Using population genetics to examine relationships of *Dirofilaria immitis* based on both macrocyclic lactone-resistance status and geography

Julie Sanchez[a], Guha Dharmarajan[b], Melissa M. George[a1], Cassan Pulaski[a], Adrian J. Wolstenholme[a], John S. Gilleard[c], and Ray M. Kaplan[a]*

a. University of Georgia College of Veterinary Medicine, Department of Infectious Diseases, Athens, Georgia

b. Savannah River Ecology Laboratory, University of Georgia, Aiken, South Carolina

c. Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada

1. Current address: Bovine Dynamics, PO Box 740, Kenmore, Qld 4069, Australia

*Corresponding author: Ray M. Kaplan; Tel: +1 (706) 542–5670; Email: rkaplan@uga.edu

Address: University of Georgia College of Veterinary Medicine, Department of Infectious Diseases 501 D.W. Brooks Dr. Athens, GA 30602

Abstract 300/300

Prevention of infection with canine heartworm (*Dirofilaria immitis*) is based on the compliant administration of macrocyclic lactone (ML) drugs. Resistance to ML drugs is well documented in *D. immitis*; however, there remains a paucity of information on the spatial distribution and prevalence of resistant isolates. This project aims to improve understanding of ML-resistance by using a population genetic approach. We developed a large panel of microsatellite loci and identified 12 novel highly polymorphic markers. These 12, and five previously published markers were used to screen pools of microfilariae from 16 confirmed drug-susceptible, 25 confirmed drug-resistant, and from 10 suspected drug-resistant field isolates. In isolates where microfilarial suppression testing indicated resistance, Spatial Principal Component Analysis (sPCoA), Neighbor Joining Trees and Bayesian clustering all revealed high genetic similarity between pre- and post-treatment samples. Somewhat surprisingly, the Neighbor Joining tree and sPCoA generated using pairwise Nei's distances did not reveal clustering for resistant isolates, nor did it reveal state-level geographic clustering from samples collected in Georgia, Louisiana or Mississippi. In contrast,

Discriminant Analysis of Principle Components was able to discriminate between susceptible, suspected-resistant and resistant samples. However, no resistance-associated markers were detected, and this clustering was driven by the combined effects of multiple alleles across multiple loci. Additionally, we measured unexpectedly large genetic distances between different passages of laboratory strains that originated from the same source infection. This finding strongly suggests that the genetic makeup of laboratory isolates can change substantially with each passage, likely due to genetic bottlenecking. Taken together, these data suggest greater than expected genetic variability in the resistant isolates, and in *D. immitis* overall. Our results also suggest that microsatellite genotyping lacks the sensitivity to detect a specific genetic signature for resistance. Future investigations using genomic analyses will be required to elucidate the genetic relationships of ML-resistant isolates.

Keywords: MFST, *Dirofilaria immitis*, macrocyclic lactones, resistance, population genetics, microsatellites

1. Introduction

Dirofilaria immitis, commonly known as the canine heartworm, is the most important helminth parasite of dogs, with the potential to cause serious and potentially fatal disease. Heartworm, a filarioid nematode parasite, is transmitted by mosquitoes and is found commonly in temperate and tropical areas of the world. In the United States, the incidence of heartworm infection is highest in the southern region, particularly along the Gulf Coast and Mississippi Delta (Bowman et al., 2009; Sacks and Caswell-Chen, 2003). To protect dogs against infections with *D. immitis*, macrocyclic lactone (ML) anthelmintics are administered prophylactically to kill L3 and L4 larval stages before they enter the vasculature. All heartworm preventive products are required by the FDA to achieve 100% efficacy in both laboratory and field trials (Hampshire, 2005). However, lack of efficacy

(LOE) complaints related to ML product ineffectiveness began to increase in the early 2000's, suggesting that resistance to MLs may have emerged (Hampshire, 2005).

Although most LOE cases in *D. immitis* are due to poor owner compliance in product administration (Atkins et al., 2014), there are also numerous proven cases of true ML-resistance. No diagnostic tests currently exist that are capable of confirming "suspected-resistant" LOE cases. Thus, resistance is difficult to diagnose definitively in clinical "suspected" cases, and can only be confirmed using one of two *in vivo* methods. In the "gold standard" method, mosquitoes are fed microfilariae (MF) from suspected-resistant isolates of *D. immitis* to produce infective L3 (iL3), laboratory dogs are infected with those iL3, and then the dogs are treated monthly with a heartworm preventive. If the infections establish and become patent then resistance is confirmed (Bourguinat et al., 2015; Maclean et al., 2017; Pulaski et al., 2014). Alternatively, microfilarial suppression tests (MFST) can be used to confirm ML-resistance in field isolates (Geary et al., 2011; Moorhead et al., 2017). This test is based on the high-expected reductions in MF following the administration of a microfilaricidal dose of an ML drug (ivermectin or milbemycin oxime), with reduction in MF counts of <75% being considered indicative of resistance (Geary et al., 2011). Moxidectin, a more potent member of the ML class has recently been approved in the USA for use as a *D. immitis* microfilaricide (Advantage Multi®, Bayer Animal Health, Leverkusen, Germany), with studies demonstrating a greater than 99.9% reduction in microfilariae (Bowman et al., 2015; McCall et al., 2014). Recent evidence using SNP markers suggests that following treatment with Advantage Multi®, a threshold of 90% reduction is indicated for a diagnosis of resistance (Ballesteros et al., 2018).

Despite an increasing awareness and concern of ML-resistance in heartworm, there remains a paucity of data on the prevalence and distribution of the drug resistant phenotype. As is true for all nematodes, the specific loci associated with resistance to ML drugs are currently unknown, and thus no simple molecular test exists to measure the allele frequencies of resistance genes or other resistance-associated markers. (Bourguinat et al., 2015) found a number of single nucleotide

78 polymorphisms (SNPs) that are highly correlated with resistance, and a study by Ballesteros et al.
79 (2018) found a significant correlation between two SNP loci frequencies and the MFST response
80 phenotype following treatment of microfilaremic dogs with moxidectin (Advantage Multi®).
81 However, almost nothing is known about the population genetic structure associated with drug-
82 resistant and drug-susceptible heartworm isolates. These gaps in our knowledge present major
83 problems for studying the epidemiology and managing the spread of drug resistance in *D. immitis*.
84 Microsatellites are highly polymorphic molecular markers consisting of tandemly repeating
85 DNA sequence motifs that range in length from 1-8 nucleotides. The suitability of microsatellites
86 for studying the population genetics of drug resistance in parasitic nematodes has been
87 demonstrated in previous studies (Chaudhry et al., 2016; Redman et al., 2015). Belanger et al.
88 (2011) used microsatellites to characterize the genetic sub-structuring of *D. immitis* populations in
89 the United States and Mexico. Using 11 polymorphic markers, they examined individual adult *D.*
90 *immitis* parasites from 9 geographic regions in the US and Mexico, and detected 4 distinct genetic
91 clusters. One of these clusters encompassed samples from the five southern states of Alabama,
92 Florida, Georgia, Louisiana, and Texas; but no further geographic sub-structuring was detected
93 among isolates in this region. In preliminary studies, we tested these 11 microsatellite markers
94 using several of our laboratory isolates and found only 7 to be polymorphic and therefore
95 informative. Given the nature of microsatellite genotyping, this number was inadequate to provide
96 the depth of analysis we desired. We therefore developed a richer set of highly polymorphic
97 markers to improve our ability to perform population genetic analysis with the aim of detecting
98 genetic sub-structuring and relationships among the parasite isolates at fine spatial scales.
99 The main objectives of this study were to: (1) determine the genetic relationships between
100 ML-susceptible and ML-resistant isolates; (2) characterize the genetic relationship between
101 resistant isolates at local (i.e., within a given state or physiographic region) and regional (i.e.,
102 between different states or physiographic regions) scales, and (3) determine if one or more

103 microsatellite markers could accurately discriminate between ML-susceptible and ML-resistant
104 isolates, thereby providing a means to perform epidemiological surveillance for resistance.
105
106 **2. Materials and methods**
107 *2.1 Isolate origin, history and naming convention*
108 Name, state of origin (if known), resistance/susceptibility status, and numbers of MF used
109 for DNA extractions for each of the 51 isolates examined in this study are listed in Table 1. This
110 included MF samples from 16 ML-susceptible, 10 suspected resistant, and 25 confirmed-resistant
111 isolates. Resistance/susceptibility status was assigned based on the following criteria: (1)
112 Susceptible: MFST yielded >99% reduction in numbers of MF (pre-treatment samples used); (2)
113 Suspected-resistant: treatment and testing history were highly compatible with resistance, however
114 no MFST was performed; and (3) Resistant: treatment and testing history were highly compatible
115 with resistance and MFST yielded <75% reduction in numbers of MF, or strain was passaged in the
116 laboratory under ML-drug pressure and yielded an active infection. All samples beginning with
117 "M" were also evaluated genetically in a recent study (Ballesteros et al., 2018). Microfilarial
118 suppression tests were performed for 7 of the source dogs. Suffixes "a" and "b" indicate samples
119 that were collected before and after treatment, respectively (Table 2). The number of days between
120 blood draws of samples "a" and "b" ranged from 7 to 42 days, and percent reduction in MF in
121 resistant samples ranged from -240.8% to 90.9% (Table 2).
122 In the course of our research, it became clear that there were distinct genetic differences in
123 different versions of the same strain; thus, calling all passages of the same source infection the same
124 strain name, as has been done in the past, is clearly scientifically inaccurate. To address this issue
125 we developed a new naming convention for lab strains of *D. immitis* based on the original strain
126 name, the passage number, and the conditions and/or methods under which they were established.
127 Though a bit cumbersome, this naming convention provides uniform and accurate biological

information that is necessary to properly interpret genotypic and phenotypic data generated using any particular strain. We recommend that this naming convention be adopted broadly.

The specifics of this naming convention are as follows: (1) strains are named starting with the original strain name; (2) if more than one line was initially established from the source infection (i.e. more than one laboratory dog infected), then each separate line is designated by a separate capital letter (e.g. A,B) following a period; (3) the passage number of the isolate is provided following another period to identify the number of passages that the isolate has undergone; (4) if a passage was established under ML-drug pressure, a "+" is added for that passage; (5) if the passage was established by worm transplantation (rather than an infection using iL3), a "t" is included for that passage; (6) if after initial establishment, a strain is later used to infect more than 1 dog, then the subsequent passages are denoted with a separate lowercase letter (e.g., a,b). For example, the strains YZO.A.1+, YZO.A.1+.2+ and YZO.B.1+.2 are three separate versions of the Yazoo strain. YZO.A.1+ is a first passage infection that was established under drug pressure. YZO.A.1+.2+ is a second passage that is derived from the YZO.A.1+ infection and again was established under drug pressure. In contrast, YZO.B.1+.2 is a separate line of YZO (established in a second dog infected from the original source infection) that was originally established under drug pressure, but was passaged a second time without drug pressure. If YZO.A.1+.2+ was passaged three more times in single dogs both with and without drug pressure, then the resulting strains would be designated YZO.A.1+.2+.3+.4+.5+ and YZO.A.1+.2+.5, respectively.

Detailed histories (and accompanying names using the naming convention described here) of the laboratory strains used in this study are provided in supplementary file S1.

2.2 MF isolation from blood and storage

Colleagues at McGill and Louisiana State University collected samples of MF with isolate names beginning with "M" or "L", respectively. MF from isolates beginning with "M" were extracted from blood using the filtration technique described in (Bourguinat et al., 2015). MF from

154 samples beginning with “L” were extracted from blood using a filtration technique (Bourguinat et
155 al., 2015) or a modified version of the saponin lysis technique (Maclean et al., 2017). Microfilariae
156 were stored in 100% isopropanol at 4°C until DNA extraction. All other MF samples were isolated
157 from canine blood at the University of Georgia using a modified version of the saponin lysis
158 technique (Maclean et al., 2017). Microfilariae were stored in 99% isopropanol at -20°C until DNA
159 extraction.
160
161 *2.3 DNA Extraction*
162 DNA samples beginning with “M” were extracted at McGill University from pools of
163 varying numbers of MF (Table 1) using previously published methods (Ballesteros et al., 2018).
164 DNA from all other samples was extracted from pools of 500 MF (unless <500 were available)
165 using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California). This same method was also
166 used to extract DNA from one adult male *D. immitis* (MO strain) in the initial studies to confirm
167 amplification of the microsatellite regions. We used pools of 500 MF based on previous work by
168 our lab where we tested a panel of previously published microsatellites (Belanger et al., 2011), and
169 found that 500 MF yielded equivalent results as compared to the genotype frequency of 30 adult
170 worms (unpublished). Additionally, we noted no discernable differences in genotype between three
171 separate pools of 500 MF. Therefore for the current study we used a single pool of 500 MF (or all
172 MF available) per isolate. The purified DNA was eluted from the DNeasy Mini spin column with
173 200 µL Tris chloride elution buffer (AE buffer) and stored at -20°C until used for PCR.
174
175 *2.4 Microsatellite development and genotyping*
176 Initially, 25 microsatellite loci were compiled using the *D. immitis* genome published by
177 (Godel et al., 2012). Forward and reverse primers were designed using Geneious version 8.1.8
178 (Kearse et al., 2012) to amplify 25 microsatellite regions. Polymerase chain reaction (PCR) was
179 performed using the forward and reverse primers on DNA from one adult male worm (MO strain).

PCR reactions consisted of 2 µl DNA, 1 µL 10 µM forward primer, 1 µL 10 µM reverse primer, 6 µL nuclease-free water, and 10 µl JumpStart ™ REDTaq® ReadyMix ™ Reaction Mix for PCR (Sigma-Aldrich) for a total volume of 20 µL. The thermocycler conditions were 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec followed by a 7 min extension at 72°C. Products were separated by 2% agarose gel electrophoresis with a Quick-Load 100bp DNA ladder marker (New England BioLabs Inc., Ipswich, MA, USA) to confirm amplification to the expected product size. For each newly developed microsatellite marker that amplified a product of the expected size, the primer in each pair with the highest GC nucleotide percentage was labeled fluorescently with one of three fluorophores: FAM, VIC, or PET. This was done originally to allow pooling of samples after PCR to reduce the number of plates submitted for analysis, however, no pooling of PCR products was performed.

These primers were then used on DNA extracted from pools of 500 MF from laboratory strains MO, MET.1+, MP3.A.a2.3, JYD.C(27).1, and GA2.A.a2t. The reaction products were run on an ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, California) using GeneScan 500 LIZ internal size standard and Hi-Di Formamide (Applied Biosystems). Individual chromatograms were manually checked and corrected for errors using Geneious version 8.1.8 (Kearse et al., 2012), and we selected 12 markers that demonstrated the highest level of polymorphism for our panel (Table 3). In addition, we also included five microsatellite markers from (Belanger et al., 2011) in our panel since these also demonstrated a high level of polymorphism (Table 3). We used these 17 markers on DNA from all samples (Table 1).

2.5 Statistical analyses

We calculated relative population allele frequencies for each sample by dividing the measured intensity of a particular allele by the sum of the intensities of all alleles measured at that locus. Calculations were made using alleles that represented greater than 5% of the total allele intensity in each sample (Blank et al., 2009).

Pairwise Nei's distances between isolates and sPCoA plots were generated using the relative population allele frequencies in R using the "adegenet" package (Jombart, 2008). Nei's distances are non-Euclidean measures of genetic distance between populations that assumes that genetic differences between two populations are caused by mutation and genetic drift (Nei, 1978). The distance matrix for all pairwise combinations of samples was then used as input into the package "ape" to generate a tree using the Neighbor Joining method (Paradis et al., 2004).

To further investigate patterns of genetic structure in each of our samples, we used a Bayesian approach for cluster analysis using the program STRUCTURE v2.3.4 (Pritchard et al., 2000). STRUCTURE uses a Markov chain Monte Carlo (MCMC) algorithm to minimize Hardy–Weinberg and linkage disequilibrium within each of k clusters, and generates a probability (Q) of an individual belonging to each cluster. For data input, allele frequencies were scored as independent dominant markers by scoring individual microsatellite bands, where microsatellite allele frequencies were converted into a presence absence matrix for each microsatellite band (Rodzen et al., 2004). Across the 51 samples, the 17 microsatellite markers yielded 96 total bands, therefore our matrix included 96 alleles that were scored as "1" if the band was present for a particular isolate, and "0" if the band was absent. We used a model assuming admixture and correlated allele frequencies between clusters to calculate likelihoods of the data given the existence of k = 1–40 distinct clusters. We performed 10 iterations per value of k, and in each iteration we used a burn-in of 10,000 MCMC simulations, and then used 50,000 MCMC simulations to find optimal groupings of individuals into k clusters. We chose the most parsimonious value of k using the Δk method (Evanno et al., 2005). The calculation of Δk and preparation of the input file for PopHelper Structure Web App v.1.0.10 were performed using STRUCTURE Harvester (Earl and vonHoldt, 2012). The creation of a combined file and graphical representation of the STRUCTURE results from the 10 replicates of the best K was performed using POPHELPER Structure Web App v.1.0.10 (Francis, 2017).

To test if our microsatellite loci could efficiently discriminate *D. immitis* subpopulations based on drug resistance status we used Discriminant Analysis of Principal Components (DAPC) as implemented in the R package ADEGENET (Jombart, 2008; Jombart and Ahmed, 2011). DAPC has been shown to provide an efficient description of genetic clusters using a few synthetic variables constructed as linear combinations of the original variables (alleles) which have the largest between-group variance and the smallest within-group variance (Jombart et al., 2010). To construct the synthetic variables DAPC first transforms the original data using Principal Component Analysis (PCA), and then performs a Discriminant Analysis (DA) on the retained principal components (Jombart et al., 2010). This approach was especially useful in our case because it does not depend on the assumption of Hardy–Weinberg or linkage equilibrium, unlike other model-based clustering methods (e.g., STRUCTURE; Pritchard et al. (2000)). To carry out the analyses we first grouped individual samples into three resistance categories: susceptible, suspected-resistant and resistant, as defined above. For some strains/isolates our sample set contained multiple samples (e.g. pre- and post-treatment MFST, samples from different passages of the same laboratory strain). Therefore, to reduce bias in the analysis that would be introduced by including multiple samples from the same strain/isolate, we used only one sample for each strain/isolate to perform the DAPC analysis. This selection criteria retained a total of 10 susceptible, 10 suspected-resistant and 13 resistant MF samples. We used the normalized frequency of each allele at each locus to carry out the PCA. We then carried out a stratified cross-validation of DAPC using varying numbers of PC axes to identify two discriminant functions that provided the most accurate clustering of individuals into the three resistance categories. We carried out 500 cross-validation replications at each level of PC axis retention, and in each replication 90% of the data was used for training and 10% for testing. The level of PC axis retention that maximized mean successful assignment was identified as the optimal number of PC axes for the final DAPC model. Using the final DAPC model we produced visual outputs of the canonical loading plot. We also used a scatter plot to represent individual pools in

DAPC axis space, and tested for statistically significant separation of the groups using 95% confidence ellipses.

3. Results

3.1 Neighbor Joining analysis using Nei's pairwise distances and Spatial PCoA analysis

The Neighbor Joining tree (Fig. 1) generated using Nei's pairwise distances as input did not demonstrate clustering for resistant isolates, or for state of origin with the exception of three resistant isolates that originated in Arkansas, which formed an easily identifiable cluster. Interestingly, strain variants, that previous to the current work would have been referred to with the same name, showed varying and sometimes rather large levels of genetic distance from one another. YZO.A.1+ and YZO.A.1+.2+, which are from the same passage line show a close relationship; however, YZO.B.1+.2 was placed very distantly from the "A" line, despite all three being derived from the same source dog/infection. Likewise, GA2.At.a2t and GA2.At.c2t.3 are more related to each other than each one is to GA2.At.b2t. Furthermore, MP3.A.a2.3 and MP3.A.b2.3 are not closely associated; MP3.A.b2.3 appears to be more closely related to M20, another sample from Georgia. Finally, JYD.A(34).2t and JYD.C(27).1, which were derived from the same source dog, but more than three years apart, also are not very closely associated. Spatial PCoA analysis was also conducted to examine genetic differences between samples, with very similar results to the Neighbor Joining method; there was no evidence of sub-structuring based on geography or resistance status (see Supplementary file S2). As might be expected in bottlenecked resistant infrapopulations, these analyses revealed little difference between the pre- and post-treatment MFST samples collected from most of the resistant isolates. Interestingly, M63a and M63b were less related as compared to all other pre- and post-treatment samples; this isolate yielded an MF reduction of 65.5%, and was classified as being a mixed sample (containing both susceptible and resistant genotypes) by Ballesteros et al. (2018).

282

283 *3.2 Bayesian clustering assignments using STRUCTURE*

284 For 51 *D. immitis* samples and 96 loci scored as independent dominant markers, results of
285 the STRUCTURE analysis (Evanno et al., 2005) detected population structure at K = 17 (Fig. 2,
286 supplementary file S3). Vertical bars represent individual samples with bars divided into multiple
287 colors when there is evidence of admixture. Samples originating from the same state show largely
288 different membership coefficients. Additionally, while some samples from the same state show
289 clustering in one predominant group, other samples show more admixed clustering assignments.
290 This is in contrast to the Neighbor Joining analysis, where samples from Arkansas formed a cluster.
291 Laboratory strains GA2.At.a2t and GA2.At.c2t.3 were predominantly one genetic group, however
292 GA2.At.b2t displays a more admixed cluster assignments. Susceptible strains MP3.A.a2.3 and
293 MP3.A.b2.3 both displayed high admixture from several different clusters, and display small
294 differences in clustering assignments. The MET.1+ strain also shows evidence of admixture.
295 Interestingly, YZO.A.1+ and YZO.A.1+.2+ show almost identical grouping with predominantly
296 one cluster, whereas YZO.B.1+.2 shows admixture from several different clusters. JYD.A(34).2t
297 and JYD.C(27).1 had similar though not identical clustering assignments, and also show similar
298 grouping with the WC strain from Kentucky. As expected based on the sPCoA and Neighbor
299 Joining analyses, most pre- and post-treatment samples from resistant isolates where MFST was
300 performed showed similar patterns. However, M63a and M63b showed different population
301 assignments. A cluster present in M63a, represented by the dark blue bar, is largely absent in M63b;
302 this is consistent with the genotyping results reported by Ballesteros et al. (2018).

303

304 *3.3 Identification of drug resistant samples using DAPC*

305 Our analyses revealed that the best DAPC model retained 14 PCA axes (Fig. 3A). Mean
306 successful assignment given 14 PC axes was 49.9%, which was significantly higher than random
307 expectation (95% CI: 17.7-48.4%) given the three resistance categories: Susceptible, Suspected-

Resistant and Resistant. The retained PC axes explain 74.18% of the variance in the genetic data (Fig. 3B), but only the first discriminant function identified had an Eigen value >20 (Fig. 3C). Our results revealed that DAPC analysis could effectively discriminate between 'Susceptible' and 'Resistant' samples, and this ability was primarily driven by Discriminant Axis 1 (Fig. 3D). Consequently, our results reveal that our microsatellite loci have the potential to differentiate drug-resistant and -susceptible samples of pooled MF. However, this approach could not reliably differentiate the 'Suspected-Resistant' and 'Resistant' groups (Fig. 3D). This is not surprising, since most if not all of the 'Suspected-Resistant' samples are likely to be truly resistant. Additionally, our analyses did not reveal any specific locus or allele as being important in differentiating between the 'Susceptible' and 'Resistant' samples, as the clustering of these was driven by the combined effects of multiple alleles across multiple loci (see Supplementary files S4, S5 and S6 for full DAPC data and figure with labels for isolates on the discriminant functions graph).

4. Discussion

The primary objectives of this study were to gain knowledge on the genetic relationships of *D. immitis* with regard to both ML-resistance status and geography. To date, virtually all research on the genetics of drug resistance in nematode parasites has been performed on Strongylid gastrointestinal nematodes of livestock; however, the life cycle, biology and epidemiology of *D. immitis* are quite different from gastrointestinal nematodes, and these differences likely have a great impact on the selective drug pressures and resulting population genetics.

Mosquitoes typically ingest a relatively small number of *D. immitis* MF during a blood meal on an infected dog, and ingestion of very high numbers of MF may be lethal to the mosquito (Russell and Geary, 1996). Thus, the numbers of iL3 transmitted per mosquito blood meal is likely rather low, though there is a dearth of data in the literature on this. The high prevalence of canine

heartworm in the southeastern U.S, in particular the Mississippi Delta region, can be attributed to environmental factors that are conducive to transmission; such as high precipitation, humidity, and temperature, among other factors (Wang et al., 2014). A previous study examining genetic polymorphism in *D. immitis* concluded that there was evidence of considerable genetic variability in *D. immitis* populations that could not be explained solely by geographic isolation (Bourguinat et al., 2011b). Consequently, over time, a dog living in a high transmission area that is not receiving an ML heartworm preventive drug, likely will be fed on by large numbers of infected mosquitoes harboring *D. immitis* iL3 with a diversity of genetic backgrounds. Additionally, *D. immitis* are polyandrous, thus, dogs with high worm burdens would be expected to harbor an infrapopulation of MF with a relatively high genetic diversity. In contrast, a very different dynamic exists in a dog that is on a (compliantly administered) heartworm preventive. In that case, the dog will be fed on by a similar assortment of infected mosquitoes, but only ML-resistant iL3 will be able to establish, and in most instances, resistant worms will make up only a very small percentage of the total yearly iL3 inoculum. Thus, the heartworm preventive is inducing a genetic bottleneck; this is expected to produce a small infra-population of adult worms, leading to a loss of heterozygosity (Bourguinat et al., 2011a), and an infrapopulation of MF with a relatively low genetic diversity compared to an infection in a dog not on an ML-preventive. Given these previously reported population genetic data, and the geographic proximity of the Mississippi Delta region where almost all of our resistant cases originate from, we expected to find a degree of genetic similarity among the resistant isolates, and an ability to readily discriminate resistant isolates/strains from susceptible isolates/strains using our panel of microsatellite markers. However, this was not the case.

Using data from our panel of 17 microsatellite markers on 51 isolates/strains of *D. immitis*, and population genetic analytical approaches such as Neighbor Joining analysis using Nei's pairwise distances, sPCoA analysis, and Bayesian clustering assignments using STRUCTURE, we were unable to detect significant clustering or population substructuring due to resistance status. Similarly we were unable to detect significant clustering or population sub-structuring due to

360 geography, with the exception of three isolates from Arkansas that clustered together. We cannot
361 readily explain this difference, however, the clustering observed for Arkansas could be due to
362 sampling bias since we only examined three isolates from Arkansas.
363 High transmission rates in the Mississippi Delta region and southeast U.S. likely produce
364 increased levels of interbreeding leading to higher levels of genetic diversity. Given that most of
365 our samples have origins in this region, this may have impaired our ability to differentiate our
366 samples by geography. Additionally, although we know the state where the dogs resided at the time
367 of sample collection, for many samples we do not have other case history data; so do not know the
368 travel history of the dogs supplying the genetic material we used. Dogs move frequently, both due
369 to the high mobility of our society, and due to displacement as a consequence of catastrophic events
370 such as hurricanes. This can further complicate the ability to detect a geographic association
371 between samples.
372 In contrast, analysis using Discriminant Analysis on Principle Components (DAPC)
373 revealed that 25 microsatellite loci could efficiently discriminate between susceptible and resistant
374 categories (Fig. 3). It is important to note that because microsatellites are most often selectively
375 neutral, and no microsatellite markers specifically associated with ML-resistance were detected, the
376 ability of our loci to discriminate between the drug resistance categories is likely driven by genetic
377 hitchhiking rather than due to direct locus and/or allele-specific effects (Smith and Haigh, 2007).
378 Additionally, the discriminatory power of the DAPC analysis was not driven by any single locus or
379 allele within locus, suggesting that ML-resistance may be driven by mutations at multiple distinct
380 loci in the genome. This finding is supported by the Neighbor Joining analysis, which showed
381 multiple branching events.
382
383 The small geographic focus of the resistance cases in the Mississippi Delta region also raises
384 the question of whether resistance emerged once and then spread as a hard selective sweep, or if
385 there were several independent origins that then spread as soft selective sweeps. Both of these
386 patterns may occur in the emergence and spread of drug resistance in parasites, but which

predominates is dependent on a variety of factors (Nair et al., 2007; Redman et al., 2015). By examining known loci responsible for benzimidazole resistance, Redman et al. (2015) detected both hard and soft selective sweeps in both *Haemonchus contortus* and *Teladorsagia circumcincta*, gastrointestinal nematode parasites of sheep. We could not use that approach to address this issue because the loci involved in ML-resistance are still unknown. Therefore, we used genetically neutral microsatellite markers and hoped that our panel was large enough to detect one or more genetic signatures that were present in some or all of the resistant strains/isolates. Our results suggest that none of our microsatellite were closely associated with an important resistance locus.

Similar to our results, other work on *D. immitis* genetics using a genomic sequencing approach also failed to identify any specific genomic markers associated with resistance (Bourguinat et al., 2017; Bourguinat et al., 2015). However, they did identify ten SNPs that demonstrated significant differences between susceptible and resistant isolates, and that were partially predictive of ML resistance. A more recent study by this group reported a correlation between SNP loci frequencies and ML-resistance phenotype using a 2-SNP model (Ballesteros et al., 2018), however the causative genomic loci of ML-resistance remain undetected.

Where MFST were performed and post-treatment blood samples contained adequate MF for DNA extraction, pre- and post-treatment MF samples clustered very closely, suggesting that treatment with microfilaricidal doses of ML drugs failed to alter the genetic composition of the MF infrapopulation of those dogs. This provides additional strong indirect evidence that those samples were indeed highly ML-resistant. One exception to this was sample M63, which did not cluster as closely as other MFST pairs, suggesting that the dog was infected with a mixed susceptible/resistant population. This an interesting finding for several reasons; (1) the MFST yielded a 65.2% reduction suggestive of a mixed population, (2) the dog was reported as not having compliant administration of heartworm preventives, and (3) the genotyping of this sample (Ballesteros et al., 2018) indicated a mixed genotype.

One surprising finding of this project was the high level of genetic variability in different passages of named laboratory strains. In our study we evaluated different passages of multiple laboratory strains, including those from Georgia-2, MP3, Yazoo, and JYD. All strains demonstrated noticeable genetic differences across different passages, and in some of the strains the level of difference was strikingly large. In terms of practical application, this finding may be the most important of this study. The most dramatic example of this are the differences between the two lines of the Yazoo strain. Originally, two laboratory dogs were infected with iL3 collected from mosquitoes that were fed with blood/MF from the same source dog from Yazoo MS, which had a history consistent with ML-resistance (Maclean et al., 2017). Both dogs were treated monthly with ivermectin and both dogs developed patent infections, establishing two independent ML-resistant lines; A and B. Two passages of Yazoo from the A line, YZO.A.1+ and YZO.A.1+.2+, showed a close genetic relationship; however, YZO.B.1+.2 was placed quite distantly from the ”A” line in the Neighbor joining tree and had very different clustering assignments in the STRUCTURE analysis. For the JYD strain, the two strain variants, JYD.A(34).2t and JYD.C(27).1 established from the same source dog approximately three years apart were located in the same branch of the Neighbor joining tree (Fig. 1), but were not very closely associated.

Based on these data, it is clear that studies using different passages and or lines of a given strain are using parasites that are genetically different. Previously, once a strain was named it was assumed to remain the same biologically over time and passage, and its name remained unchanged no matter how many passages it underwent. This assumption led to serious discussions in the heartworm community regarding the cause of disparate efficacy results in two studies using the MP3 strain (Blagburn et al., 2011; Snyder et al., 2011). Similarly, two different isolations of the JYD strain produced greatly different efficacy results (McTier et al., 2017). This issue was addressed in a recent paper, where using a parametric bootstrap model the authors demonstrated that the differences in efficacy in these studies could not be explained by variability, and therefore must be due to true biological differences (Vidyashankar et al., 2017). Similarly, the JYD strains

used in this study had disparate clustering assignments in sPCoA and Neighbor Joining analysis, with only small variations in Bayesian clustering probabilities using STRUCTURE. Given those statistical data and the genetic data presented here, it seems clear that heartworm strains can change, sometimes relatively dramatically, in both phenotype and genotype with each passage. Here we refer to phenotype not as a qualitative trait of resistant vs. susceptible, but rather as a quantitative trait on a continuum of susceptibility from highly susceptible to highly resistant. This is likely due to the inherent bottlenecking that occurs in the establishment of experimental infections (Vidyashankar et al., 2017). The amount of change would then depend on the level of genetic diversity present in the original infection and the number of worms that establish in the new passage. Consequently, what we have considered in the past as a single heartworm strain should no longer be considered so. YZO.A.1+.2+ and YZO.A.1+ clustered closely in all analyses; however, when compared to its very close relative YZO.B.1+.2 there was a great deal of variation observed. The most likely explanation for this observation is genetic bottlenecking during the establishment of the first two infection lines from Yazoo, YZO.A.1+ and YZO.B.1+. Although DNA from YZO.B.1+ was not available for this study and its genetic profile is unknown, it is also possible that the passage YZO.B.1+.2 is distantly related to the other Yazoo passages because it was established without drug pressure. However, this seems unlikely because even without further drug selection, YZO.B.1+.2 yielded a MFST reduction of 12.76% (unpublished), indicating it is still ML-resistant.

Consequently, we propose the implementation of a new naming convention in order to establish a standard paradigm for naming new laboratory strains, and for naming subsequent passages of a given strain. This would require using a naming protocol that includes the name of the original strain, the passage number, whether it was established via transplantation or by inoculation with iL3, and for resistant strains, whether the isolate was established under drug pressure (see methods section for complete details). We believe strongly that the naming scheme suggested here will provide improved transparency regarding the origin of the strain and enable proper comparisons to be made between studies. This will be especially important in the future, as new

drug classes and products developed for heartworm prevention will need to be tested against both ML-susceptible and ML-resistant strains. Knowing what is being compared among studies will be extremely important for proper interpretation of the resulting data.

5. Conclusion

To conclude, data from this study suggest that there is low level of spatial population structure among *D. immitis* isolates in the southern United States, suggesting that there is extensive gene flow, and greater genetic diversity than previously assumed. These genetic data also provide further insight into previously observed phenotypic differences seen in discrete studies using heartworm strains with the same name. Our data also reveal that there is measurable genetic differentiation between susceptible and resistant samples. However, this genetic differentiation is linked, likely through genetic hitchhiking, to allelic mutations at multiple microsatellite loci. This study has highlighted several relevant issues regarding the population genetics of ML-resistance in *D. immitis*; however, there is still a great deal to learn. If we are to truly begin to understand the population genetics, and to identify the genomic loci involved with ML-resistance in *D. immitis*, additional studies using a variety of genomic approaches are needed.

Acknowledgments

Funding: This work was supported in part by the National Center for Veterinary Parasitology (NCVP). GD is supported by the U.S. Department of Energy Financial Assistance Award no. DE-EM0004391 to the University of Georgia Research Foundation. The authors wish to thank TRS labs Inc., and the veterinarians and dog owners who provided many of the blood samples used in this project. Additionally, the authors would like to express their appreciation to Dr. Roger Prichard and Dr. Cristina Ballesteros at McGill University for providing some of the DNA samples used in this study.

Figure Legends:

Figure 1: Neighbor joining tree based on Nei (1978) unbiased genetic distance for 51 *D. immitis* isolates and strains. The state of origin is included in parentheses except for samples labeled as LSU, which all were from Louisiana. The dashed circle highlights isolates from Arkansas.

Figure 2: Population genetic analyses for 51 *D. immitis* isolates and strains based on 96 dominant loci. Each color represents Bayesian assignment probabilities using the software STRUCTURE for 17 clusters. Each vertical bar represents an individual *D. immitis* sample. Vertical bars containing multiple colors indicate an isolate's membership probability in each of the K inferred clusters. The length of each bar segment represents the probability of membership to each of the genetic clusters identified, with the overall bar summing to 1 (i.e., 100%). Isolates are grouped by state of origin (from left to right: GA, AL, LA, LA/MS, MO, MS, SC, TX, AR, IL, and KY).

Figure 3: Discriminant Analysis of Principal Components (DAPC) was used to cluster genotypes of *Dirofilaria immitis* samples (pools of microfilariae) into three drug resistance categories: Susceptible, Suspected Resistant and Resistant. (A) Proportion of successful prediction outcomes (prediction accuracy) based on stratified cross-validation of DAPC (500 replicates) to identify two discriminant functions using varying numbers of PCA axes (5-40). The background represents a smoothed 2-dimension kernel density scatterplot, with darker colors representing higher occurrence frequencies, and red points and lines represent the observed average prediction accuracy across all cross-validation replicates, with outlier points from areas of lowest regional densities indicated by black symbols. Also shown are the expected prediction accuracies based on random expectation (white solid line) and 95% confidence intervals (dashed white lines). The optimal DAPC model (i.e., model with maximum prediction accuracy) retained 25 Principal Component (PC) axes. (B) Cumulative proportion of variance in the genetic data based on number of PCA axes retained (red

lines indicate the number of axes retained in the optimal DAPC model); (C) Eigen values of the two discriminant functions identified by the optimal DAPC model; (D) Scatter plot of genotypes based on the two discriminant functions identified by DAPC. Symbols are colored based on their *a priori* membership to the three drug resistance categories. Also represented are 95% confidence ellipses (solid lines), with non-overlapping ellipses showing significant clustering in DAPC axis space.

References

Atkins, C.E., Murray, M.J., Olavessen, L.J., Burton, K.W., Marshall, J.W., Brooks, C.C., 2014. Heartworm 'lack of effectiveness' claims in the Mississippi delta: Computerized analysis of owner compliance – 2004–2011. Veterinary Parasitology 206, 106-113.

Ballesteros, C., Pulaski, C.N., Bourguinat, C., Keller, K., Prichard, R.K., Geary, T.G., 2018. Clinical validation of molecular markers of macrocyclic lactone resistance in *Dirofilaria immitis*. International Journal for Parasitology: Drugs and Drug Resistance 8, 596-596-596.

Belanger, D.H., Perkins, S.L., Rockwell, R.F., 2011. Inference of population structure and patterns of gene flow in canine heartworm (*Dirofilaria immitis*). J. Parasitol. 97, 602-609.

Blagburn, B.L., Dillon, A.R., Arther, R.G., Butler, J.M., Newton, J.C., 2011. Comparative efficacy of four commercially available heartworm preventive products against the MP3 laboratory strain of *Dirofilaria immitis*. Veterinary Parasitology 176, 189-194.

Blank, W.A., Reis, E.A., Thiong'o, F.W., Braghiroli, J.F., Santos, J.M., Melo, P.R., Guimaraes, I.C., Silva, L.K., Carmo, T.M., Reis, M.G., Blanton, R.E., 2009. Analysis of *Schistosoma mansoni* population structure using total fecal egg sampling. J. Parasitol. 95, 881-889.

Bourguinat, C., Keller, K., Blagburn, B., Schenker, R., Geary, T.G., Prichard, R.K., 2011a. Correlation between loss of efficacy of macrocyclic lactone heartworm anthelmintics and P-glycoprotein genotype. Veterinary Parasitology 176, 374-381.

Bourguinat, C., Keller, K., Prichard, R.K., Geary, T.G., 2011b. Genetic polymorphism in *Dirofilaria immitis*. Veterinary Parasitology 176, 368-373.

Bourguinat, C., Keller, K., Xia, J., Lepage, P., McTier, T.L., Woods, D.J., Prichard, R.K., 2017. Genetic profiles of ten Dirofilaria immitis isolates susceptible or resistant to macrocyclic lactone heartworm preventives. Parasit Vectors 10, 504.

Bourguinat, C., Lee, A.C.Y., Lizundia, R., Blagburn, B.L., Liotta, J.L., Kraus, M.S., Keller, K., Epe, C., Letourneau, L., Kleinman, C.L., Paterson, T., Gomez, E.C., Montoya-Alonso, J.A., Smith, H., Bhan, A., Peregrine, A.S., Carmichael, J., Drake, J., Schenker, R., Kaminsky, R., Bowman, D.D., Geary, T.G., Prichard, R.K., 2015. Macrocyclic lactone resistance in *Dirofilaria immitis*: Failure of heartworm preventives and investigation of genetic markers for resistance. Veterinary Parasitology 210, 167-178.

Bowman, D., Little, S.E., Lorentzen, L., Shields, J., Sullivan, M.P., Carlin, E.P., 2009. Prevalence and geographic distribution of *Dirofilaria immitis*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Anaplasma phagocytophilum* in dogs in the United States: results of a national clinic-based serologic survey. Veterinary Parasitology 160, 138-148.

Bowman, D.D., Charles, S.D., Arther, R.G., Settje, T., 2015. Laboratory Evaluation of the Efficacy of 10 % Imidacloprid+2.5 % Moxidectin Topical Solution (Advantage (R) Multi, Advocate (R)) for the Treatment of *Dirofilaria immitis* Circulating Microfilariae in Dogs. Parasitology Research 114, S165-S173.

Chaudhry, U., Redman, E.M., Ashraf, K., Shabbir, M.Z., Rashid, M.I., Ashraf, S., Gilleard, J.S., 2016. Microsatellite marker analysis of *Haemonchus contortus* populations from Pakistan suggests that frequent benzimidazole drug treatment does not result in a reduction of overall genetic diversity. Parasites & Vectors 9, 349.

Earl, D.A., vonHoldt, B.M., 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources 4, 359-361.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611-2620.

Francis, R.M., 2017. pophelper: an R package and web app to analyse and visualize population structure. Molecular Ecology Resources 17, 27-32.

Geary, T.G., Bourguinat, C., Prichard, R.K., 2011. Evidence for Macrocyclic Lactone Anthelmintic Resistance in *Dirofilaria immitis*. Topics in Companion Animal Medicine 26, 186-192.

Godel, C., Kumar, S., Koutsovoulos, G., Ludin, P., Nilsson, D., Comandatore, F., Wrobel, N., Thompson, M., Schmid, C.D., Goto, S., Bringaud, F., Wolstenholme, A., Bandi, C., Epe, C., Kaminsky, R., Blaxter, M., Maser, P., 2012. The genome of the heartworm, *Dirofilaria immitis*, reveals drug and vaccine targets. Faseb Journal 26, 4650-4661.

Hampshire, V.A., 2005. Evaluation of efficacy of heartworm preventive products at the FDA. Veterinary Parasitology 133, 191-195.

Jombart, T., 2008. adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24, 1403-1405.

Jombart, T., Ahmed, I., 2011. ADEGENET 1.3-1: New tools for the analysis of genome-wide SNP data. Bioinformatics 27, 3070-3071.

Jombart, T., Devillard, S., Balloux, F., 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genetics 11, 94.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647-1649.

590 Maclean, M.J., Savadelis, M.D., Coates, R., Dzimianski, M.T., Jones, C., Benbow, C., Storey, B.E.,
591 Kaplan, R.M., Moorhead, A.R., Wolstenholme, A.J., 2017. Does evaluation of in vitro microfilarial
592 motility reflect the resistance status of *Dirofilaria immitis* isolates to macrocyclic lactones?
593 Parasites & Vectors 10, 480.

594 McCall, J.W., Arther, R., Davis, W., Settje, T., 2014. Safety and efficacy of 10%
595 imidacloprid+2.5% moxidectin for the treatment of *Dirofilaria immitis* circulating microfilariae in
596 experimentally infected dogs. Veterinary Parasitology 206, 86-92.

597 McTier, T.L., Six, R.H., Pullins, A., Chapin, S., McCall, J.W., Rugg, D., Maeder, S.J., Woods, D.J.,
598 2017. Efficacy of oral moxidectin against susceptible and resistant isolates of *Dirofilaria immitis* in
599 dogs. Parasites & Vectors 10, 482.

600 Moorhead, A.R., Evans, C.C., Kaplan, R.M., 2017. A diagnostic algorithm for evaluating cases of
601 potential macrocyclic lactone–resistant heartworm. Parasites & Vectors 10, 479.

602 Nair, S., Nash, D., Sudimack, D., Jaidee, A., Barends, M., Uhlemann, A.C., Krishna, S., Nosten, F.,
603 Anderson, T.J.C., 2007. Recurrent gene amplification and soft selective sweeps during evolution of
604 multidrug resistance in malaria parasites. Molecular Biology and Evolution 24, 562-573.

605 Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of
606 individuals. Genetics 89, 583-590.

607 Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of Phylogenetics and Evolution in R
608 language. Bioinformatics 20, 289-290.

609 Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using
610 multilocus genotype data. Genetics 155, 945-959.

611 Pulaski, C.N., Malone, J.B., Bourguinat, C., Prichard, R., Geary, T., Ward, D., Klei, T.R., Guidry,
612 T., Smith, G.B., Delcambre, B., Bova, J., Pepping, J., Carmichael, J., Schenker, R., Pariaut, R.,
613 2014. Establishment of macrocyclic lactone resistant *Dirofilaria immitis* isolates in experimentally
614 infected laboratory dogs. Parasites & Vectors 7, 1-9.

615 Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P.J., Jackson, F., Gilleard, J.S.,
616 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of
617 livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Neglected
618 Tropical Diseases 9, e0003494-e0003494.

619 Rodzen, J.A., Famula, T.R., May, B., 2004. Estimation of parentage and relatedness in the
620 polyploid white sturgeon (Acipenser transmontanus) using a dominant marker approach for
621 duplicated microsatellite loci. Aquaculture 232, 165-182.

622 Russell, R.C., Geary, M.J., 1996. The influence of microfilarial density of dog heartworm
623 *Dirofilaria immitis* on infection rate and survival of *Aedes notoscriptus* and *Culex annulirostris*
624 from Australia. Medical and Veterinary Entomology 10, 29-34.

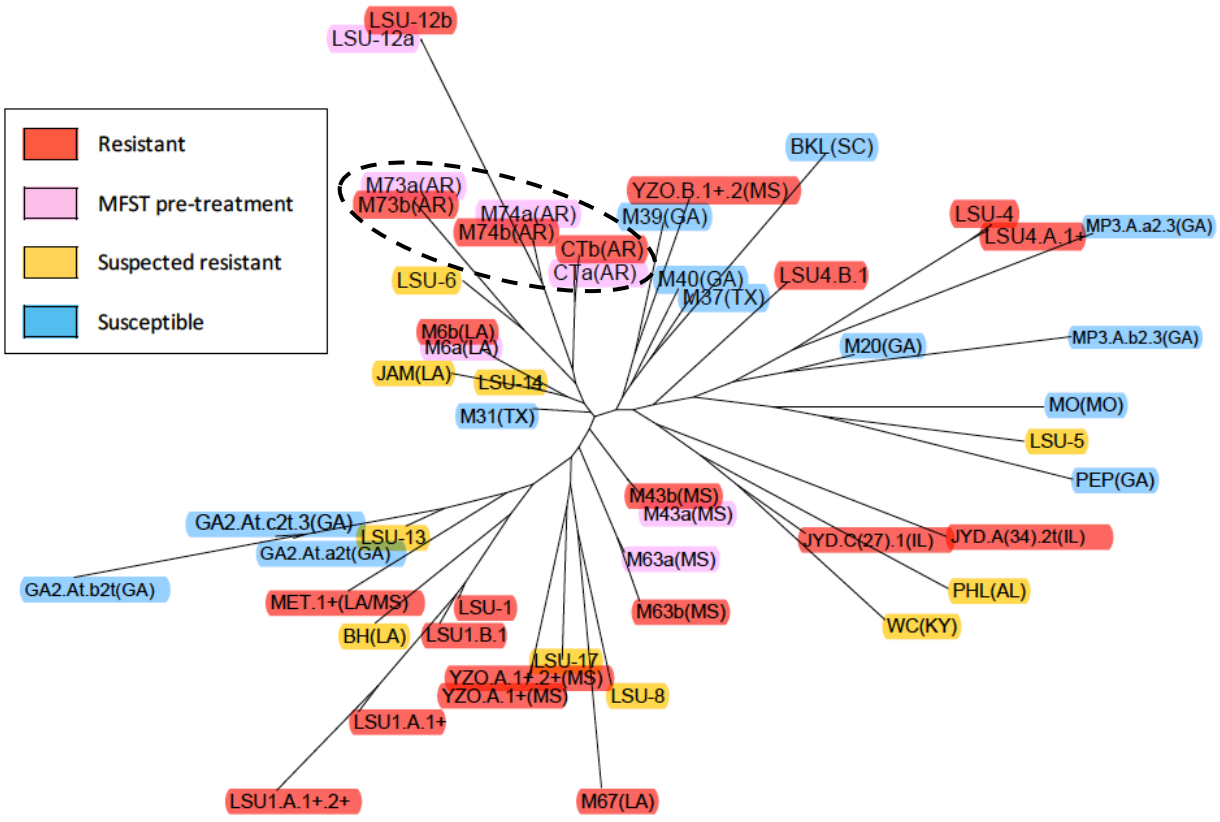
625 Sacks, B.N., Caswell-Chen, E.P., 2003. Reconstructing the spread of *Dirofilaria immitis* in
626 California coyotes. J. Parasitol. 89, 319-323.

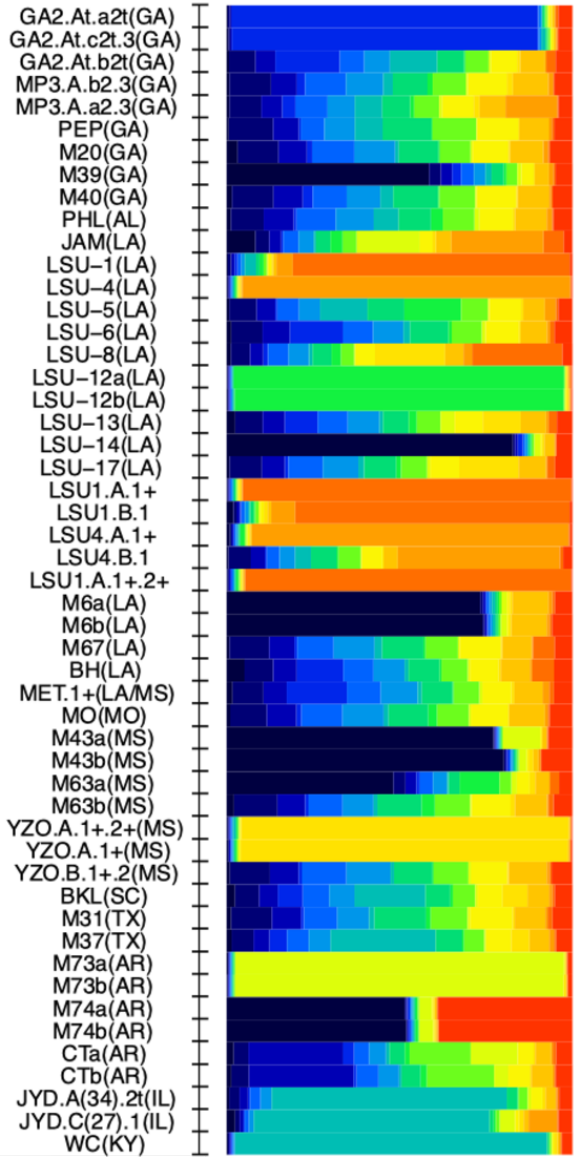
627 Smith, J.M., Haigh, J., 2007. The hitch-hiking effect of a favourable gene. Genetics Research 89,
628 391-403.

629 Snyder, D., Wiseman, S., Cruthers, L., Slone, R., 2011. Ivermectin and milbemycin oxime in
630 experimental adult heartworm (Dirofilaria immitis) infection of dogs. J Vet Intern Med 25, 61-64.

631 Vidyashankar, A.N., Jimenez Castro, P.D., Kaplan, R.M., 2017. A statistical approach for
632 evaluating the effectiveness of heartworm preventive drugs: what does 100% efficacy really mean?
633 Parasites & Vectors 10, 516.

634 Wang, D.M., Bowman, D.D., Brown, H.E., Harrington, L.C., Kaufman, P.E., McKay, T., Nelson,
635 C.T., Sharp, J.L., Lund, R., 2014. Factors influencing US canine heartworm (*Dirofilaria immitis*)
636 prevalence. Parasites & Vectors 7.
637


Resistant
MFST pre-treatment
Suspected resistant
Susceptible
LSU-12b
LSU-12a
BKL(SC)
M73a(AR)
M73b(AR)
YZO.B.1+.2(MS)
M74a(AR)
M39(GA)
LSU-4
LSU4.A.1+
MP3.A.a2.3(GA)
M74b(AR)
CTb(AR)
CTa(AR)
LSU-6
M40(GA)
LSU4.B.1
M37(TX)
M6b(LA)
M6a(LA)
M20(GA)
MP3.A.b2.3(GA)
JAM(LA)
LSU-14
MO(MO)
M31(TX)
LSU-5
PEP(GA)
M43b(MS)
M43a(MS)
GA2.At.c2t.3(GA)
LSU-13
JYD.C(27).1(IL)
JYD.A(34).2t(IL)
GA2.At.a2t(GA)
M63a(MS)
GA2.At.b2t(GA)
PHL(AL)
MET.1+(LA/MS)
LSU-1
M63b(MS)
WC(KY)
BH(LA)
LSU1.B.1
LSU-17
YZO.A.1+.2+(MS)
YZO.A.1+(MS)
LSU-8
LSU1.A.1+
LSU1.A.1+.2+
M67(LA)


GA2.At.a2t(GA)
GA2.At.c2t.3(GA)
GA2.At.b2t(GA)
MP3.A.b2.3(GA)
MP3.A.a2.3(GA)
PEP(GA)
M20(GA)
M39(GA)
M40(GA)
PHL(AL)
JAM(LA)
LSU−1(LA)
LSU−4(LA)
LSU−5(LA)
LSU−6(LA)
LSU−8(LA)
LSU−12a(LA)
LSU−12b(LA)
LSU−13(LA)
LSU−14(LA)
LSU−17(LA)
LSU1.A.1+
LSU1.B.1
LSU4.A.1+
LSU4.B.1
LSU1.A.1+.2+
M6a(LA)
M6b(LA)
M67(LA)
BH(LA)
MET.1+(LA/MS)
MO(MO)
M43a(MS)
M43b(MS)
M63a(MS)
M63b(MS)
YZO.A.1+.2+(MS)
YZO.A.1+(MS)
YZO.B.1+.2(MS)
BKL(SC)
M31(TX)
M37(TX)
M73a(AR)
M73b(AR)
M74a(AR)
M74b(AR)
CTa(AR)
CTb(AR)
JYD.A(34).2t(IL)
JYD.C(27).1(IL)
WC(KY)

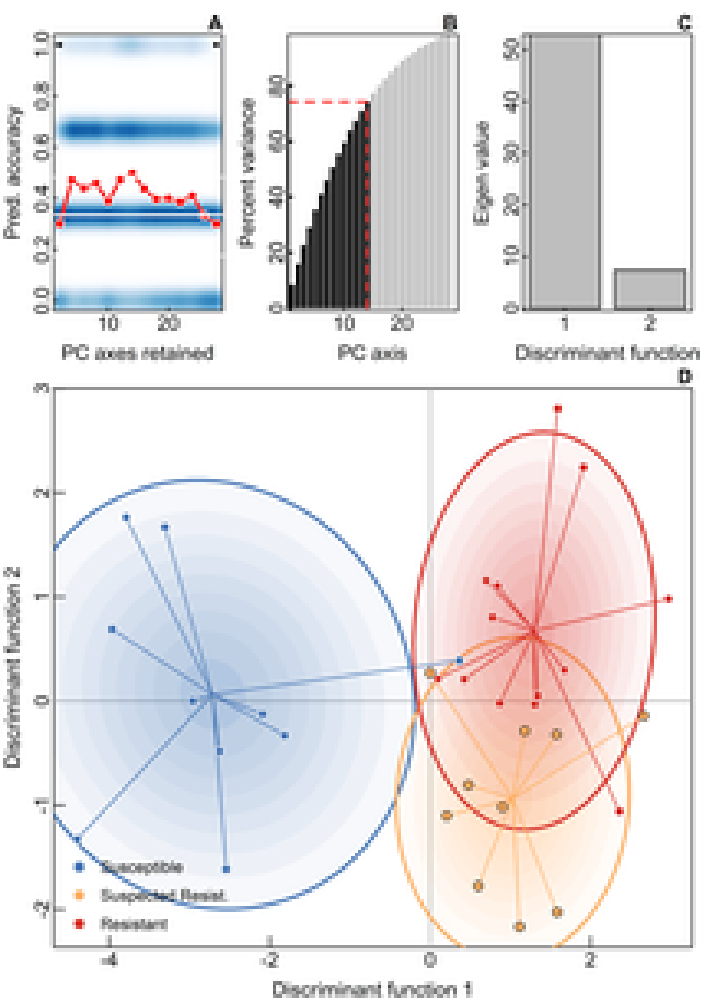

A
Pred. accuracy
1.0
0.8
0.6
0.4
0.2
0.0
10
20
PC axes retained
B
Percent variance
80
60
40
20
0
10
20
PC axis
C
Eigen value
50
40
30
20
10
0
1
2
Discriminant function
D
Discriminant function 2
3
2
1
0
-1
-2
Susceptible
Suspected Resist.
Resistant
-4
-2
0
2
Discriminant function 1

Table 1. Isolate name, state of origin, resistance status, and number of microfilariae included in the pool used for DNA extraction.

Strain/Isolate name[1]	Origin	Status[2]	# Microfilariae in pool
MO	MO	Susceptible (L)	500
MP3.A.a2.3	GA	Susceptible (L)	500
MP3.A.b2.3	GA	Susceptible (L)	500
MET.1+	LA/MS	Resistant (L)	500
GA2.At.a2t	GA	Susceptible (L)	500
GA2.At.b2t	GA	Susceptible (L)	500
GA2.At.c2t.3	GA	Susceptible (L)	500
JYD.A(34).2t	IL[3]	Resistant (L)	500
JYD.C(27).1	IL[3]	Resistant (L)	500
YZO.A.1+	MS	Resistant (L)	500
YZO.A.1+.2+	MS	Resistant (L)	500
YZO.B.1+.2	MS	Resistant (L)	500
WC	KY	Suspected resistant (L)	500
BH	LA	Suspected resistant (L)	500
PEP	GA	Susceptible (L)	500
BKL	SC	Susceptible (L)	500
PHL	AL	Suspected resistant	500
JAM	LA	Suspected resistant	500
LSU-1	LA	Resistant (S)	470
LSU-4	LA	Resistant (S)	366
LSU-5	LA	Suspected resistant	300
LSU-6	LA	Suspected resistant	500
LSU-8	LA	Suspected resistant	500
LSU-12a	LA	Resistant - pre treatment	181
LSU-12b	LA	Resistant - post treatment	81
LSU-13	LA	Suspected resistant	500
LSU-14	LA	Suspected resistant	500
LSU-17	LA	Suspected resistant	170
LSU1.A.1+	LA	Resistant (L)	500
LSU1.B.1	LA	Resistant (L)	500

LSU4.A.1+	LA	Resistant (L)	500
LSU4.B.1	LA	Resistant (L)	500
LSU1.A.1+.2+	LA	Resistant (L)	500
M6a	LA	Resistant - pre treatment	6,100
M6b	LA	Resistant - post treatment	43,700
M20	GA	Susceptible	57,700
M31	TX	Susceptible	8,200
M37	TX	Susceptible	27,000
M39	GA	Susceptible	40,800
M40	GA	Susceptible	42,867
M43a	MS	Resistant - pre treatment	260,400
M43b	MS	Resistant - post treatment	78,752
M63a	MS	Resistant - pre treatment	146,252
M63b	MS	Resistant - post treatment	50,852
M67	LA	Resistant	2,142
M73a	AR	Resistant - pre treatment	158,742
M73b	AR	Resistant - post treatment	6,400
M74a	AR	Resistant - pre treatment	106,700
M74b	AR	Resistant - post treatment	220,617
CTa	AR	Resistant - pre treatment	500
CTb	AR	Resistant - post treatment	500

[1] Isolate names with the same number but different lowercase letters represent pre-treatment (a) and post-treatment (b) samples from the same isolate, in cases where a microfilarial suppression test (MFST) was performed. In cases where a MFST was not performed, or the treatment was highly effective so that post-treatment MF could not be collected and analyzed, then the name appears only once with an uppercase letter and number only.

[2] Resistance status for laboratory isolates was confirmed by in vivo testing, and for field isolates, status was confirmed by performing a MFST. In cases where resistance was suspected based on case history, but MFST was not performed, the isolate was classified as suspected-resistant.

[3] The original JYD dog originated from Illinois, however, given the minimal amount of case history, the infection may be derived from a different state

(L) = Laboratory strain

(S) = Source isolate used to establish a laboratory strain

Table 2. Case information and results for microfilarial suppression tests (MFST) for 7 ML-resistant isolates of *D. immitis*. Counts of microfilaria (MF/ml of blood) before (a) and after (b) macrocyclic lactone treatment, percent reduction in MF, and details of MFST are provided.

Isolate	Fully compliant in past 24 months[1]	(a) MF/ml	(b) MF/ml	Percent reduction in MF[2]	Resistance Status	Drug used	Timing between blood draws (days)
LSU-12	Yes	184	627	-240.8	R	Ivermectin[3]	22
M6	Yes	1,525	10,925	-616.4	R	Moxidectin[4]	14
M43	No	65,100	19,688	69.8	R	moxidectin	42
M63	No	36,563	12,713	65.2	R	moxidectin	22
M73	Yes	17,638	1,600	90.9	R	moxidectin	14
M74	No	26,675	24,513	8.1	R	moxidectin	14
CT	Yes	2,356	885	62.4	R	moxidectin	7

[1] Based on the case history, heartworm prevention was provided to the dog each month for the past 24 months in a compliant manner

[2] Percent reduction in MF was calculated for each isolate where both pre- and post-treatment MF counts were performed as part of a MFST using the following formula: [((pre-Tx MF_{count} – post-Tx MF_{count})/(pre-Tx MF_{count})) x 100].

[3] ivermectin was administered at a dose of 0.2 mg/kg, orally

[4] moxidectin was administered at a dose of 2.5 mg/kg, topically

R = Resistant

Table 3. Primer sequences of 17 polymorphic microsatellite loci for *D. immitis*.

Locus	Primer sequences (5'-3')	Allele sizes (bp)	Repeat type
Dims3	F: AAGACGCGGAGGAGAAAAGG R: TGAACGGGGAATGGATCTGC	354-394	tetra
Dims4	F: TAGCAATGCAAACGACCACC R: AGCTGGAGCAAAAATCAACG	331-364	tri
Dims6	F: GTGAGGTGCGAGTAGAGTGG R: ACACCACCATACACCACACC	308-338	penta
Dims7	F: GGTCATCTACATTATTGCCGAAGC R: CCCTGGAATGATGTCTCACCG	291-300	tri
Dims10	F: GTTAACTGATTGATAGTACCGCC R: ACCGTGAAGGACTATTATAATTAAGGG	369-377	tetra
Dims11	F: AAGCGTAGTCAGCCTCATCC R: AGTAGGAAAGCGAAACGGGG	208-211	tri
Dims12	F: GCGAGGGCCATTAAGCATCC R: TTTCCATCCATCCACCCAGC	240-252	hexa
Dims15	F: CTGCAAGTAGGAAATGTGGCG R: ATCATGTCATGTCGCATCGC	337-352	penta
Dims16	F: CGTGGTTAGCTGCTGATACG R: TCATCCGTGGTTGTTGTTGC	210-216	tri
Dims19	F: TTTGTCTTAATATCTAACCTTCGCG R: GCGTTGTTGGATTACCGTTCC	159-171	tri
Dims21	F: ATAAATGAAGGCAAGCGCGC R: CACTTCCAGACCTCCTCTGC	321-349	tetra
Dims25	F: TCTACACCCTCCTTTCCCCC R: GGTGTTTGTAAGATGATGAAGGTAGG	139-163	octa
D2*	F: CGAATTATTACTACTATCGCCG R: GTTTCTTTGAGGAGGAGAAGAAGAAGAGA	105-112	tri
A05*	F: CATTGTTGTCGTGATCGCT R: GTTTCTTAGCAACAGCAGCATTAGCA	190-318	tri
A5*	F: TTCATTTCAAGCCACAGCAG R: GTTTCTTGGGAATCCCAGGTGTTGTAG	194-209	tri
E4*	F: GCTTGCACTTCGTCCTTTTC R: GTTTCTTGTATGTGTGTGTAAGCGTGTG	138-152	di
H4*	F: GAATACAACGCAAACCGTCC R: GTTTCTTCTGCGCTAAACAATGCAAAA	191-197	tri

* Primers from Belanger et al. (2011)