INFERENCE OF POPULATION STRUCTURE AND PATTERNS OF GENE FLOW IN CANINE HEARTWORM (*DIROFILARIA IMMITIS*)

Diana H. Belanger, Susan L. Perkins, and Robert F. Rockwell*
Sackler Institute for Comparative Genomics, American Museum of Natural History, New York, New York 10024. *e-mail: dbelanger@amnh.org*

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 opportunistically sampled from 9 geographic regions in the United States 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.

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 on both the level of dispersal and the genetic effective sizes of local populations. The pattern and degree of sub-structure 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. Many mosquito species are competent vectors, and transmission rates follow a seasonal cycle (Sacks et al., 2003; Vezzani and Carbajo, 2006; Lee et al., 2007). In the contiguous United States, the infection is most prevalent along the Gulf Coast and Mississippi River and continues to advance along the Pacific Coast (Sacks and Caswell-Chen, 2003). 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 1950s (Drudge et al., 1957; Leathwick et al., 2001; Mortensen et al., 2003; Kaplan, 2004; McKellar 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 1 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 in the United States and Mexico between June 2007 and September 2009 (Fig. 1). Adult heartworms were fixed in 70–95% ethanol or 20% dimethylsulfoxide saturated with sodium chloride. Samples were stored in 100% ethanol at −20 C until DNA

FIGURE 1. Geographic distribution of *Dirofilaria immitis* specimens. 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 sampling locales. In some instances, more than 1 nematode was collected in a given locality.

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 hand-picked, submerged in 50 µl of 10 mM Tris-HCl and 0.1 mM EDTA, and boiled at 95 C for 5 min; 1 µl of the supernatant was 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 http://dna.uga.edu/protocols/capillary-genotyping). 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 MgCl₂, 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

TABLE I. Distribution of *D. 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.

(Thermo Fisher Scientific), and 16.6 µl H₂O 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 ABI3730xl 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 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 8 loci out of HWE. These alternate primers 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 10 mM forward and reverse primer, 2.5 µl 25 mM MgCl₂, 2 µl PCR buffer A (Thermo Fisher Scientific), 1 µl 8 mM dNTP mixture, 1 µl 10× bovine serum albumin, 0.2 µl *Taq* polymerase (Thermo Fisher Scientific), and 15.3 µl H₂O. 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 that of Meirmans (2006). Data were recoded to maximize the divergence between populations and used to 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

TABLE II. Primer sequences and characteristics of 11 polymorphic microsatellite loci developed for *D. immitis*.

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

populations helps 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 $\Pr(X \mid K)$ 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). Individual nematodes and their collection data were analyzed using the spatial clustering option. We ran 5 replicates of K ranging from 2 to 15. 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 identified genetic clusters. This method assumes microsatellite loci are unlinked and estimates recent bidirectional 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 2,000.

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 ($P < 0.001$).

TABLE III. Descriptive statistics by locus and population for 11 microsatellites of *D. 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

* Abbreviations: N, total number of samples; A, number of alleles; A$_U$, number of unique alleles; H$_O$, observed heterozygosity; H$_E$, expected heterozygosity.
† Loci in HWE calculated over all sampled regions.
‡ Total number of alleles per locus.

For 192 heartworms and 11 loci, results of the STRUCTURE analysis showed an improvement in $\Pr(X \mid K)$ 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 $\Pr(X \mid K)$ (Fig. 2A). AIC values suggested the greatest weight always corresponded to the largest K (data not shown). The ΔK statistic of Evanno et al. (2005) 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 $\Pr(X \mid K)$ 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 cluster together with a significant amount of admixture elsewhere.

As the Mexico samples appeared to have a profound effect on STRUCTURE's prediction of K, we reran the STRUCTURE analysis without those samples to determine if they were driving a potential artificial effect. Results showed population structure in

TABLE IV. Pairwise F_{ST} values for 9 *D. immitis* sampling regions based on 11 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.

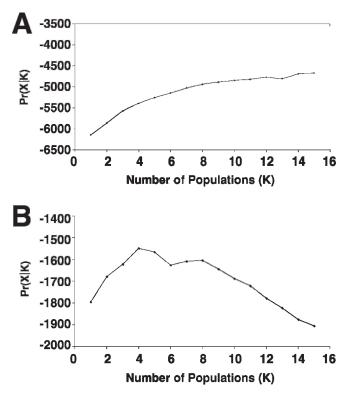| Region	NJ	GA	FL	AL	LA	TX	MX	WI	CA
New Jersey (NJ)	—	0.07	0.153	0.229	0.223	0.256	0.554	0.232	0.369
Georgia (GA)	0.028	—	0.061	0.246	0.111	0.081	0.469	0.077	0.302
Florida (FL)	0.057	0.021	—	0.225	0.069	0.074	0.437	0.135	0.349
Alabama (AL)	0.095	0.093	0.079	—	0.287	0.229	0.523	0.391	0.451
Louisiana (LA)	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 2.   (A) Mean $\Pr(X \mid K)$ ($\pm$ SD) over 5 runs for each value of K for 11 loci. (B) Mean $\Pr(X \mid K)$ ($\pm$ SD) over 5 runs for each value of K for 3 loci in HWE.

FIGURE 4.   Population genetic analyses for 192 *D. 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 4 clusters: blue (eastern cluster), green (Mexico), red (western cluster 1), and white (western cluster 2).
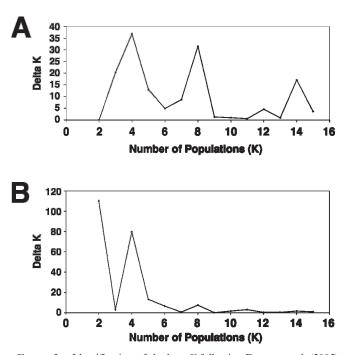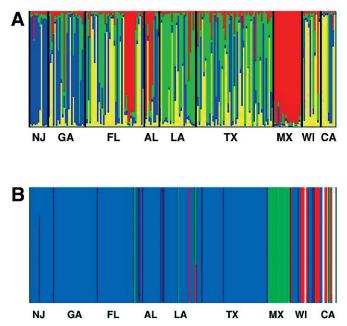

FIGURE 3.   Identification of the best K following Evanno et al. (2005). (A) Data from 11 microsatellite loci. (B) Data from 3 microsatellite loci that do not deviate from HWE.

the form of 2 broad genetic groups, i.e., an eastern cluster (samples from New Jersey, Georgia, Florida, Louisiana, Alabama, and Texas) and a western cluster (California samples). Wisconsin samples were a mixture of genotypes from both the eastern and the western regions.

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 formed 2 separate clusters. The eastern cluster included nematodes from New Jersey, Georgia, Florida, Louisiana, Alabama, and Texas with several individuals displaying admixed genotypes. California samples were predominantly 1 genetic group; however, BAPS analyses indicated the presence of a second genetic cluster in that sampling area. 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 (samples from Georgia, Florida, Alabama, Louisiana, and Texas). Coordinate 2 separates Wisconsin from New Jersey and supports New Jersey's association with the Gulf
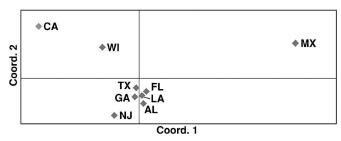
FIGURE 5. Spatial principle component analysis of multilocus genotypes from *D. immitis* samples. Eleven microsatellite loci were used, and each point represents a sampling region. Coordinates 1 and 2 account for 40.3% and 25.6% of the variance, respectively. Loadings show a strong clustering of Gulf 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).

Coast samples. Relationships among the population clusters are depicted in Figure 5.

Estimates of recent migration, expressed as a proportion of the total population, 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 apparently restrict the amount of gene flow between these areas.

DISCUSSION

Population genetic structure in nematodes runs the gamut from panmixia (Johnson et al., 2006; Grillo et al., 2007; Webster et al., 2007) to moderately (Hawdon et al., 2001) and highly structured (Blouin et al., 1999; Criscione et al., 2007; Redman et al., 2008). Our data suggest that *D. immitis* has moderately structured populations. In terms of levels of diversity both within and among populations, the genetic structure of canine heartworm is similar to that of *Wuchereria bancrofti*, a related vector-borne filarial nematode of humans (Schwab et al., 2006; Thangadurai et al., 2006; 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, but not necessarily sylvatic hosts in others. Studies of coyote population genetics indicate high levels of gene flow throughout their range and genetic structuring that corresponds to habitat breaks (Roy et al., 1994; Sacks et al., 2004). The movement of domestic/companion animals might also contribute to connectivity between sampled regions.

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 substructuring 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
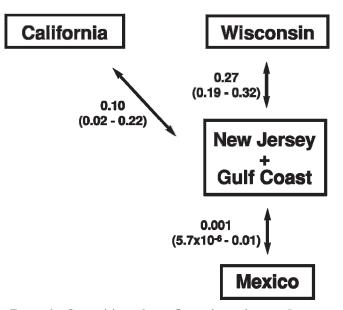

FIGURE 6. Connectivity and gene flow estimates between *D. immitis* genetic clusters as computed by BAYESASS+. The 95% confidence intervals are indicated in parentheses.

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 the method used by Evanno et al. (2005) 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

sub-structures 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.

Our opportunistic sampling scheme may have affected the population structure and migration rate analyses in this research. Schwartz and McKelvey (2009) showed that STRUCTURE's prediction of the number of genetic clusters was influenced not only by the distribution of alleles, but also by sampling scheme. They found STRUCTURE produced misleading results in scenarios where neighbor mating caused a genetic gradient and suggested an examination of local relatedness prior to population genetic analysis.

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. In an area where there is a significant amount of gene flow, such as the Gulf Coast, the dispersal of drug resistance alleles would occur rapidly. Those resistance alleles would not necessarily need to arise in that geographic region, but could arrive there via dispersal from some other area. Bi-directional gene flow might affect the ability of drug resistance to arise in the first place.

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 epizootiology 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.

ACKNOWLEDGMENTS

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