Craig and D. McIntosh (Texas Agriculture and Mining University), T. Nolan (University of Pennsylvania), B. Sacks, J. Theis, and S. Nadler (University of California Davis), E. Howerth (University of Georgia), P. Payne (Kansas State University), J. M. Lockhart (Valdosta State University), D. Paulsen (Louisiana State University), N. Heininger (Princeton University), and the following people from USDA-

APHIS: A. Piaggio, Y. Toxqui, S. Swafford, M. Pipas, M. Milleson, D. Emanueli, K. Van Why, G. Wiscomb, S. Woodruff, T. Felix, T. Puckett, B. Leland, L. Tschirhart-Hejl, W. Gaston, D. Hughes, D. LeBlanc, K. Shively and B. Zscheile. R. F. Rockwell provided useful comments on the manuscript. Without their help, this research would not have been possible.

Table 1. Primer sequences for *Wolbachia pipientis* and *Dirofilaria immitis*.

Locus	Primer Sequence (5′-3′)	Fragment size (bp)
*cox1**	F: GTCTGCCTGTGATGTTTATAATGTAT mid-F: GGGTCAGCCTGAGTTATCTT R: CCCAAACACATAGAATAATAACATCA mid-R: CCAATACCAACAGTATGAAGACCTAA	1644
wsp¶	F: GTCCAATA(AG)STGATGA(AG)GAAAC R: C(CT)GCACCAA(CT)AG(CT)(AG)CT(AG)TAAA	328
ftsZ§	F: GG(CT)AA(AG)GGTGC(AG)GCAGAAGA R: ATC(AG)AT(AG)CCAGTTGCAAG	661

* Previously unpublished; four primers were required to sequence the entire *cox1* gene.
¶ Baldo et al. (2006)
§ Casiraghi et al. (2001)

Table 2. Regional nucleotide diversity estimates for *cox1* sequences from 247 *Dirofilaria immitis* samples.

Region	N	h	H_d	π
New Jersey	22	3	0.40	0.001
Georgia	36	7	0.50	0.004
Florida	34	4	0.51	0.001
Alabama	18	3	0.52	0.001
Louisiana	31	4	0.63	0.002
Texas	54	4	0.56	0.001
Mexico	19	3	0.50	0.001
Wisconsin	22	2	0.53	0.001
California	11	4	0.76	0.003

N, number of sequences; h, number of haplotypes; H_d, haplotype diversity; and π, nucleotide diversity.

Table 3. Nematode and insect nucleotide diversity estimates and tests for departure from neutrality for *cox1*.

Species	N	L	h	H_d	S	θ_W	π	D_T	D^*
Dirofilaria immitis	247	451	14	0.57	16	0.0058	0.0017	-1.843†	-3.028†
*Nasonia vitripennis**	52	399	9	–	7	–	0.0031	-0.880	–
Drosophila recens‡	58	1411	23	–	25	0.0038	0.0014	-2.040†	-3.422†
Necator americanus¶	100	395	59	0.95	75	0.0398	0.0250	-1.120	-1.505
Mecistocirrus digitatus§	12	830	12	1.00	84	0.0335	0.0360	0.105	0.629
Drosophila subquinaria‡	26	1411	25	–	83	0.0150	0.0140	-0.297	-0.390

N, number of sequences; L, average length of sequences in basepairs; h, number of haplotypes; H_d, haplotype diversity; S, number of polymorphic sites; D_T, Tajima's *D*; and *D**, Fu and Li's *D**. †$P<0.05$

¶ Hu et al. (2008)

§ GenBank accession numbers AB245047-58

‡ Shoemaker et al. (2004)

* Raychoudhury et al. (2010)

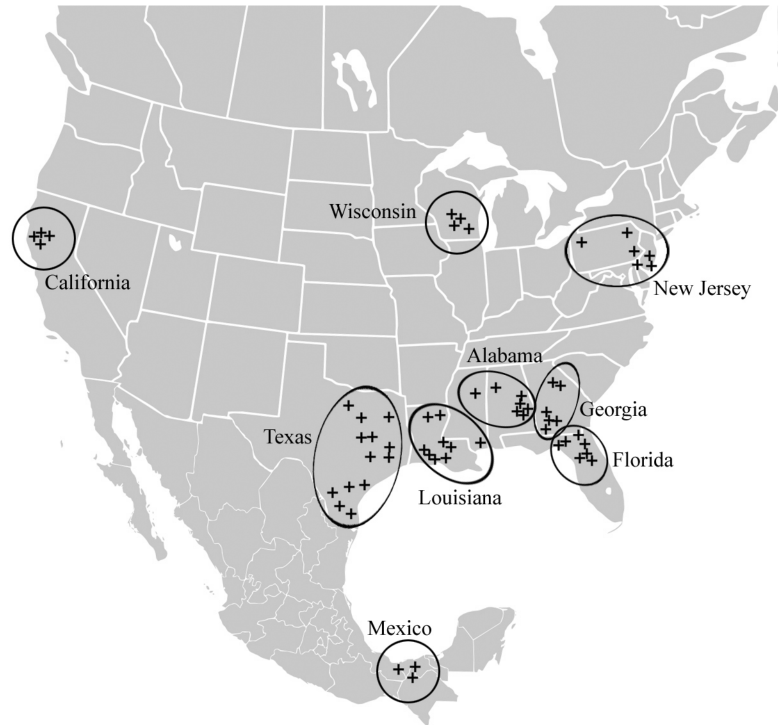

Figure 1. Geographic distribution of *Dirofilaria immitis* samples. Ovals represent sampled regions.

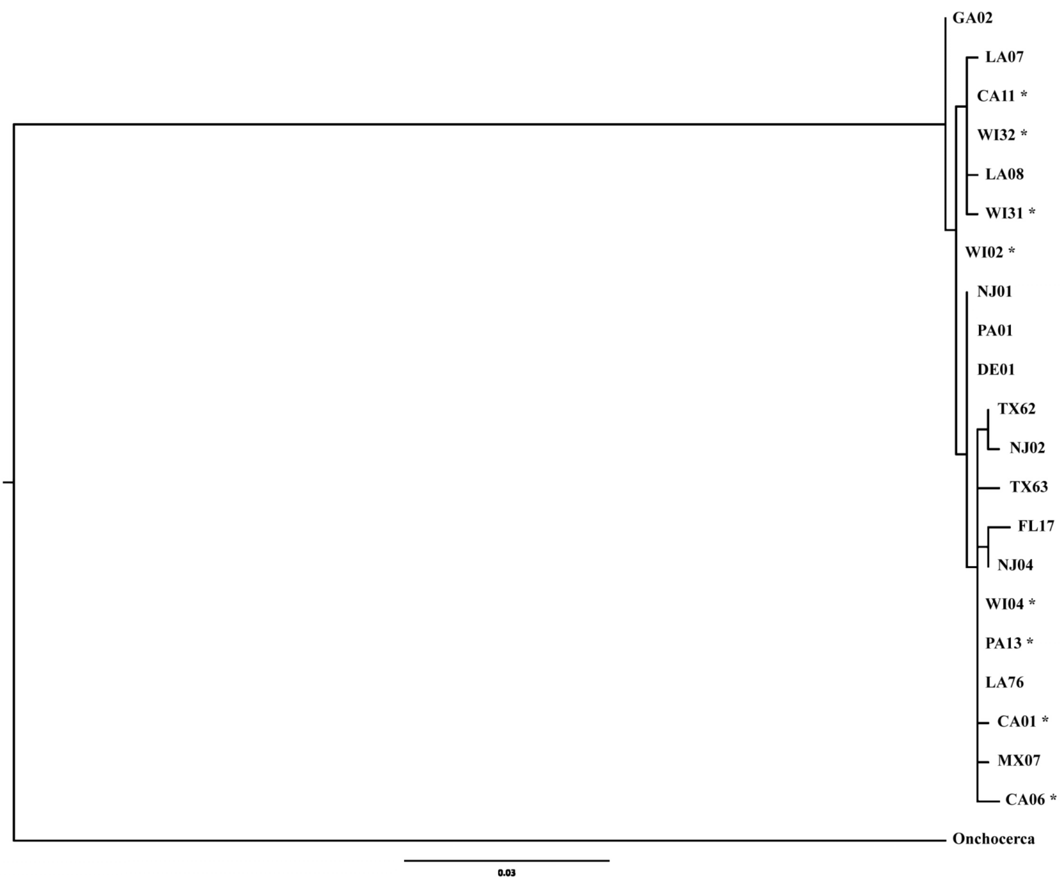

Figure 2. Maximum likelihood phylogenetic analysis of *cox1* sequences from 37 *Dirofilaria immitis* samples. Twenty-one unique haplotypes were identified. The scale indicates an estimate of substitutions per site. Samples from wild hosts are marked with an asterisk. All bootstrap values were <50% (not shown). Collection locality name abbreviations: CA, California; DE, Delaware; FL, Florida; GA, Georgia; LA, Louisiana; MX, Mexico; NJ, New Jersey; PA, Pennsylvania and WI, Wisconsin.

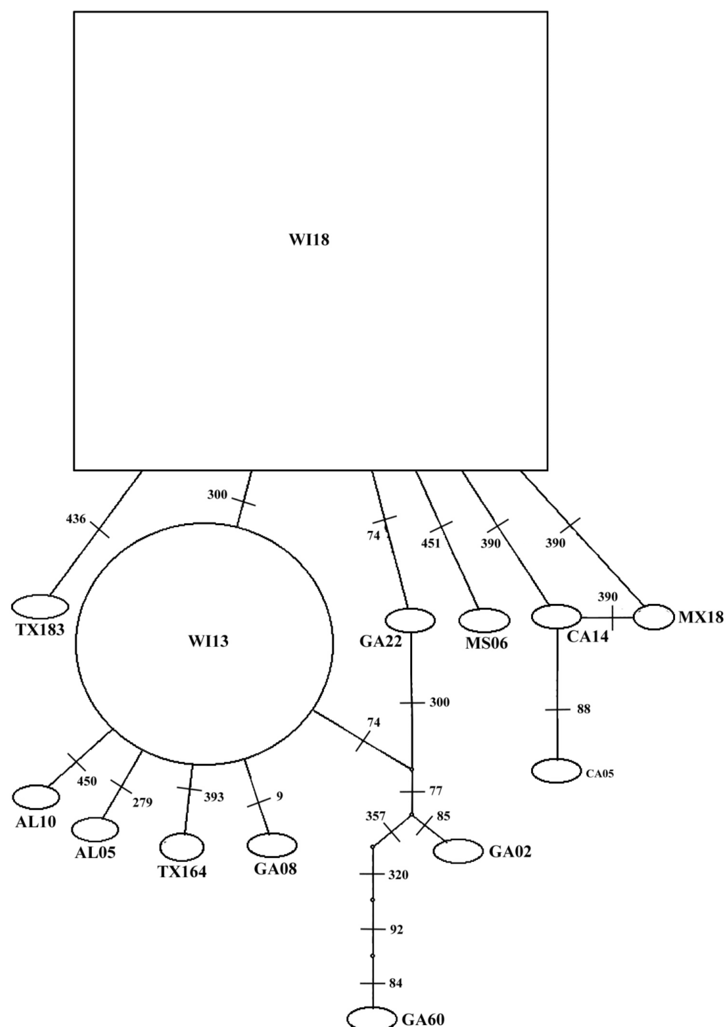

Figure 3. Haplotype network of *cox1* sequences within *Dirofilaria immitis*. The frequency of each haplotype is proportional to its area. The numbers on the network indicate nucleotide positions of mutations within the *cox1* sequences. For locality name abbreviations in the haplotype, see Figure 2.

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Chapter 3: Inference of population structure and patterns of gene flow in canine heartworm (*Dirofilaria immitis*)

Abstract

Understanding the genetic variation within a parasitic species is crucial to implementing successful control programs and preventing the dispersal of drug resistance alleles. We examined the population genetics and structure of canine heartworm (*Dirofilaria immitis*) by developing a panel of 11 polymorphic microsatellite loci for this abundant parasite. In total**,** 192 individual nematodes were sampled from 9 geographic regions in North America and Mexico and genotyped. Population genetic analyses indicate the presence of 4 genetic clusters. The canine heartworm samples used in this study were characterized by low heterozygosity with eastern and central North America experiencing high levels of reciprocal gene flow. Geographic barriers impede the movement of vectors and infected hosts west of the Rocky Mountains and south of the Central Mexican Plateau. This, combined with corridors of contiguous habitat, could influence the spread of drug resistance alleles.

Belanger DH, Perkins SL, Rockwell RF. Inference of population structure and patterns of gene flow in canine heartworm (*Dirofilaria immitis*). Journal of Parasitology (in press).

Introduction

Local differentiation can occur when a species occupies a large territory. Although geographic barriers favor the formation of local colonies, some differentiation can occur without them. In the case of parasites, low dispersal rates of either the vector or host may prevent a species from forming a panmictic unit and result in a structured metapopulation. The extent of sub-structuring depends both on the level of dispersal and the genetic effective sizes of local populations. The pattern and degree of substructure can have profound effects on the response of the parasite to selection in general and on the spread of drug resistance in particular.

Heartworm disease is a mosquito-borne, parasitic infection by *Dirofilaria immitis* nematodes found in temperate and tropical animal populations worldwide. In the contiguous United States, the infection is most prevalent along the Gulf Coast and Mississippi River. Most commonly found infecting domestic dogs and other species of *Canis*, *D. immitis* also parasitizes cats, foxes, ferrets, and other mammals, including humans (Pence et al., 2003; Riley et al., 2004; Sacks et al., 2004). Adult worms live in the pulmonary arteries of the host, where mature females release microfilariae into the blood stream. Microfilariae are ingested by vectors during the course of a blood meal, and develop into third stage larvae within the malpighian tubules (Anderson 2000). The larvae migrate to the salivary glands in preparation for transmission, and are inoculated into the dermis of the final host with subsequent vector bites. Larvae reach the pulmonary arteries after several months of migration and maturation. Adult nematodes eventually compromise circulation through the entire right side of the heart and its associated blood vessels.

Parasitic nematodes, like canine heartworm, infect humans, livestock, companion animals, and wild populations worldwide. Current treatment regimes rely on a few broad-spectrum anthelmintics to eliminate infection; however, the use of these compounds can select for drug resistance in parasite populations under strong coverage. This scenario is exacerbated by a lack of population genetic information for many parasitic species. Knowledge of population genetic structure and molecular epidemiology allows for control measures to be designed for maximum impact while concurrently mitigating the evolution of resistance (Gilleard and Beech, 2007). Information about population genetic structure underpins the study of drug resistance and its dispersal as well as being crucial to its prevention.

Anthelmintic resistance is a well-studied problem that has been documented in small ruminants since the 1950's (Drudge, 1957; Leathwick, 2001; Mortensen et al., 2003; Kaplan, 2004; McKeller and Jackson, 2004; Prichard, 2005; Garretson et al., 2009; Traversa et al., 2009). Selection plays an important role in the advent, dispersal, and maintenance of resistance alleles. Studies suggest that population genetic processes also influence the evolution of drug resistance (Cornell et al., 2003; Gilleard and Beech, 2007). Many parasitic species possess genetic features that favor the development of resistance (Kaplan, 2004). Rapid rates of nucleotide substitution, combined with large effective population sizes, translate into high levels of genetic diversity (Blouin et al., 1995; Schwenkenbecher et al., 2006; Grillo et al., 2007). Additionally, many studies of nematodes have shown a population structure with high levels of gene flow (Johnson et al., 2006; Webster et al., 2007). Thus, these species possess the genetic potential to respond to chemical attacks and the ability to spread resistance alleles (Kaplan, 2004).

Before one can predict the spread of drug resistance in *D. immitis*, at least 2 fundamental questions about this parasite's population genetic structure must be answered. First, are heartworms in the United States one large continuous population, or do they show geographic population structure? Second, what patterns of gene flow are consistent with any such population structure? To address these questions, we developed a panel of 11 polymorphic microsatellite markers for *D. immitis* and used them to analyze the population genetics of this common nematode. We hypothesize that *D. immitis* experiences some degree of reduced dispersal whereby forming regional genetic clusters.

Materials and Methods

Source of parasites and DNA extraction

Veterinarians and wildlife officials opportunistically collected *D. immitis* specimens from 9 geographic regions between June 2007 and September 2009 (see acknowledgements; Fig. 1). Adult heartworms were fixed in 70-95% ethanol or 20% dimethylsulfoxide (DMSO) saturated with sodium chloride. Samples were stored in 100% ethanol at -20 C until DNA extraction. Genomic DNA was extracted from a segment (~15 mg muscle tissue) of each nematode using the DNeasy Tissue kit (Qiagen, Valencia, California), following the manufacturer's protocol for tissue samples. With female specimens, we took care to avoid reproductive tissue that might contain male DNA (Anderson et al., 2003). Extracted DNA was stored at -20 C until used for PCR. We sampled 1 nematode from each vertebrate host. Of the specimens used in this study, 111 were collected from domestic hosts (*Canis familiaris* and *Felis catus*) and 81 from

wild hosts (*Canis latrans*, *Nasua narica*, *Mustela nigripes*, and *Urocyon cinereoargenteus*) (Table I).

Microsatellite development

The 11 loci given in Table II were compiled from 2 genomic libraries constructed following Hamilton et al. (1999). The libraries were enriched using biotinylated oliogonucleotides (e.g., CA, AAC, and CAGC) and fragments were recovered with streptavidin-coated beads (Dynabeads, Invitrogen, Carlsbad, California). These fragments were reamplified and cloned using a TOPO-TA cloning kit (Invitrogen) according to the manufacturer's instructions. In total, 576 positive colonies were handpicked, submerged in 50 μl 10 mM Tris-HCl and 0.1 mM EDTA, boiled at 95 C for 5 min**;** 1 μl of the supernatant used as template for PCRs. Inserted fragments were amplified using M13(-20)F and M13R primers and sequenced in both directions using BigDye v3.1 on an ABI3730x1 capillary sequencer. Forward and reverse sequences were aligned using Sequencher v4.2 (Gene Codes Corp, Ann Arbor, Michigan). Candidate loci were identified using the Tandem Repeat Occurrence Locator (Castelo et al., 2002).

Microsatellite genotyping

Microsatellite analysis was performed using an M13-tailed microsatellite protocol adapted from Boutin-Ganache et al. (2001) where each forward primer was augmented on the 5' end with a CAG tag (5'-CAGTCGGGCGTCATCA-3'; see www.uga.edu/srel/DNA_LAB/protocols.htm). This altered primer was used in combination with fluorescently labeled forward CAG primer. Reactions consisted of 1 μl

DNA, 0.1 μl 10 μM forward primer, 0.6 μl 10 μM reverse primer, 2 μl 25 mM $MgCl_2$, 2 μl PCR buffer A (Thermo Fisher Scientific, Hampton, New Hampshire), 1 μl 8 mM dNTP mixture, 1 μl 10× bovine serum albumin, 0.1 μl *Taq* polymerase (Thermo Fisher Scientific) and 16.6 μl H_2O for a total volume of 25 μl. The thermocycler conditions were 94 C for 2 min followed by 35 cycles of 94 C for 15 sec, 55 C for 30 sec, and 72 C for 45 sec followed by a 5 min extension at 72 C. Allele sizes were separated and measured on an ABI3730 × l DNA Analyzer (Applied Biosystems, Foster City, California) using GeneScan LIZ600 internal size standard (Applied Biosystems). Individual chromatograms were analyzed using GeneMapper (Applied Biosystems) to determine the genotype of each individual. All genotypes were checked by eye with 20% of male specimens and all female specimens being genotyped twice to ensure accurate results.

Null alleles

We used MICROCHECKER to identify loci that might be harboring null alleles (Van Oosterhout et al., 2004). In most cases, null alleles are caused by a mutation in 1 of the primer binding sites, which prevents proper DNA amplification (Holm et al., 2001). To further examine this issue, we created an alternate panel of primers for the eight loci out of HWE, which lay outside the original priming sites. We selected 8 homozygous samples from across the study area to sequence. Reactions contained 1 μl extracted DNA, 1 μl of each 10mM forward and reverse primer, 2.5 μl 25mM $MgCl_2$, 2 μl PCR buffer A (Thermo Fisher Scientific), 1 μl 8mM dNTP mixture, 1 μl 10× bovine serum

albumin, 0.2 μl *Taq* polymerase (Thermo Fisher Scientific) and 15.3 μL H_2O. The same thermocycler conditions were used as for microsatellite genotyping.

Data analyses

Expected heterozygosity, observed heterozygosity, allelic diversity, Fisher's exact tests, and linkage disequilibrium were calculated by region and over all regions using GENEPOP v4.0.10 (Rousset, 2008). Pairwise F_{ST} values (Weir and Cockerham, 1984) were calculated using GENALEX 6 (Peakall and Smouse, 2006). We used a sequential Bonferroni correction to account for multiple comparisons (Rice, 1989). For highly variable loci the maximum F_{ST} among populations may be far less than 1 (Hedrick 2005); therefore, we standardized F_{ST} values following Meirmans (2006). Data were recoded to maximize the divergence between populations and calculate $F_{MAXIMUM}$. Wright's guidelines, as referenced by Conner and Hartl (2004), were used to classify F_{ST} values as low, moderate or high. Analysis of molecular variance (AMOVA) was conducted to test for population differentiation in GENODIVE v2.01b (Meirmans and Van Tienderen, 2004) and genotypes were permuted 1,000 times among the populations to determine significance.

Population structure among *D. immitis* samples was assessed using STRUCTURE v2.2 (Pritchard et al., 2000; Falush et al., 2003, 2007). To obtain a representative value of K for modeling the data, 5 independent runs of K from 1 to 15 were used to estimate the probable number of clusters. Multiple runs of the program across a large number of possible populations help evaluate the strength of evidence for the inferred groups (Pritchard et al. 2000). The burn-in period was set to 200,000 steps and probability

estimates were obtained using 10^6 MCMC iterations. An admixture model with correlated frequencies was used in all runs. To mitigate the increased variance between runs as K increased, the second order rate of change in Pr(X | K) was calculated as ΔK (Evanno et al., 2005). AIC weights, penalized for the number of parameters, were also computed for each model using the calculated likelihood of the data following Burnham and Anderson (2002). Data were also analyzed with BAPS v5.4. This program uses Bayesian assignment to determine the number of genetically distinct populations present in a sample based on allele frequencies (Corander et al., 2003; Corander, Marttinen et al., 2008; Corander, Siren, and Arjas, 2008; Tang et al., 2009). The maximum number of genetic clusters (K) ranged from 1 to 15 and we used 5 replicates for all values. Individual nematodes and their collection data were analyzed using the spatial clustering option. Spatial principal component analysis (sPCA) was conducted in GENALEX 6 (Peakall and Smouse, 2006). This multivariate method creates uncorrelated, synthetic variables and plots the major axes of variation in conjunction with geographic data.

BAYESASS+ v1.2 (Wilson and Rannala, 2003) was used to estimate the amount of migration between the genetic clusters identified by STRUCTURE, BAPS, and sPCA. This method assumes microsatellite loci are unlinked and estimates recent bidirectional, asymmetric migration rates. The burn-in period was set to 100,000 steps and estimates were obtained using 5×10^6 MCMC iterations. The sampling frequency was set at 2000.

Results

Microsatellite polymorphism, linkage disequilibrium, Hardy-Weinberg equilibrium, and null alleles

Preliminary analysis revealed no significant genetic difference (as measured by F_{ST}) between the heartworm samples of wild and domestic hosts or between heartworms of different genders; therefore, all data were pooled by region. The number of allelic variants ranged from 6 (D2 and H4) to 17 (C2), with the observed heterozygosity per loci per region being lower than expected (Table III). The Mexico samples harbored the greatest number of unique alleles ($A_U = 6$). All loci were tested for linkage disequilibrium. After a sequential Bonferroni correction, there were no significant associations between pairs of loci. Exact tests showed 8 loci departing from Hardy-Weinberg equilibrium (HWE) expectations.

Initially, we hypothesized that departure from HWE was most likely due to null alleles. Eight homozygous specimens from across the study area were genotyped and sequenced. Although this was a small sub-sampling of our total data set, the original genotypes were replicated with only minor discrepancies. Sequencing results showed no mutations to the original primer binding sites.

Pairwise genetic differentiation was significant among the sampling regions ($P < 0.01$ after Bonferroni correction). Standardized F_{ST} values ranged from 0.069 to 0.59 with 6 of 36 comparisons being relatively low ($F_{ST} < 0.077$). The majority of these low values were found between the sampling areas of the Gulf Coast and New Jersey. This is consistent with minimal genetic differentiation in these regions (Table IV). The Mexico population showed the highest level of genetic differentiation when compared to the other populations. Its F_{ST} values ranged from 0.437 to 0.59. Moderate to high levels of differentiation (F_{ST} values from 0.214 to 0.59) were also found between California samples and all others.

Population genetic structure

Among the sampled regions, AMOVA results provided evidence for genetic structure. *P*-values indicate that the null hypothesis, i.e., no partitioning of variance between hosts and individuals, is rejected in favor of a hypothesis of population structure. While genetic variation within individual samples accounted for 53.1% of the total, 35% of the variance occurred within the 9 geographic regions, and 11.9% of the variance was found between the regions ($P < 0.001$).

For 192 heartworms and 11 loci, results of the STRUCTURE analysis showed an improvement in Pr(X | K) when 2 population clusters were assumed rather than 1. There was a slight improvement for higher numbers of populations; however, no value of K produced a definitive peak in Pr(X | K) (Fig. 2A). AIC values suggested the greatest weight always corresponded to the largest K (data not shown). Evanno et al.'s (2005) ΔK statistic detected population structure at K = 4 and showed additional peaks at K = 8 and K = 14 (Fig. 3A).

To improve STRUCTURE's performance, the data set was censored to include only those loci not significantly departing from HWE. Figure 2B shows a more usual trend in Pr(X | K) values. A small, but identifiable, plateau at K = 4 can be seen and this value had the greatest AIC weight. The ΔK statistic showed a large peak at K = 2 and another at K = 4 (Fig. 3B). Figure 4A shows STRUCTURE's population assignments for K = 4. The Mexico samples group together with a significant amount of admixture elsewhere.

Analyzing the data with BAPS revealed 4 population clusters and a more specific distribution of genotypes. In Figure 4B, each color corresponds to an ancestral

population. Vertical bars represent individual nematodes and bars are divided into several colors when there is evidence of admixture. In North America, samples from east and west of the Rocky Mountains form 2 separate clusters. The eastern cluster included nematodes from New Jersey, Georgia, Florida, Alabama, and Texas with several individuals displaying admixed genotypes. Likewise, the western cluster (CA samples) was predominantly 1 genetic group, with a few admixed individuals. Heartworm samples from Wisconsin were an exception. This region harbored a fairly even mixture of genotypes from east and west of the Rockies. Heartworms from Mexico appear genetically isolated from all other samples.

Spatial PCA analysis was conducted to express genetic differences between populations in conjunction with geographic distance. The analysis revealed marked population subdivision at the extreme ends of the sample range. The first and second principle components describe 65.9% of the variation between the populations. The loadings for coordinate 1 are consistent with a split of California, Mexico, Wisconsin, and New Jersey from the Gulf Coast group. Coordinate 2 separates Wisconsin from New Jersey and supports New Jersey's association with the Gulf Coast samples. Relationships among the 4 distinct population clusters are depicted in Figure 5.

Estimates of recent migration (in migrants per generation) show the Gulf Coast population to be the largest emigration source, with strong ties to Wisconsin (Fig. 6). The Gulf region also exchanges migrants with California and Mexico, but geographic barriers reduce migration. Additionally, there is a genetic connection between California and Wisconsin.

Discussion

Population genetic structure in nematodes runs the gamut from panmixia (Johnson et al., 2006) to moderately and highly structured (Hawdon et al., 2001; Schwenkenbecher et al., 2006; Criscione et al., 2007; Grillo et al., 2007; Redman et al., 2008). Our data suggest that *D. immitis* has structured populations, but they are not as highly structured as some nematodes that parasitize insects (Blouin et al., 1999). In terms of levels of diversity within, and among, populations, the genetic structure of canine heartworm is similar to that of *Wuchereria bancrofti*, a vector-borne filarial parasite of humans (Churcher et al., 2008).

As a working hypothesis, we suggest that population structure in heartworm may be characterized by substantial gene flow between some geographic areas, while habitat barriers limit the dispersal of vectors and hosts in others. Eleven microsatellite markers and 3 methodologies were used to explore population structure. Traditional *F*-statistics, Bayesian modeling, and spatial PCA were able to uncover the relationships between populations and the results showed general agreement between the different methods.

Descriptive statistics indicate that canine heartworm exhibits a lower than expected heterozygosity. This reduction in genetic diversity could be due to any number of processes, including null alleles, indirect selection of microsatellite loci linked to coding genes, demography, or population sub-structuring undetected in this study. Although only a small number of samples were examined for null alleles, the results suggest that this is not the reason heartworm samples are more homozygous than expected. Intense localized sampling could potentially identify further nested population structure and provide a more definitive reason for heartworm's decreased heterozygosity.

Descriptive statistics provide evidence of population structure on a large geographic scale. Geographic barriers, such as the Rocky Mountains, Central Mexican Plateau, and Mojave-Sonoran desert transition mitigate vector dispersal, as they are inhospitable mosquito habitats (Barker et al., 2009; Venkatesan and Rasgon, 2010). *F*-statistics for California and Mexico heartworm samples support this statement. In other regions of the sampling range, vertebrate host movement and viable vector habitat translate into higher levels of gene flow and lower levels of genetic differentiation. For example, the Mississippi River provides a movement corridor for vectors and hosts that connects Gulf Coast populations to Wisconsin. *F*-statistics indicate heartworm samples from the Gulf Coast and New Jersey are less differentiated than those from California and Mexico.

Bayesian modeling in STRUCTURE returned mixed results. When utilizing the entire data set of 192 individuals and 11 loci, STRUCTURE was unable to recover a specific number of population clusters. Although Evanno et al.'s (2005) method did show a peak at K = 4, AIC values indicated that the greatest weight always corresponded to the largest value of K. When the analysis was run with a truncated data set of 192 individuals and the 3 loci in HWE, STRUCTURE's performance improved dramatically. It was able to recover 4 populations. This model had the greatest AIC weight.

STRUCTURE minimizes departures from HWE and there has been debate about its ability to analyze systems that have high levels of gene flow (Latch et al., 2006; Waples and Gaggiotti, 2006). In the case of canine heartworm, it seems unlikely that STRUCTURE's initial failure can be attributed to gene flow alone. The censored data set demonstrated that the software was able to identify populations using only 3 loci that did

not depart significantly from HWE. This suggests that STRUCTURE has difficulties minimizing departures from HWE in systems where there are substantial and, in this case biologically real, departures from HWE.

BAPS analysis also recovered population structure in canine heartworm. It utilizes geographic sampling information to assess the distribution of genotypes and determine which population substructures are empirically plausible (Corander et al., 2003). This analysis identified 4 population clusters and clearly indicates the great similarity of eastern regions (Fig. 4B). *Dirofilaria immitis* population genetic structure is most easily visualized with sPCA. Samples from the Gulf Coast strongly cluster together, while samples from California, Wisconsin, Mexico, and New Jersey are more removed.

The genetic evidence is persuasive that there are at least 4 genetically distinct *D. immitis* clusters in the sampling area. One might argue that New Jersey warrants its own population grouping as it is spatially removed from the rest of the Gulf Coast samples (Fig. 5). This ambiguity could be resolved with additional sampling between New Jersey and Georgia. Coordinate loadings place New Jersey close to, but separate from, Gulf Coast samples. Although geographic barriers prevent panmixia between eastern and western clusters, there is a significant amount of gene flow east of the Rocky Mountains (Fig. 6). The east coast of the United States and the Mississippi River provide contiguous habitats for host and vector movement. These corridors may promote high levels of gene flow between the Gulf Coast and these regions. Geographic barriers greatly restrict this movement to California and Mexico. This pattern of gene flow could certainly influence the spread of alleles beneficial to canine heartworm. A population, such as the Gulf

Coast, could disperse drug resistance alleles rapidly throughout the United States regardless of whether they arose in that geographic region or arrived there via dispersal.

This research not only illuminates population structure in canine heartworm, it also suggests patterns and levels of gene flow consistent with that structure. This information is vitally important to understanding the parasite's epidemiology and can inform the implementation of control measures that mitigate the onset of drug resistance. Additionally, it sets the stage for building a predictive model for the spread of any such resistance in canine heartworm, information that could save many canine lives.

Acknowledgements

We are grateful to E. Greiner (University of Florida), T. Craig, and D. McIntosh (Texas A&M University), T. Nolan (University of Pennsylvania), B. Sacks, J. Theis, and S. Nadler (University of California-Davis), E. Howerth (University of Georgia), P. Payne (Kansas State University), J. M. Lockhart (Valdosta State University), D. Paulsen (Louisiana State University), N. Heininger (Princeton University), and the following people from USDA-APHIS: A. Piaggio, Y. Toxqui, S. Swafford, M. Pipas, M. Milleson, D. Emanueli, K. Van Why, G. Wiscomb, S. Woodruff, T. Felix, T. Puckett, B. Leland, L. Tschirhart-Hejl, W. Gaston, D. Hughes, D. LeBlanc, K. Shively, and B. Zscheile for their generous assistance in sample collection. Mark Siddall provided valuable comments on an earlier draft of this manuscript. Financial support for this work was provided in part by the Sackler Institute for Comparative Genomics, American Museum of Natural History, New York, New York.

Table I. Distribution of *Dirofilaria immitis* samples by region and host type.

Region	Domestic Hosts	Wild Hosts
New Jersey (NJ)	4	-
Delaware*	1	-
Pennsylvania*	3	3
Georgia (GA)	6	19
Florida (FL)	26	13
Alabama (AL)	-	10
Louisiana (LA)	16	5
Mississippi†	3	2
Texas (TX)	33	13
Mexico (MX)	19	-
Wisconsin (WI)	-	6
California (CA)	-	10
Total	111	81

* pooled with New Jersey samples

† pooled with Louisiana samples

Table II. Primer sequences and characteristics of 11 polymorphic microsatellite loci developed for *Dirofilaria immitis*.

Locus	Motif	Primer sequences (5′-3′)	Allele sizes (bp)
A2	ATTA	F: GAATCAATCGGGGAAATG R: GTTTCTTAAATGCAAATGCTCCGTTGT	252-292
A5	TAA	F: TTCATTTCAAGCCACAGCAG R: GTTTCTTGGGAATCCCAGGTGTTGTAG	193-217
B5	TC	F: TTTGGTTATAAAAAGAATGGACA R: GTTTCTTTCGCCTAAAAAGATAGTGCAA	271-317
G9	TGT	F: GATGTTGCTGCGATTGTTGT R: GTTTCTTCCTCAACAACGATTACGTTT	135-159
H4	CAA	F: GAATACAACGCAAACCGTCC R: GTTTCTTCTGCGCTAAACAATGCAAAA	200-218
E4	AC	F: GCTTGCACTTCGTCCTTTTC R: GTTTCTTGTATGTGTGTGTAAGCGTGTG	141-171
D2	GTA	F: CGAATTATTACTACTATCGCCG R: TGAGGAGGAGAAGAAGAAGAGA	113-125
A4	CA	F: CATGTTATACAGGGGCGTGA R: ATTCGGGACAATACACTGCC	225-253
C2	TC	F: TTTGGTTATAAAAAGAATGGACA R: TTCGCCTAAAAAGATAGTGCAA	265-305
H5	TAG	F: CACCAACGAATATCACCGTTT R: GCTTCAACAAAACAACAAACACA	272-314
A05	CAG	F: CATTGTTGTCGTGATCGCT R: AGCAACAGCAGCATTAGCA	199-226

Table III. Descriptive statistics by locus and population for 11 microsatellites of *Dirofilaria immitis*.

Region (N)	Locus	A2	A5*	B5	G9	H4*	E4	D2*	A4	C2	H5	A05
NJ (11)	A (A_U)	8	4	3	5	5	2	3	7	4	11(2)	2
	H_O	0.385	0.385	0.077	0.385	0.636	0.167	0.182	0.700	0.083	0.727	0.154
	H_E	0.812	0.698	0.218	0.791	0.701	0.290	0.498	0.753	0.308	0.861	0.148
GA (25)	A (A_U)	6	4	8	4	4	5	4	4	8	6	3(1)
	H_O	0.381	0.357	0.143	0.316	0.476	0.13	0.294	0.231	0.087	0.238	0.056
	H_E	0.700	0.585	0.726	0.552	0.619	0.633	0.483	0.625	0.754	0.798	0.338
FL (39)	A (A_U)	6	6	11	5	4	6(1)	3	8	9(1)	9	5
	H_O	0.389	0.455	0.258	0.355	0.444	0.188	0.382	0.094	0.107	0.257	0.129
	H_E	0.721	0.664	0.850	0.722	0.673	0.563	0.403	0.680	0.875	0.816	0.369
AL (10)	A (A_U)	7	3	5	2	4	2	3	5	4	3	2
	H_O	0.444	0.125	0.400	0	0.500	0.100	0.286	0	0	0	0.100
	H_E	0.771	0.642	0.816	0.442	0.700	0.268	0.538	0.800	0.737	0.615	0.100
LA (26)	A (A_U)	7	6	9	5	3	4	4	9(1)	11(2)	10	4
	H_O	0.542	0.458	0.391	0.348	0.417	0.167	0.238	0.545	0.409	0.478	0.250
	H_E	0.812	0.642	0.779	0.753	0.494	0.524	0.301	0.634	0.734	0.829	0.384
TX (46)	A (A_U)	6	4	12	7	4	3	3	10(1)	11(1)	6	3
	H_O	0.444	0.304	0.341	0.220	0.320	0.341	0.417	0.467	0.298	0.676	0.140
	H_E	0.678	0.461	0.854	0.766	0.589	0.522	0.500	0.732	0.852	0.800	0.167
MX (19)	A (A_U)	5	4(1)	9(1)	4	2	4	4(2)	4	6(1)	8	6(1)
	H_O	0.105	0.421	0.421	0.133	0.389	0.111	0.421	0.368	0.316	0.167	0.474
	H_E	0.477	0.559	0.777	0.644	0.322	0.387	0.580	0.622	0.657	0.533	0.754
WI (6)	A (A_U)	5	5	7	4	3	6	2	4	8(1)	4	2
	H_O	0.500	0.750	0.417	0.583	0.500	0.417	0.583	0.250	0.167	0.583	0
	H_E	0.692	0.754	0.819	0.757	0.562	0.815	0.518	0.489	0.855	0.859	0.159
CA (10)	A (A_U)	4	4	7(1)	5(1)	4	5(1)	3	4	4	4	2(1)
	H_O	0.400	0.300	0.200	0.200	0.300	0.600	0.111	0.300	0	0.800	0.100
	H_E	0.595	0.558	0.800	0.800	0.489	0.653	0.451	0.563	0.758	0.700	0.100
Total†		8	7	16	9	6	9	6	13	17	14	8

N, number of samples; A, number of alleles; A_U, number of unique alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; * Loci in HWE; †Total number of alleles per locus.

Table IV. Pairwise F_{ST} values for nine *Dirofilaria immitis* sampling regions based on eleven microsatellite markers. Both raw (below diagonal) and standardized (above diagonal) F_{ST} values are included. All values are significant (p<0.01) after a sequential Bonferroni correction.

Region	NJ	GA	FL	AL	LA	TX	MX	WI	CA
New Jersey (NJ)	-	0.07	0.153	0.229	0.223	0.256	0.554	0.232	0.369
Georgia (GA)	0.028	-	0.061	0.246	0.111	0.081	0.469	0.077	0.302
Florida (FL)	0.057	0.021	-	0.225	0.069	0.074	0.437	0.135	0.349
Alabama (AL)	0.095	0.093	0.079	-	0.287	0.229	0.523	0.391	0.451
Louisiana (LA)	0.089	0.041	0.024	0.109	-	0.077	0.494	0.154	0.368
Texas (TX)	0.101	0.03	0.026	0.087	0.028	-	0.441	0.156	0.278
Mexico (MX)	0.243	0.189	0.164	0.22	0.198	0.174	-	0.546	0.59
Wisconsin (WI)	0.089	0.027	0.044	0.14	0.054	0.055	0.212	-	0.214
California (CA)	0.155	0.115	0.123	0.178	0.14	0.105	0.248	0.077	-

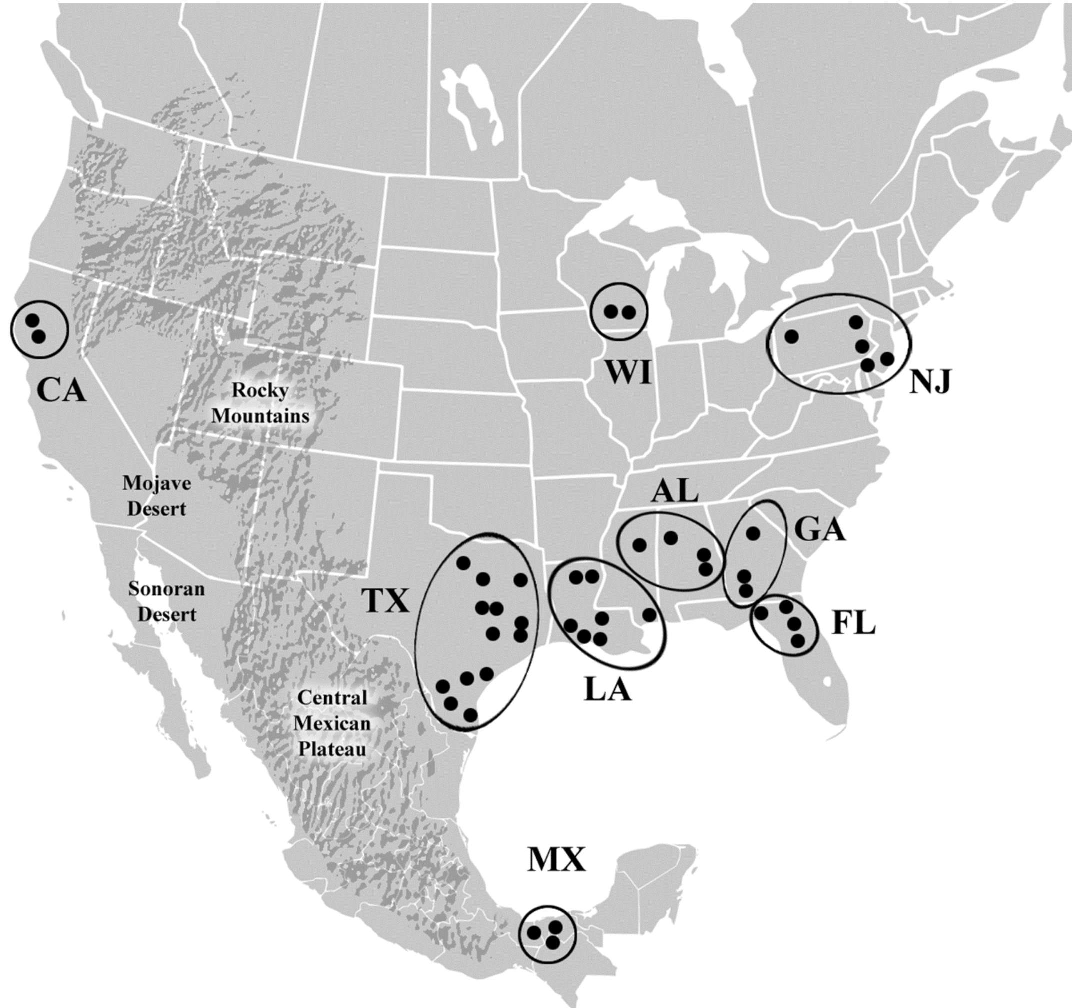

FIGURE 1. Geographic distribution of sampling locales for *Dirofilaria immitis*. Sampled regions are circled and include: New Jersey (NJ), Georgia (GA), Florida (FL), Alabama (AL), Louisiana (LA), Texas (TX), Mexico (MX), Wisconsin (WI), and California (CA). The dots indicate specific locales and some contributed more than 1 nematode sample to this study.

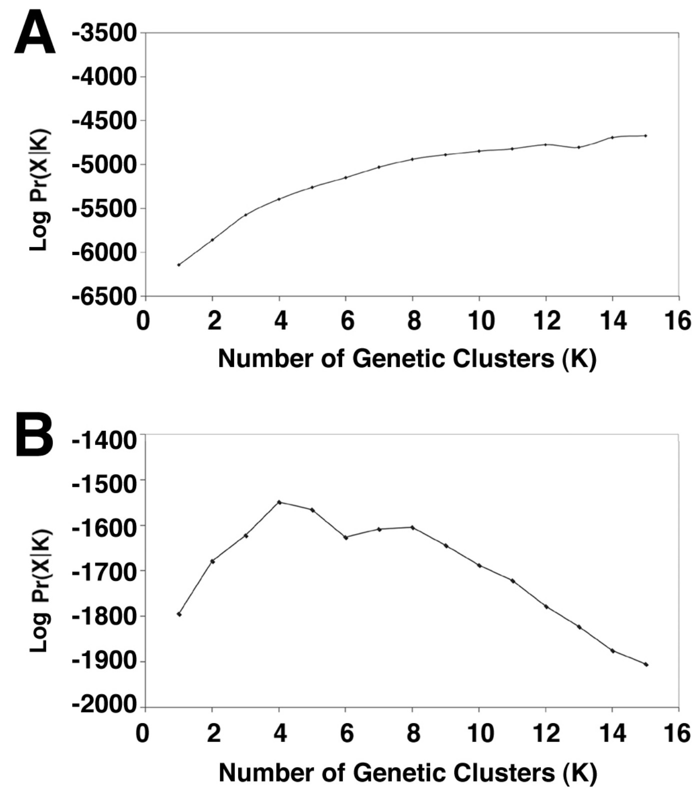

FIGURE 2. (A) Mean Pr(X | K) (± SD) over 5 runs for each value of K for eleven loci (B) Mean Pr(X | K) (± SD) over 5 runs for each value of K for three loci in HWE.

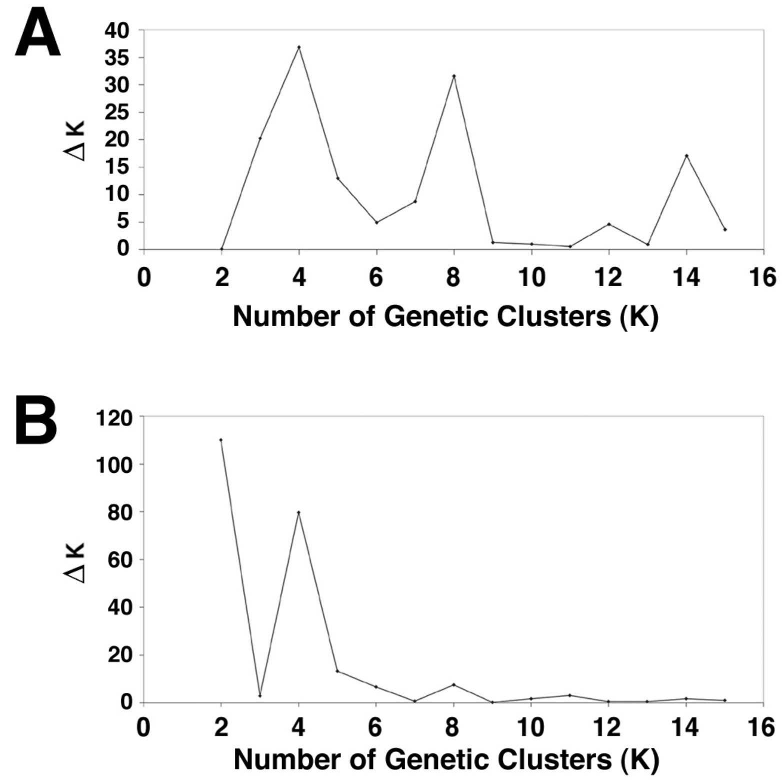

FIGURE 3. Identification of the best K following Evanno et al. (2005) (A) Data from 11 microsatellite loci (B) Data from 3 microsatellite loci that do not deviate from HWE.

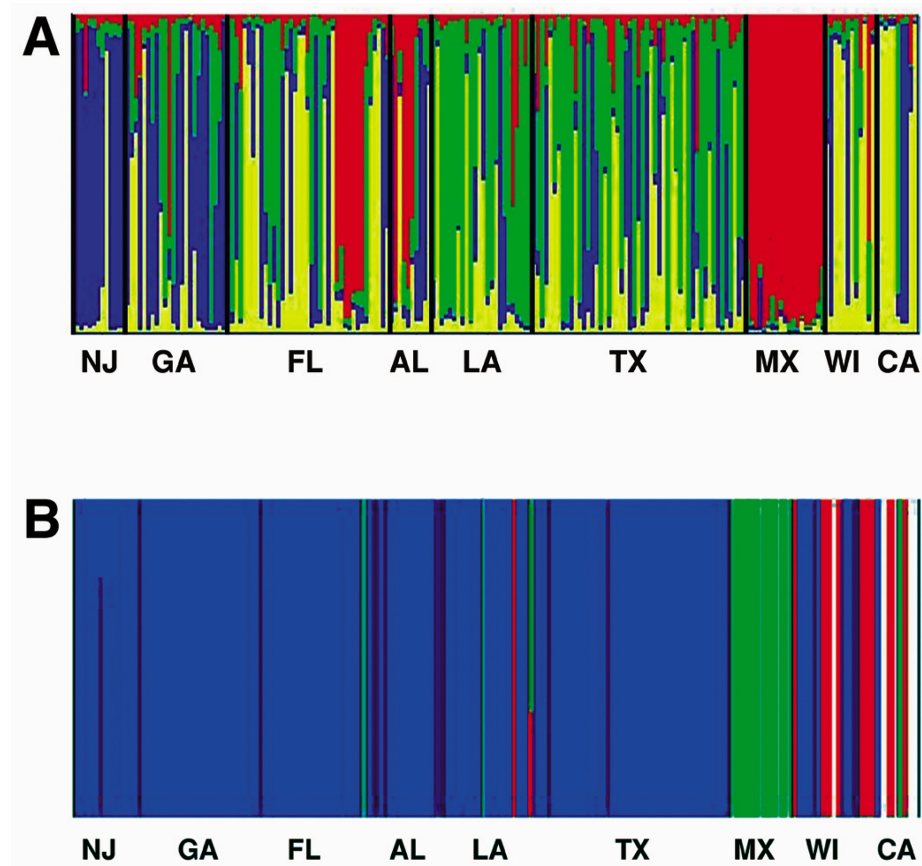

FIGURE 4. Population genetic analyses for 192 *Dirofilaria immitis* nematodes and 11 microsatellite loci. Each color represents a population group based on allele frequency. Vertical bars containing multiple colors indicate individuals of mixed ancestry. (A) STRUCTURE population assignments for K = 4 (B) BAPS admixture analysis of four genetic clusters.

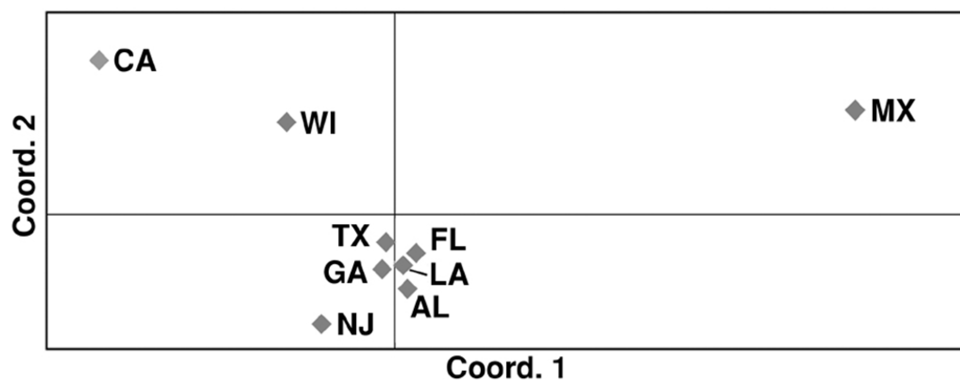

FIGURE 5. Spatial principle component analysis of multilocus genotypes from canine heartworm samples. Eleven microsatellite loci were used and each point represents a sampling region. Coordinates 1 and 2 account for 40.25% and 25.63% of the variance respectively. Loadings show a strong clustering a Gulf Coast samples ((-0.031, -0.005, 0.008, 0.005, 0.003, -0.004, 0.199, -0.047, -0.127) (-0.081, -0.040, -0.029, -0.054, -0.036, -0.021, 0.078, 0.069, 0.114) for NJ, GA, FL, AL, LA, TX, MX, WI and CA respectively).

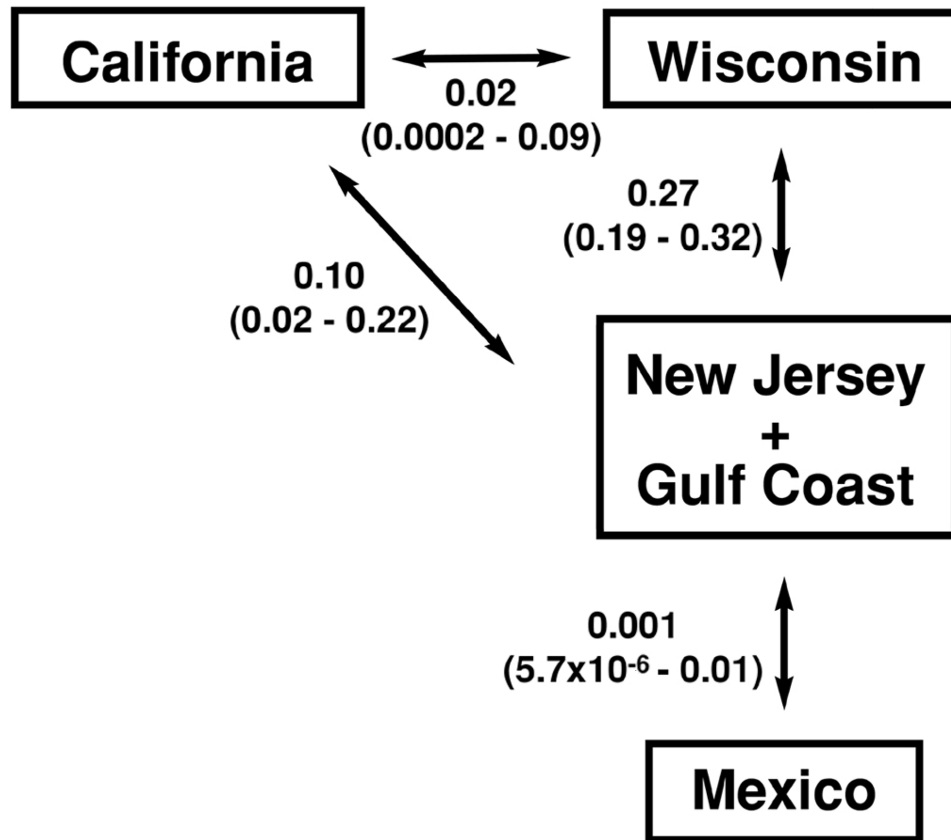

FIGURE 6. Connectivity and gene flow estimates between *Dirofilaria immitis* genetic clusters as computed by BAYESASS+. The 95% confidence intervals are indicated in the parentheses.

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Chapter 4: Dispersal of drug resistance alleles in canine heartworm (*Dirofilaria immitis)* populations

Abstract

When a species occupies a large territory, local differentiation can occur. Although the existence of geographic barriers favors the formation of local colonies, differentiation can occur without them. Low dispersal rates may prevent a species from forming a panmictic unit and result in isolation by distance. If gene exchange occurs primarily between adjacent populations, then a stepping stone model may be the best way to approximate overall population structure. Gene flow can occur among populations that are not adjacent, but probabilities of gene exchange decrease as a function of geographic distance. Here we present a simple model of gene flow that can be used to address relevant questions about the potential spread of drug resistance alleles in canine heartworm, *Dirofilaria immitis*, a nematode that infects both wild and companion animals.

Introduction

Twenty years ago, many scientists considered drug resistance in helminths to be an unimportant phenomenon (Geerts and Gryseels 2000). Today, veterinarians and physicians must deal with the reality of not only drug resistance, but also of multi-drug resistant parasites (Schwab et al. 2006, Schwab et al. 2007, Mottier and Prichard 2007). The evolution of drug resistant parasites threatens the health of both domestic and wild animals. Unfortunately, veterinary experiences have shown that the problem often becomes apparent only when it is too late and reversion to susceptibility to current drug regimens is no longer possible (Leathwick 2001, Mortensen et al. 2003, Kaplan 2004, McKeller and Jackson 2004, Wolstenholme et al. 2004, Prichard 2005, Geary 2005, Condi et al. 2009). The most common anthelmintic resistance problems have occurred in the nematodes of ruminants and horses (Ghisi et al. 2007, Blackhall et al. 2008). It is difficult to classify resistance in human filarial nematodes such as *Onchocerca volvulus* as they have no alternate hosts; however, suboptimal treatment responses have been documented in parts of West Africa where strong chemotherapeutic coverage has occurred for two decades (Bourguinat et al. 2007).

Dirofilaria immitis, a parasitic nematode infecting wild and domestic species worldwide. Similar to other nematodes, it may possess genetic features that favor the development of drug resistance including rapid mutation rates, large population sizes, and high levels of gene flow between different geographic regions (Blouin et al. 1995, Kaplan 2004, Grillo et al. 2007). Heartworm uses mosquito vectors, several definitive canid hosts, and “dead end” hosts such as domestic cats and other carnivores. Similar to other filarial worms, its pathogenesis is greatly influenced by a *Wolbachia* endosymbiont,

which is required for basic metabolic function. Canine heartworm is currently treated with macrocyclic lactone anthelminthics (milbemycin oxime, selamectin, moxidectin and ivermectin) and while it has not yet shown evidence for evolving resistance to these present treatments, resistance or a significant decrease in treatment efficacy to these compounds has been documented in related nematodes infecting ruminants and humans (Leathwick et al. 2001, Bourguinat et al. 2007, Lustigman and McCarter 2007, Prichard 2007).

The likelihood of drug resistance emerging in a population and the rate at which it is dispersed depends upon the genetics of resistance, the generation time of the parasite, the degree of gene flow between parasite populations, and the strength of the selection (Prichard 2005, Vuilleumier et al. 2008). We developed a projection model that integrates these factors and used it to examine the potential spread of drug resistance through a four-unit *D. immitis* metapopulation (Belanger et al. submitted). Specifically, we explored the dynamics of an emerging drug resistance allele with respect to dispersal pattern, point of origin, mode of inheritance and fitness differential. We focused primarily on the boundary conditions of the model.

Model Development

We used a stepping-stone model (Kimura and Weiss 1964) to examine the potential spread of a resistance allele throughout the canine heartworm metapopulation in North America. We assumed that resistance was based on a single autosomal locus with two alleles. At the beginning of each projection series each panmictic subpopulation, of equal effective size, was initially fixed for a susceptible allele. The resistance allele

originated in one of the subpopulations by mutation or immigration from outside the metapopulation and was assumed to have a frequency of 0.01. While this frequency is higher than anticipated from mutation rate alone, preliminary studies indicate that its value does nothing more to the dynamics than reduce the initial lag period.

Current research shows that drug resistance is commonly dominant; however, recessive variants do exist (Cornell et al. 2003, Schwab et al. 2006, Blackhall et al. 2008, Mottier and Pritchard 2008). We examined both scenarios. In each case, we assumed that the expressed resistant allele conferred a higher fitness than the susceptible allele, and explored several levels of fitness differential. We projected the spread of the new allele throughout the metapopulation using the timeline depicted in Figure 1. Dispersal occurred during a short time interval at the start of each one-year projection period and was followed by selection. The dynamics of an emerging dominant allele were examined over a 50-year time span while the recessive allele required a much longer projection period.

The dispersal portion of the projection took the form of

$$\mathbf{g}_{t+\Delta} = \mathbf{M} \times \mathbf{g}_i \qquad (1)$$

where $\mathbf{g}_i$ is a 4-element vector of the frequency of the susceptible alleles in each of the populations (i = 1,…, 4 corresponding to New Jersey and the Gulf Coast, Mexico, Wisconsin, and California) and **M** is a 4 × 4 projection matrix wherein the diagonal elements m_{ij} define the proportion of each population that does not disperse and the off-diagonal elements m_{ij} ($i \neq j$) define the proportion of individuals from population j that move to population i during the interval t to $t+\Delta$. Except for the possible external origin

of the initial resistant allele, we assume the system is strictly closed and the matrix is therefore constrained so that each row and column sums to 1.

The baseline parameterization of the migration matrix was defined using non-equilibrium estimates of recent migration (Chapter 3; Belanger et al. submitted). To better understand the potential spread of the resistant allele and its sensitivity to the pattern and extent of dispersal, we examined the system's dynamics under projection matrices with both reduced and enhanced dispersal, but under the same basic stepping stone pattern, and with a fully random pattern of dispersal. As the best estimate of the dispersal pattern is not symmetrical, we examined the effect of point of origin by comparing the dynamics of projections wherein the initial resistant alleles arose in each unit in turn.

The selection portion of the projection was accomplished using the relationship

$$\mathbf{g}_{t+1} = \mathbf{g}_{\mathbf{t+\Delta}} - \Delta\mathbf{q}_{\mathbf{i}} \qquad (2)$$

where $\Delta\mathbf{q}_{\mathbf{i}}$ is a vector whose elements are the reductions in the susceptible allele when the resistant allele is dominant

$$\Delta\mathbf{q}_{\mathbf{dom}} = (-sq^2(1-q)/(1-sq^2) \qquad (3)$$

or when the resistant allele is recessive

$$\Delta\mathbf{q}_{\mathbf{rec}} = (sq(1-q)^2)/(1-sq(2-q)) \qquad (4)$$

and where s is the selection coefficient against the susceptible allele (Hedrick 2005). We examined a range of selection values from 0, a neutral model where spread dynamics are only influenced by host and vector dispersal, through an extreme selection coefficient of 0.9. All projections were conducted using MATLAB 7.1.0.246 (R14).

Results and Discussion

Antihelminthic resistance, a heritable change that enables parasite survival after being treated with a formerly effective chemotherapeutic compound, impacts companion animals, livestock, wild populations, and humans (Prichard 2007). Resistant phenotypes establish a selective advantage and spread throughout a metapopulation via selection and dispersal (Webster et al. 2008). Mathematical models can be invaluable for predicting the spread of these alleles and provide insight into the evolutionary ramifications of chemotherapies (Churcher et al. 2005, Schwab et al. 2006, Schwab et al. 2007). A rare recessive allele experiences a long lag time between its advent and the threshold value above which natural selection can increase its frequency. This is known as the "q^2 constraint" as the recessive resistant phenotype only occurs in a vanishingly small frequency (Cornell et al. 2003). In contrast, dominant phenotypes occur at a much higher frequency and are easily spread. Therefore, dominant resistance alleles have a much greater short-term impact than their recessive counterparts and will be addressed first.

We began by looking at the system under its initial conditions using the baseline estimated gene flow matrix. In the absence of selection, gene flow between populations determines how quickly a resistant allele will disperse and reach fixation. When a dominant mutation arises in a well-connected population (e.g. New Jersey and the Gulf Coast), fixation occurs relatively quickly. Conversely, when a resistant allele arises in a less connected population, it disperses and reaches fixation more slowly (e.g. Mexico). Figure 2 shows the effect of point of origin and illustrates the difference in fixation time when the selection coefficient increases from 0 (no selection) to 0.5 (moderate selective pressure).

Figure 3 shows that selection pressure is inversely proportional to fixation time. When a resistant allele originates in Mexico and the selection pressure is small (s = 0.1), its frequency gradually increases in that population without escaping to the rest of the population network. In contrast, resistant alleles arising in the Gulf Coast rapidly spread throughout the population network. We can conclude that as selection pressure increases so do the frequencies of rare alleles and the probability that gene flow will carry resistant alleles to a naive population.

The final step in looking at the dominant allele scenario involved adjusting the migration matrix to reflect reduced, enhanced, and random gene flow. Figures 4 and 5 illustrate that the dispersal patterns under reduced and enhanced conditions are similar with regard to point of origin, but the time to fixation reflects the differing amounts of dispersal. If all subpopulations exchange migrants equally and at high migration rates, the fixation time is at a minimum. Figure 6 shows the frequency trajectory of a resistant allele arising in the Gulf Coast under maximum gene flow and selection coefficients of 0, 0.1, 0.5, and 0.9. When the migration matrix is totally symmetrical and random, point of origin plays little to no role in the dispersal of the resistant allele.

Due to significant lag time associated with the advent of a recessive allele and its threshold value, our examination of the recessive allele scenario focused on its origin in the New Jersey and Gulf Coast population. Altering the selection pressure as well as the amount of gene flow between the units of the metapopulation model showed fixation occurring in excess of the 50-year projection period for the dominant allele (see supplementary data for details).

The New Jersey and Gulf Coast population exerts the greatest influence on the model. Once a resistant allele arrives in that region, either arising through mutation or entering via immigration, it rapidly spreads to the other populations. The Mexico population is exactly the opposite. Due to the small amount of gene flow between Mexico and the Gulf Coast, a resistant allele arising there would take significantly more time to spread throughout North America. The influence Wisconsin and California falls below that of the Gulf Coast and above that of Mexico.

An important question remains: how do we keep ourselves informed about the emergence of these alleles? We suggest periodic monitoring of heartworm populations. Specifically, it would be prudent to regularly update baseline genetic information about Gulf Coast heartworms as they drive this system. This is not to suggest that resistant alleles could not arise in a less connected population; however, due to decreased gene flow there would be more time for preventative measures to be effected.

Fortunately, to date, there is no evidence to suggest that *D. immitis* has developed or established drug resistance. Current prophylactic regimes kill microfilaria at a low concentration threshold (Hampshire 2005), selective pressure is intermittent, and many wild hosts shelter refugia populations that provide a pool of susceptible parasites available to dilute resistant alleles.

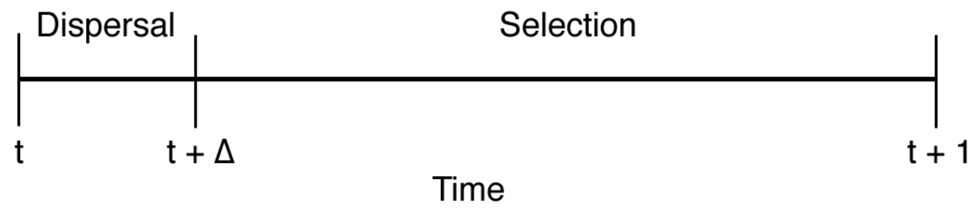


Figure 1. Timeline for projection model. We assumed that dispersal among populations occurred during a short interval at the beginning of each projection period. Selection occurred throughout the rest of the period.

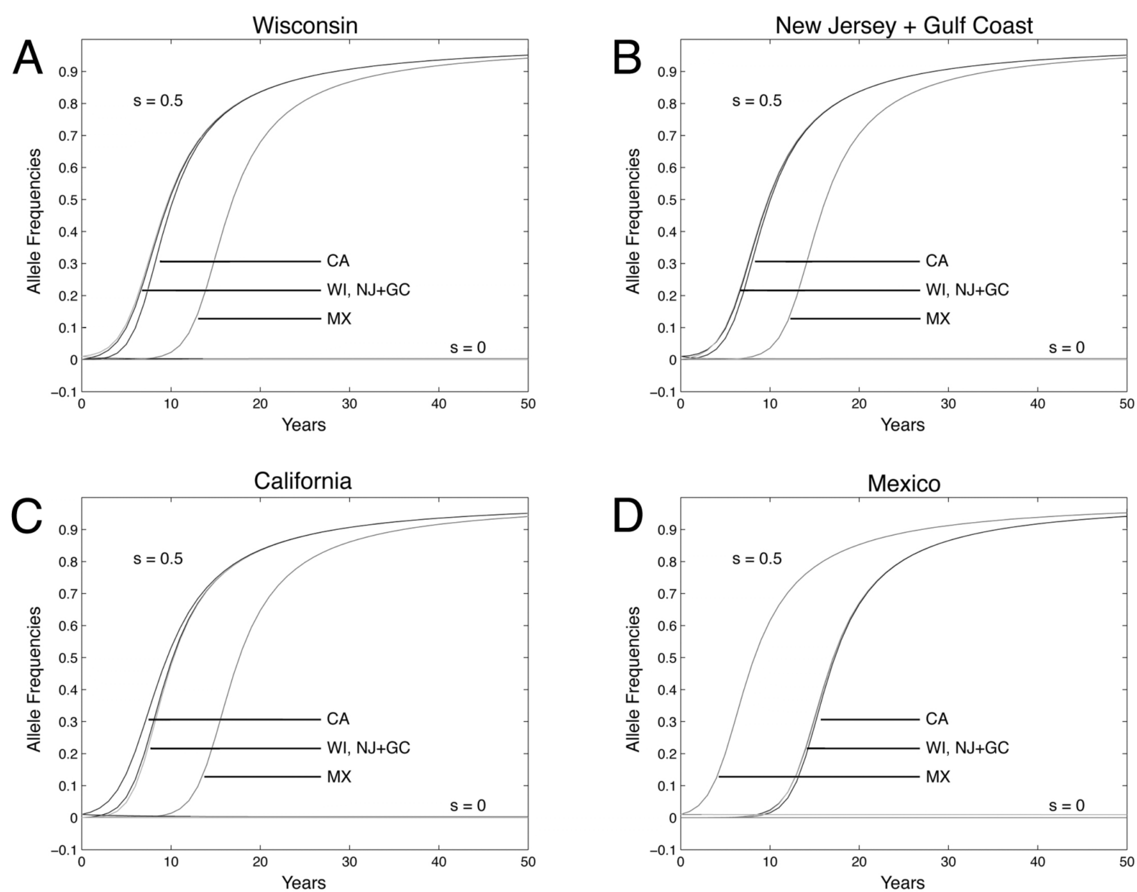


Figure 2. Dominant allele trajectories calculated using the baseline migration matrix and selection coefficients of 0 and 0.5. The resistant allele originated in (A) Wisconsin (B) New Jersey and Gulf Coast (C) California and (D) Mexico.

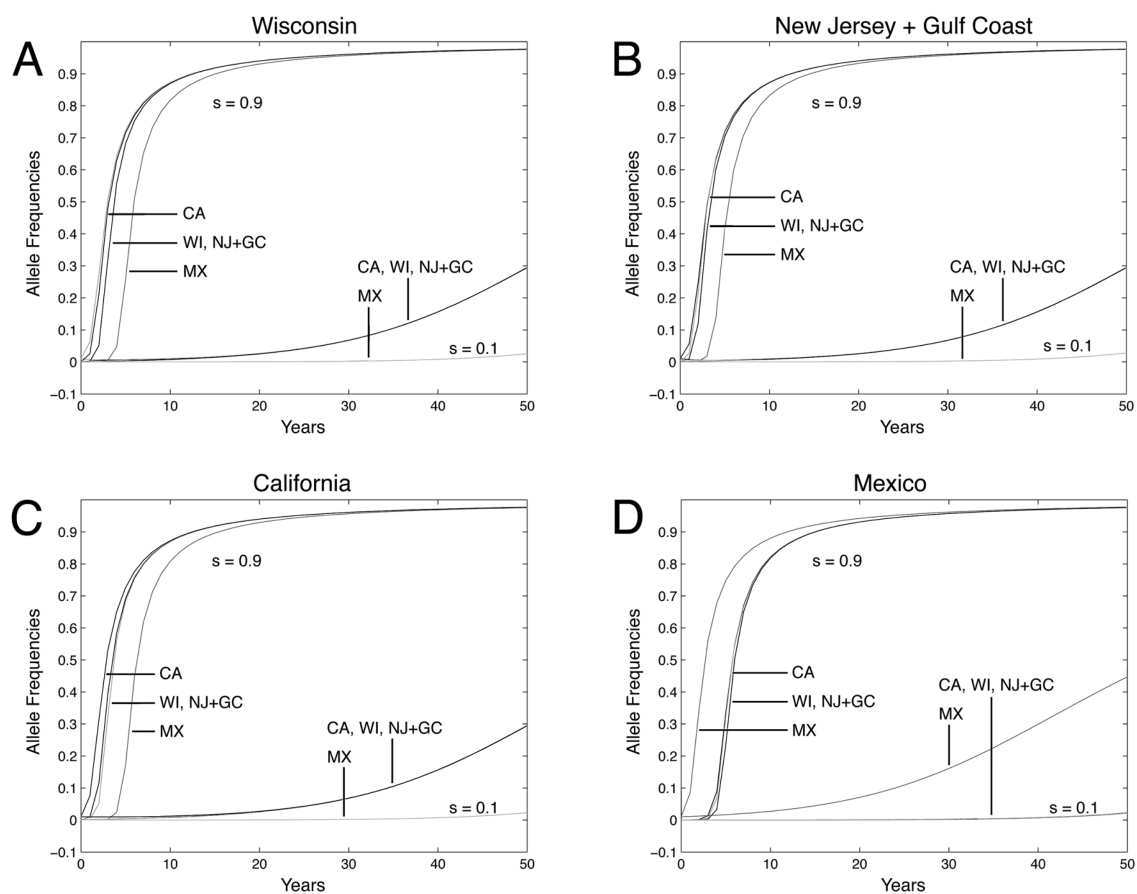


Figure 3. Dominant allele trajectories calculated using the baseline migration matrix and selection coefficients of 0.1 and 0.9. The resistant allele originated in (A) Wisconsin (B) New Jersey and Gulf Coast (C) California and (D) Mexico.

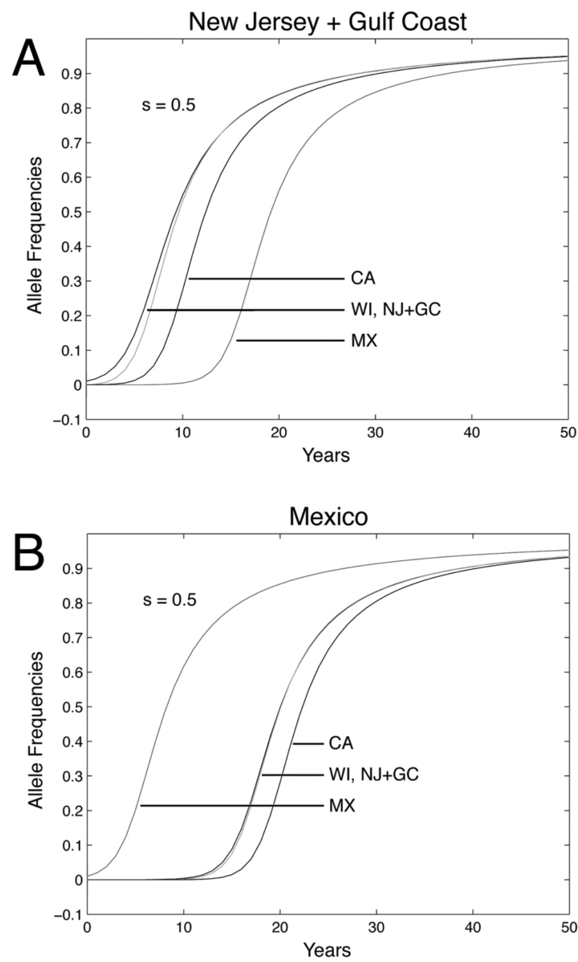


Figure 4. Dominant allele trajectories calculated using the reduced migration matrix and a selection coefficient of 0.5. The resistant allele originated in (A) New Jersey and Gulf Coast and (B) Mexico.

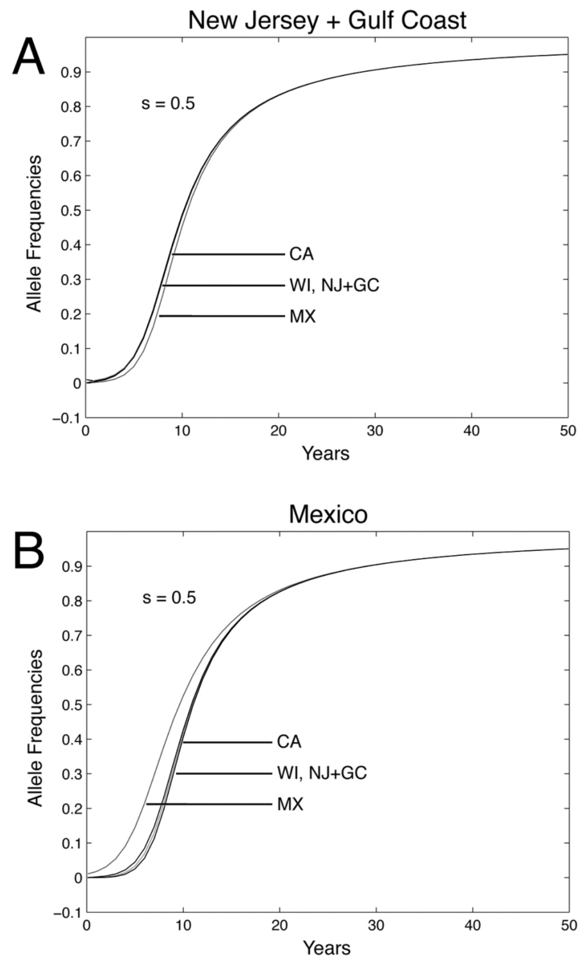


Figure 5. Dominant allele trajectories calculated using the enhanced migration matrix and a selection coefficient of 0.5. The resistant allele originated in (A) New Jersey and Gulf Coast and (B) Mexico.

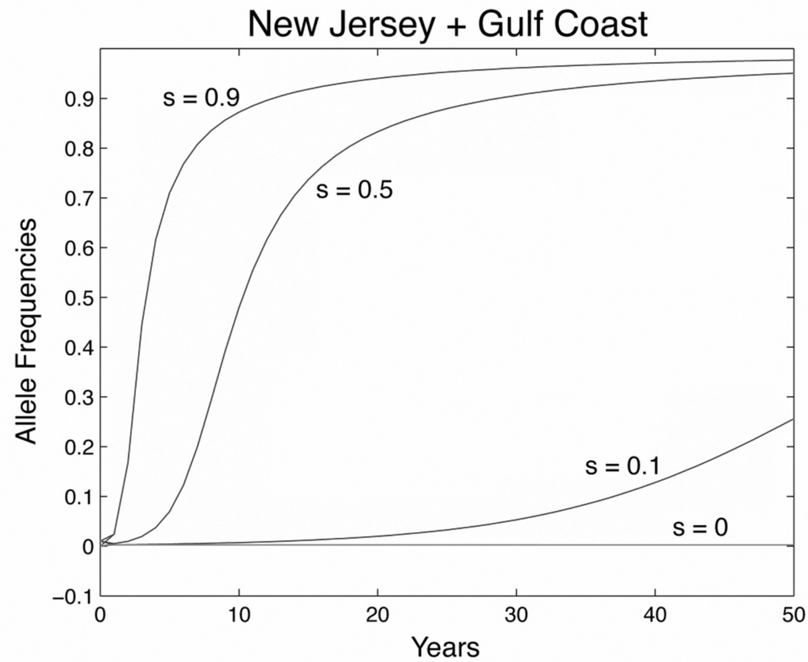
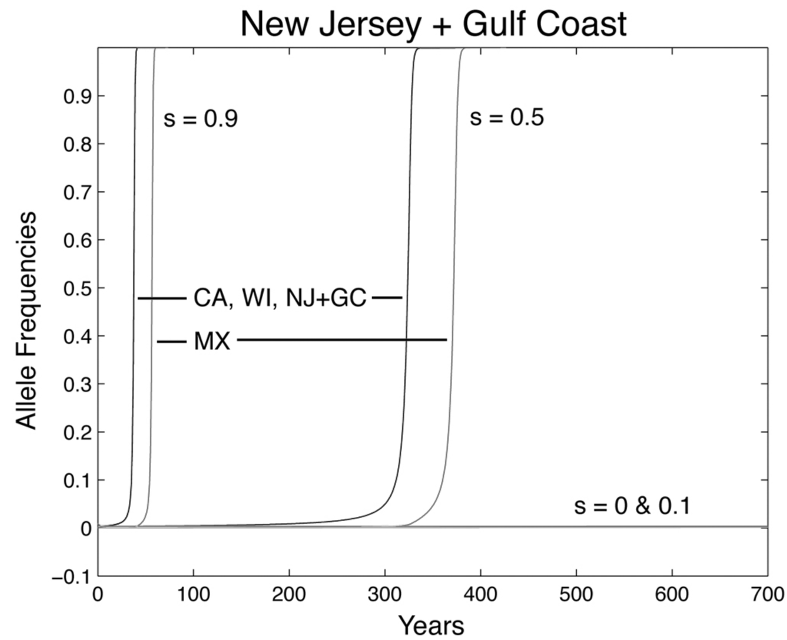
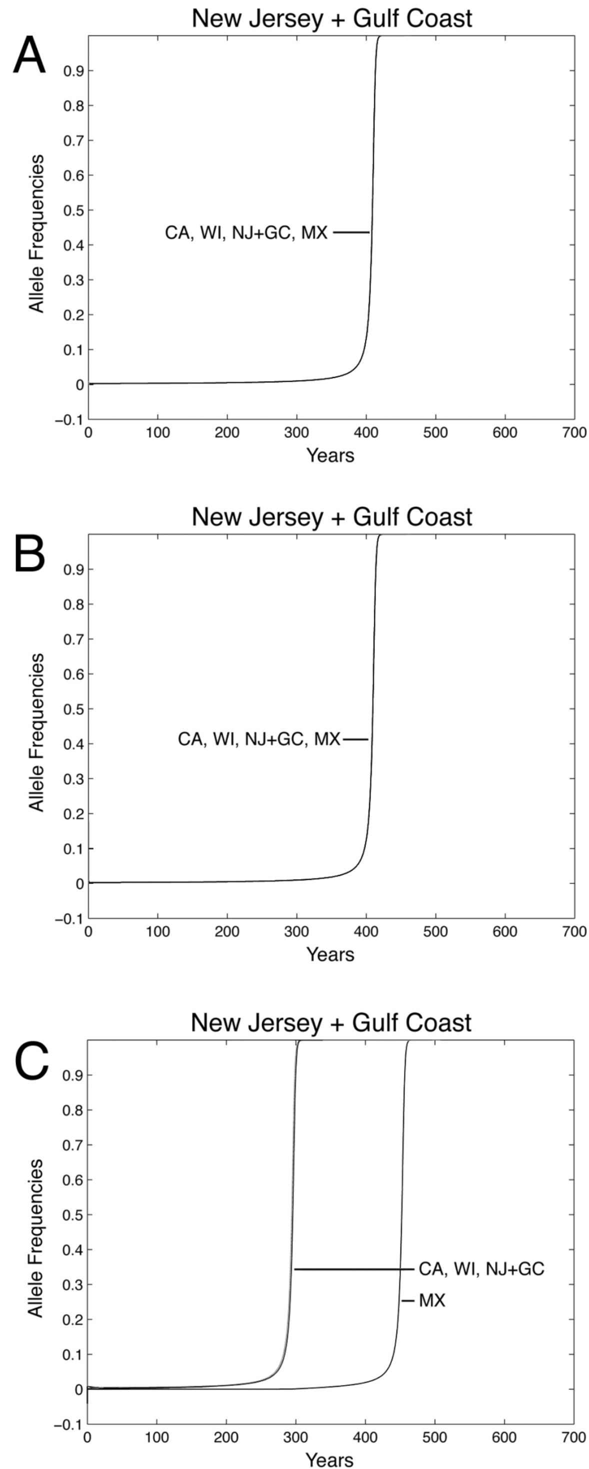


Figure 6. Dominant allele frequency trajectories calculated using the random migration matrix. This represents maximum gene flow and selective pressure ranged from 0 to 0.9. The resistant allele originated in the New Jersey and Gulf Coast population.



Supplementary Figure 1. Recessive allele frequency trajectories were calculated using the baseline migration matrix and selection coefficients ranged from 0 to 0.9. The resistant allele originated in the New Jersey and Gulf Coast population. During the 700-year projection period, there was no increase in the frequency of the resistance allele when the selective pressure was small. Under scenarios of moderate and maximum selection, fixation of the recessive allele occurred after 300 and 50 years, respectively.



Supplementary Figure 2. Recessive resistant allele frequency trajectories were calculated using (A) reduced (B) enhanced and (C) random migration matrices. The selection coefficient was 0.5 and the resistant allele originated in the New Jersey and Gulf Coast population. Fixation of the resistant allele occurred after 400 years.

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Chapter 5: Conclusions

The ability of parasites to rapidly evolve drug resistance is of concern to human health, animal welfare, and agriculture. Control measures have been established to prevent the dispersal of resistance alleles, but the effectiveness of these barriers depends on understanding population genetics. The increasing prevalence of anthelmintic resistance threatens the sustainability of parasite control using current practices. It is crucial that we understand how parasite genotypes are distributed among hosts and between host populations as well as which forces - gene flow, genetic drift, or selection - are impacting this distribution.

While mitochondrial sequences and *Wolbachia* sequence data were unable to shed light on the question of population structure in canine heartworm, they did allow for a unique examination of mitochondrial diversity in light of a *Wolbachia* infection. *D. immitis* is characterized by low mitochondrial diversity and no discernable geographic distribution pattern based on taxon or host domesticity. Tajima's D and Fu and Li's D*, which test departures from neutral evolutionary expectations, show that *D. immitis* populations contain an excess of low frequency mtDNA polymorphisms unlike some *Wolbachia*-free invertebrate species. In order to more fully understand this phenomenon in filarial nematodes, appropriate comparison studies are needed.

The development of rapidly evolving microsatellite markers is the first step to understanding the genetic structure of an organism. In the case of canine heartworm, these microsatellites illuminate a picture of population structure that is defined by geographic and habitat barriers. *D. immitis* populations in eastern and central North

America experience high levels of reciprocal gene flow while geographic barriers impede the movement of vectors and infected hosts west of the Rocky Mountains and south of the Central Mexican Plateau. Three different analysis methodologies all showed concordance and support this conclusion.

Finally, armed with information about population structure, a simple predictive model was used to answer questions about the potential spread of drug resistance alleles. The advent of drug resistance in a source population, such as the Gulf Coast, would rapidly disseminate resistance alleles to the rest of the metapopulation. The amount of time to fixation of those alleles is influenced by gene flow and selection pressure. A sequestered population, such as Mexico, presents a lesser problem. Alleles are disseminated slowly, providing time for implementation of new control and treatment policies. This information has real-world applications for pet owners as well as for wild species. Given the level of gene flow estimated between heartworm populations and the dispersal capabilities of the host species, the spread of resistance alleles would be rapid if it were to arise.

Ultimately, the data collected in this research will benefit both domestic and wild populations. It answers questions about heartworm population structure, identifies geographic regions which could potentially acquire drug resistance, and provides valuable information about the dispersal of genetic information within the heartworm population. All of this information can be used to better inform veterinarians about effective treatments, help create appropriate control measures for wild populations, and perhaps guide drug development to new drug targets.

Appendix 1: Canine heartworm collection data

Table 1. Locale information for heartworms collected from domestic hosts for population genetic analysis.

Sample ID	Sex	Locale	Host Species
DE02	M	DE	*C. familiaris*
FL01	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL02	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL03	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL04	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL05	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL06	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL07	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL08	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL17	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL20	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL21	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL22	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL23	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL24	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL25	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL26	F	FL, Alachua Co., Gainsville	*C. familiaris*
FL35	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL36	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL37	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL38	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL39	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL40	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL41	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL42	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL44	M	FL, Alachua Co., Gainsville	*C. familiaris*
FL45	M	FL, Alachua Co., Gainsville	*C. familiaris*
GA01	F	GA, Clarke Co., Athens	*C. familiaris*
GA02	M	GA, Clarke Co., Athens	*C. familiaris*
GA03	M	GA, Clarke Co., Athens	*C. familiaris*
GA07	F	GA, Clarke Co., Athens	*C. familiaris*
GA15		GA, Clarke Co., Athens	*C. familiaris*
LA01	F	LA, Gulf Coast	*C. familiaris*
LA05	M	LA, Gulf Coast	*C. familiaris*
LA07	M	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA09	F	LA, East Baton Rouge Co., Baton Rouge	*F. catus*
LA10	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA12	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*

LA14	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA17	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA21	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA28	M	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA40	M	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA50	M	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA61	F	LA, Gulf Coast	*C. familiaris*
LA64		LA, Gulf Coast	*C. familiaris*
LA71		LA, Gulf Coast	*C. familiaris*
MS01	F	MS, Gulf Coast	*C. familiaris*
MS02	F	MS, Gulf Coast	*C. familiaris*
MX01	F	Mexico, Centro	*C. familiaris*
MX03	M	Mexico, Tabasco, Teapa	*C. familiaris*
MX04	F	Mexico, Tabasco, Teapa	*C. familiaris*
MX05	M	Mexico, Centro	*C. familiaris*
MX06	F	Mexico, Centro	*C. familiaris*
MX07	M	Mexico, Tabasco, Jalapa	*C. familiaris*
MX08	F	Mexico, Tabasco, Jalapa	*C. familiaris*
MX09	M	Mexico, Tabasco, Teapa	*C. familiaris*
MX10	M	Mexico, Centro	*C. familiaris*
MX11	M	Mexico, Tabasco, Macuspana	*C. familiaris*
MX12	F	Mexico, Tabasco, Macuspana	*C. familiaris*
MX13	M	Mexico, Tabasco, Teapa	*C. familiaris*
MX14	M	Mexico, Tabasco, Tenosique	*C. familiaris*
MX15	F	Mexico, Tabasco, Tenosique	*C. familiaris*
MX16	F	Mexico, Centro	*C. familiaris*
MX17	F	Mexico, Tabasco, Teapa	*C. familiaris*
MX18	F	Mexico, Tabasco, Teapa	*C. familiaris*
MX19	F	Mexico, Tabasco, Jalapa	*C. familiaris*
MX24	F	Mexico, Tabasco, Cunduacan	*C. familiaris*
NJ01	F	NJ	*C. familiaris*
NJ03	F	NJ	*C. familiaris*
NJ05		NJ	*C. familiaris*
NJ10	M	NJ	*C. familiaris*
NJ16	F	NJ	*C. familiaris*
PA01	M	PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA03		PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA04	M	PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA05	M	PA, Philadelphia Co., Philadelphia	*C. familiaris*
TX01	F	TX, Austin Co., Belleville	*C. familiaris*
TX03	F	TX, Brazos Co., College Station	*C. familiaris*
TX06	M	TX, Harris Co., Houston	*C. familiaris*
TX07	F	TX, Austin Co., Bellville	*C. familiaris*
TX09	F	TX, Brazos Co., Bryan	*C. familiaris*
TX15	F	TX, Brazos Co., College Station	*C. familiaris*
TX19	F	TX, Hill Co., Hillsboro	*C. familiaris*

TX20	M	TX, Parker Co., Weatherford	*C. familiaris*
TX26	F	TX, Brazos Co., Bryan	*C. familiaris*
TX28	F	TX, Austin Co., Belleville	*C. familiaris*
TX29	F	TX	*C. familiaris*
TX31	F	TX, Harris Co.	*C. familiaris*
TX36	F	TX, Brazos Co.	*C. familiaris*
TX41	M	TX, Montgomery Co.	*C. familiaris*
TX43	F	TC, San Jacinto Co.	*C. familiaris*
TX46	F	TX, Brazos Co., College Station	*C. familiaris*
TX49	F	TX, Brazos Co., Bryan	*C. familiaris*
TX55	M	TX, Brazos Co., College Station	*C. familiaris*
TX59	F	TX, Brazoria Co., Manvel	*C. familiaris*
TX61	M	TX, Brazos Co., Bryan	*C. familiaris*
TX66	M	TX, Limestone Co., Mexia	*C. familiaris*
TX70	F	TX, Harris Co., Spring	*C. familiaris*
TX80	F	TX, Brazos Co., College Station	*C. familiaris*
TX81	M	TX, Burleson Co., Caldwell	*C. familiaris*
TX87	F	TX, Harris Co., Waller	*C. familiaris*
TX88	F	TX, Brazos Co., College Station	*C. familiaris*
TX95	F	TX, Brazos Co., College Station	*C. familiaris*
TX98	M	TX, Brazos Co., College Station	*C. familiaris*
TX111	F	TX, Burleson Co., Somerville	*C. familiaris*
TX128	F	TX, Burleson Co., Somerville	*C. familiaris*
TX130	M	TX, Harris Co., Spring	*C. familiaris*
TX131	F	TX, Brazos Co., Bryan	*C. familiaris*
TX136	F	TX, Montgomery Co.	*C. familiaris*
TX142	F	TX, Grimes Co., Iola	*F. catus*
TX160	F	TX, McLennan Co.	*C. familiaris*
TX164	F	TX, McLennan Co.	*C. familiaris*

Table 2. Locale information for heartworms collected from wild hosts for population genetic analysis.

Sample ID	Sex	Locale	Host Species
AL01	F	AL, Russell Co., Seale	*C. latrans*
AL04	M	AL, Russell Co., Seale	*C. latrans*
AL05		AL, Lee Co. Salem	*C. latrans*
AL06	F	AL, Lee Co., Spring Villa	*C. latrans*
AL14	F	AL, Russell Co., Sandfort	*C. latrans*
AL18	M	AL, Lee Co., Beehive	*C. latrans*
AL33	F	AL, Lee Co., Farmville	*C. latrans*
AL41	F	AL, Lee Co., Opelika	*C. latrans*
AL48	F	AL, Lee Co., Opelika	*C. latrans*
AL59		AL, Macon Co., Fort Davis	*C. latrans*
CA01	F	CA, Mendocino Co.	*C. latrans*
CA02	F	CA, Mendocino Co.	*C. latrans*
CA03	F	CA, Mendocino Co.	*C. latrans*
CA04	F	CA, Mendocino Co.	*C. latrans*
CA05	F	CA, Mendocino Co.	*C. latrans*
CA09	F	CA, Mendocino Co.	*C. latrans*
CA16	M	CA, Mendocino Co.	*C. latrans*
CA17	M	CA, Mendocino Co.	*C. latrans*
CA18	M	CA, Mendocino Co.	*C. latrans*
CA20	M	CA, Mendocino Co.	*C. latrans*
FL53	F	FL, Columbia Co., Benton	*C. latrans*
FL54	M	FL, Columbia Co., Ellisville	*C. latrans*
FL55	F	FL, Columbia Co., Winfield	*C. latrans*
FL56	M	FL, Columbia Co., Ellisville	*C. latrans*
FL58	F	FL, Columbia Co., Ellisville	*C. latrans*
FL64	M	FL, Columbia Co., Winfield	*C. latrans*
FL74	M	FL, Leon Co., Tallahassee	*C. latrans*
FL84	F	FL, Leon Co., Tallahassee	*C. latrans*
FL85		FL, Leon Co., Tallahassee	*C. latrans*
FL86	F	FL, Leon Co., Tallahassee	*C. latrans*
FL87	F	FL, Leon Co., Tallahassee	*C. latrans*
FL88	F	FL, Leon Co., Tallahassee	*C. latrans*
FL89	F	FL, Leon Co., Tallahassee	*C. latrans*
GA34	M	GA, Thomas Co., Thomasville	*C. latrans*
GA40	F	GA, Baker Co., Albany	*C. latrans*
GA44	F	GA, Baker Co., Albany	*C. latrans*
GA52	F	GA, Baker Co., Albany	*C. latrans*
GA53	F	GA, Baker Co., Albany	*C. latrans*
GA55	F	GA, Baker Co., Albany	*C. latrans*
GA56	F	GA, Baker Co., Albany	*C. latrans*
GA57	F	GA, Thomas Co., Thomasville	*C. latrans*

GA58	M	GA, Thomas Co., Thomasville	*C. latrans*
GA59	F	GA, Thomas Co.	*C. latrans*
GA60	F	GA, Randolf Co.	*C. latrans*
GA61	M	GA, Thomas Co.	*C. latrans*
GA62	M	GA Baker Co.	*U. cinereoargenteus*
GA63	F	GA, Thomas Co., Thomasville	*C. latrans*
GA64	F	GA, Thomas Co., Thomasville	*C. latrans*
GA65		GA, Thomas Co.	*C. latrans*
GA66	F	GA, Thomas Co., Thomasville	*C. latrans*
GA67	M	GA, Randolf Co.	*C. latrans*
GA68	F	GA, Baker Co.	*C. latrans*
LA78	M	LA	*C. latrans*
LA81	F	LA, Vermillion Pa., Kaplan	*C. latrans*
LA82		LA, Vermillion Pa., Forked Island	*C. latrans*
LA84		LA, Richland Pa., Delhi	*C. latrans*
LA85		LA, Ouachita Pa., Fairbanks	*C. latrans*
MS06	M	MS, Oktibbeha Co.	*C. latrans*
MS12	F	MS, Oktibbeha Co.	*C. latrans*
PA12	F	PA, Pike Co., Hawley	*C. latrans*
PA13		PA, Mercer Co.	*C. latrans*
PA19	F	PA	*C. latrans*
TX44	F	TX, Brazos Co.	*M. nigripes*
TX75	F	TX, Nueces Co., Corpus Christi	*C. latrans*
TX118	M	TX, Kleberg Co., Kingsville	*C. latrans*
TX120	F	TX, Willacy Co.	*C. latrans*
TX122	F	TX, Nueces Co., Corpus Christi	*C. latrans*
TX123	F	TX, Kleberg Co., Kingsville	*C. latrans*
TX126	F	TX, Kleberg Co., Kingsville	*C. latrans*
TX144	F	TX, Smith Co., Tyler	*N. narica*
TX145	F	TX, Jim Hogg Co., Ranado	*C. latrans*
TX147	F	TX, Cameron Co., Port Isabel	*C. latrans*
TX152	F	TX, Nueces Co., Corpus Christi	*C. latrans*
TX174	M	TX, Nueces Co., Corpus Christi	*C. latrans*
TX178	M	TX, Jefferson Co.	*C. latrans*
TX180	F	TX, Williamson Co.	*C. latrans*
TX183	F	TX, Jefferson Co.	*C. latrans*
WI01	M	WI	*C. latrans*
WI02	F	WI	*C. latrans*
WI06	M	WI	*C. latrans*
WI07	F	WI	*C. latrans*
WI08	M	WI	*C. latrans*
WI09	M	WI	*C. latrans*

Table 3. Locale information for heartworms used in mitochondrial (*cox1*) phylogenetic analysis.

Sample ID	Sex	Locale	Host Species
AL59		AL, Macon Co., Fort Davis	*C. latrans*
CA01	F	CA, Mendocino Co.	*C. latrans*
CA06	F	CA, Mendocino Co.	*C. latrans*
CA09	F	CA, Mendocino Co.	*C. latrans*
CA11	M	CA, Mendocino Co.	*C. latrans*
DE01	F	DE	*C. familiaris*
GA01	F	GA, Clarke Co., Athens	*C. familiaris*
GA02	M	GA, Clarke Co., Athens	*C. familiaris*
KS01		KS, Harvey Co., Newton	*C. familiaris*
LA07	M	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA08	F	LA, East Baton Rouge Co., Baton Rouge	*C. familiaris*
LA76		LA, Gulf Coast	*C. familiaris*
MI01		MI	*C. familiaris*
MS02	F	MS, Gulf Coast	*C. familiaris*
MS04	M	MS, Gulf Coast	*C. familiaris*
MX03	M	Mexico, Tabasco, Teapa	*C. familiaris*
MX04	F	Mexico, Tabasco, Teapa	*C. familiaris*
MX05	M	Mexico, Centro	*C. familiaris*
MX07	M	Mexico, Veracruz, Jalapa	*C. familiaris*
MX21	F	Mexico, Centro	*C. familiaris*
NJ01	F	NJ	*C. familiaris*
NJ02		NJ	*C. familiaris*
NJ03	F	NJ	*C. familiaris*
NJ04	M	NJ	*C. familiaris*
PA01	M	PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA03		PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA04	M	PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA13	F	PA, Mercer Co.	*C. latrans*
QU03	F	Canada, Quebec	*C. latrans*
TX20	M	TX, Parker Co., Weatherford	*C. familiaris*
TX62	F	TX, Brazos Co., Bryan	*C. familiaris*
TX63	F	TX, Brazos Co., Bryan	*C. familiaris*
WI01[a]		WI	*C. latrans*
WI02	F	WI	*C. latrans*
WI04	M	WI	*C. latrans*
WI31		WI	*C. latrans*
WI32	F	WI	*C. latrans*

Table 4. Locale information for heartworms used in *Wolbachia* phylogenetic analysis.

Sample ID	Sex	Locale	Host Species
AL13	F	AL, Russell Co., Sandfort	*C. latrans*
AL18	F	AL, Lee Co., Beehive	*C. latrans*
AL33	F	AL, Lee Co., Farmville	*C. latrans*
AL41	F	AL, Lee Co., Opelika	*C. latrans*
CA01	F	CA, Mendocino Co.	*C. latrans*
CA08	F	CA, Mendocino Co.	*C. latrans*
DE01	F	DE	*C. familiaris*
FL53	F	FL, Columbia Co., Benton	*C. latrans*
FL55	F	FL, Columbia Co., Winfield	*C. latrans*
FL58	F	FL, Columbia Co., Ellisville	*C. latrans*
FL72	F	FL, Columbia Co., Winfield	*C. latrans*
GA35	F	GA, Thomas Co., Thomasville	*C. latrans*
GA59	F	GA, Thomas Co.	*C. latrans*
GA60	F	GA, Randolf Co.	*C. latrans*
GA63	F	GA, Thomas Co., Thomasville	*C. latrans*
GA66	F	GA, Thomas Co., Thomasville	*C. latrans*
LA02	F	LA, Gulf Coast	*C. familiaris*
LA61	F	LA, Gulf Coast	*C. familiaris*
LA68	F	LA, Gulf Coast	*C. familiaris*
LA81	F	LA, Vermillion Pa., Kaplan	*C. latrans*
MS11	F	MS, Oktibbeha Co.	*C. latrans*
MX08	F	Mexico, Tabasco, Jalapa	*C. familiaris*
MX12	F	Mexico, Tabasco, Macuspana	*C. familiaris*
MX15	F	Mexico, Tabasco, Tenosique	*C. familiaris*
MX16	F	Mexico, Centro	*C. familiaris*
NJ03	F	NJ	*C. familiaris*
NJ16	F	NJ	*C. familiaris*
PA07	F	PA, Philadelphia Co., Philadelphia	*C. familiaris*
PA12	F	PA, Pike Co., Hawley	*C. latrans*
PA13	F	PA, Mercer Co.	*C. latrans*
PA19	F	PA	*C. latrans*
QU02	F	Canada, Quebec	*C. latrans*
QU03	F	Canada, Quebec	*C. latrans*
QU04	F	Canada, Quebec	*C. latrans*
TX71	F	TX, Nueces Co., Corpus Christi	*C. latrans*
TX74	F	TX, Nueces Co., Corpus Christi	*C. latrans*
TX120	F	TX, Willacy Co.	*C. latrans*
TX142	F	TX	*F. catus*
TX147	F	TX, Cameron Co., Port Isabel	*C. latrans*
WI02	F	WI	*C. latrans*
WI07	F	WI	*C. latrans*
WI10	F	WI	*C. latrans*
WI16	F	WI	*C. latrans*

Appendix 2: Alternate microsatellite primers

Table 1. Primers used for investigating presence of null alleles.

Locus	Primer sequences (5′-3′)[a]	T_a °C
A2	R: AAATGCAAATGCTCCGTTGT	55
B5	R: TTTCGCCTAAAAAGATAGTGCAA	55
G9	F: GATGTTGCTGCGATTGTTGT	55
E4	R: TGTGTGTATGTGTGTGTAAGCG	55
A4	F: TTGCATTCGGGACAATACAC	55
	R: TAAGCGGTAACCACACGACA	
C2	R: TCGCCTAAAAAGATAGTGCAA	55
H5	F: GACACCAACGAATATCACCG	55
	R: TCAACAAAACAACAAACACATCA	
A05	F: GCAACAGCAGCATTAGCAGA	55
	R: CGTGATCGCTATCGATGTTC	

[a] M13 tail, CAGTCGGGCGTCATCA**,** attached to 5′ prime end of forward primers.

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