# The Use of Molecular Markers to Investigate Possible Resistance to Heartworm Preventives in *Dirofilaria Immitis* Samples from Heartworm Positive Dogs in Europe

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# Abstract

**Background:** *Dirofilaria immitis* is a parasitic filarial nematode. It is the causative agent of dirofilariosis, a potentially fatal pulmonary infection which primarily infects canids and felines. dirofilariosis infections are primarily controlled with a prophylactic macrocyclic lactone (ML) regimen. Recent evidence has confirmed the development of ML-resistant isolates in the US which are genetically distinct from wild-type populations. Previous research clinically validated 9 single nucleotide polymorphism (SNP) molecular markers associated with these ML resistance phenotypes isolated from the USA.

**Methods:** In this study, three *D. immitis* US laboratory-maintained isolates: two putative susceptible isolates, Berkeley, and Georgia II, one putative resistant isolate, WildCat; and eleven European *D. immitis* clinical samples, from Italy, Spain, and Hungary were analyzed. The samples tested were fresh microfilaria (mf) in blood or adult female worms shipped in ethanol and rehydrated in phosphate buffered saline (PBS). After DNA extraction, each sample underwent MiSeq sequencing of regions encompassing the 9 SNP sites previously correlated with ML resistance. The nucleotide frequencies of the 9 SNP sites were analyzed and the pairwise fixation index ($F_{ST}$) of the top 2 SNP molecular markers were calculated in order to estimate the probability of identity with known resistant isolates.

**Results:** In the three laboratory-maintained US *D. immitis* isolates Berkeley had a 2-SNP pairwise $F_{ST}$ of 0.00, indicating a ML-susceptible genotype, WildCat had a 2-SNP pairwise $F_{ST}$ of 0.33 indicating a ML-resistant genotype, and Georgia II had a 2-SNP pairwise $F_{ST}$ of 0.07, which may indicate early selection for ML resistance. The genotype analysis of the European clinical samples showed that all eleven European samples had 2-SNP pairwise $F_{ST}$ of 0.00, which indicates their genotypes are consistent with ML susceptibility.

**Conclusions:** Prior to genotyping the European samples, it was possible that the positive heartworm infections could have arisen because of the development of ML-resistance or due to lack of owner compliance or incomplete use of heartworm preventives. Our results indicate no genomic evidence of ML-resistance and suggests that resistance has not developed, so far, in Europe, or been introduced via movement of infected dogs. However, vigilance is needed to maintain susceptibility to heartworm preventives in regions of the world so far without resistance.


# Background

*Dirofilaria immitis* is a veterinary parasitic filarial nematode and the cause of heartworm disease, a potentially fatal pulmonary infection which primarily affects canids and felids, with humans occasionally acting as an incidental host. Macrocyclic lactones (MLs) were first approved as a monthly prophylactic treatment for dirofilariosis in 1987 and remain the standard of care [1–5]. In *D. immitis* the MLs target and kill the infective L3 and developing L4 larvae, can reduce the fecundity of adults for up to 6 months, and help clear blood-circulating microfilariae (mf). This class of drugs has been used as an effective and generally safe treatment for preventing dirofilariosis. Unfortunately, complaints of heartworm preventive

product ineffectiveness were brought to the US FDA Center for Veterinary Medicine (FDA/CVM) as early as 1998, only 11 years after being placed on the market [6]. In 2005, ML drug loss of efficacy (LOE) cases, documented in dirofilariosis hotspots throughout the Southern United States, were brought to public attention [6]. The heritability of ML resistant isolates was established in 2014 by experimentally infecting laboratory dogs with *D. immitis* field LOE isolates [7].

Whole genome analysis elucidated a number of single nucleotide polymorphisms (SNPs) apparently associated with a resistant phenotype [8–10]. The 10 SNPs which best differentiated the ML-resistant phenotype from the ML-susceptible phenotype were selected for analysis in clinical infections collected from the continental USA [11]. A significant correlation of the SNP loci frequencies and the ML microfilaricidal response phenotype was observed in 9 of the 10 SNPs. The clinical validation of the molecular markers for ML resistance in *D. immitis* provides the first genetic test to confirm the development of ML-resistant isolates which are genetically distinct from wild-type populations. These markers can be used to differentiate between ML-resistant *D. immitis* isolates versus cases of owner non-compliance.

The prevalence of *Dirofilaria* infections is on the rise in Europe [12]. The rise in *Dirofilaria* infections is likely to be the result of the increased temperatures due to climate change and the increased movement of companion animals across borders [13–15]. Hundreds of thousands of dogs are relocated internationally each year in Europe, with more than 300,000 entering the United Kingdom via the EU Pet Travel Scheme (PETS) alone [16]. Large numbers of dogs are also relocated throughout North America, with a recent report from Canada demonstrating that dogs originating in USA are positive for heartworm at double the frequency of Canadian dogs [17]. *D. immitis* infections were reported in 109 dogs in Austria, with the dogs originating from Hungary, Greece, the western Balkans, the Iberian Peninsula, Romania, USA, or Bulgaria [18]. As temperatures continue to rise *D. immitis* infections are expected to spread north from the Mediterranean [19]. Autochthonous transmission of canine dirofilariosis has encroached on regions previously untouched by naturally occurring infections such as Hungary and Balkan countries [20–23]. Given this geographical expansion of dirofilariosis it will be important to monitor the effectiveness of MLs in Europe and the possible emergence of resistance. To date there is no background information on the genetic make-up of European strains and the prevalence of SNPs associated with resistance to MLs. These insights are needed to help distinguish LOE cases caused by poor owner treatment compliance from those caused by the emergence of resistance

# Methods

# US Samples

Three US laboratory-maintained isolates were analyzed, two putative susceptible isolates, Berkeley, and Georgia II, and one putative resistant isolate, WildCat (Table 1). The Berkeley *D. immitis* isolate originated in Berkeley County, South Carolina, and has been maintained under laboratory conditions since April, 2014. The Georgia II isolate originated in Vidalia, Georgia and has been maintained under laboratory

conditions since April, 2013. The putative ML resistant Wildcat isolate originated in West Liberty, Kentucky and has been maintained under laboratory conditions since August, 2012. The 3 isolates were provided by TRS Lab Inc., Athens, GA, USA.

Table 1
*Dirofilaria immitis* sample identification, life stage, treatment history and origin for the 3 US laboratory-maintained isolates and the 11 European clinical samples which underwent MiSeq Illumina Sequencing.

| Sample | Life Stage | Isolate | Dog Type | ML Treatment | Origin |
|---|---|---|---|---|---|
| **US laboratory-maintained isolates** | | | | | |
| WildCat | Blood mf | WildCat | Unknown | Untreated | West Liberty, KY, USA |
| Berkeley | Blood mf | Berkeley | Unknown | Untreated | Berkeley County, SC, USA |
| Georgia II | Blood mf | Georgia II | Unknown | Untreated | Vidalia GA, USA |
| **European clinical samples** | | | | | |
| T2 | Adult □ | Unknown | Unknown | Unknown | Lombardy Region, Italy |
| T3 | Adult □ | Unknown | Unknown | Unknown | Lombardy Region, Italy |
| T4 | Adult □ | Unknown | Unknown | Unknown | Lombardy Region, Italy |
| T9 | Adult □ | Unknown | Unknown | Unknown | Hungary |
| T10 | Adult □ | Unknown | Unknown | Unknown | Hungary |
| T11 | Adult □ | Unknown | Unknown | Unknown | Hungary |
| C1 | Blood mf | Unknown | Canary Mastiff | Untreated | Canary Island, Spain |
| C2 | Blood mf | Unknown | Canary Mastiff | Untreated | Canary Island, Spain |
| C4 | Blood mf | Unknown | Canary Hound | Untreated | Canary Island, Spain |
| C5 | Blood mf | Unknown | Canary Mastiff | Untreated | Canary Island, Spain |
| M | Blood mf | Unknown | Spanish Greyhound | Ivermectin | Huelva, Andalusia, Spain* |

***** Dog adopted and relocated to Savona, Italy.

# European Sample Details

Eleven European clinical samples were collected from 7 different canines (Fig. 1; Table 1). Four blood samples were collected from the Canary Islands, Spain. One blood sample was from a dog from Huelva, Andalusia, Spain adopted and relocated to Savona, Italy. Six adult female samples were collected from

infected canines: three female worms from an infected dog in Lombardia, Italy, and three adult female worms from an infected dog in Hungary (Table 1). All adult worms were preserved in ethanol.

# Sample Processing and DNA Extraction

The canine venous blood samples of the 3 US laboratory-maintained isolates and the 5 European clinical samples were shipped to McGill University for immediate processing. The mf were extracted from the blood by filtration [8]. The blood was diluted 1:1 with $NaHCO_3$ solution and passed throughpolycarbonate membrane filters (3.0 µm; 25 mm; Sterlitech® Corporation, Kent, WA, USA) to isolate mf. The 6 adult worms from Italy and Hungary were shipped to McGill University and rehydrated in PBS prior to genomic DNA extraction.

Genomic DNA from the mf samples and the rehydrated adult worms were extracted using the QIAamp® DNA Micro kit (Qiagen Inc., Toronto, ON). DNA concentrations were determined with the Quant-iT™ PicoGreen DNA Assay Kit (Invitrogen®, Life Technologies Inc., Burlington, ON, Canada). The 14 samples were stored at -80ºC prior to being sent to Génome Québec for sequencing.

# 9 SNP Markers

The 9 SNP markers used to analyse the status of ML resistance in the European samples were the top 9 markers clinically validated in 2018 to best differentiate ML-susceptible and ML-resistant phenotypes [11] (Table S1). The SNP 10 marker on nDi.2.2.scaf00597 at position 12915 was not chosen for further analysis as it was not considered a reliable indicator for susceptible versus resistant genotyping.

# Sequencing

The regions encompassing the 9 SNPs of interest were sequenced on an Illumina MiSeq Platform, at a coverage of 2000X. The Fluidigm Access Array system performed target enrichment using array-based PCR amplification of the genomic target regions. The 14 samples underwent parallel amplification using custom primers with added CS1 and CS2 tails, as described in Ballesteros et al. [11] (Table S1). The samples were barcoded during target enrichment which allowed for multiplexed sequencing, and adapter sequences were added during the PCR amplification reaction.

# Data Analysis

Trimmomatic was used to trim for minimal trailing quality (30 PHRED score) and filter for minimum read length by removing the Illumina sequencing adapters from read and adapter clippings [24]. The resulting read pairs were aligned to the *D. immitis* reference genome nDi.2.2 (http://www.nematodes.org/genomes/dirofilaria_immitis) using BWA-mem (http://bio-bwa.sourceforge.net/) resulting in binary alignment map files (BAM) [25]. The alignments were processed with Picard (https://broadinstitute.github.io/picard) for the realignment of indels, mate fixing, and marking of duplicate reads. BVATools (https://bitbucket.org/mugqic/bvatools/src) was used to extract base frequencies at each of the 9 SNP positions and the read frequencies were assimilated to the allele frequencies (Table S2). The allele frequency variance at the 9 SNP positions were compared to the

previously described allele frequencies of the ML susceptbile populations by calculating the Fixation Index ($F_{ST}$) [8–11]. The average $F_{ST}$ of each sample was calculated for the top 2 SNP-$F_{ST}$ values for the 3 US laboratory-maintained isolates and the 11 European clinical samples from 7 dogs. The $F_{ST}$ value is the average coefficient of inbreeding in a population and measures the difference in allele frequencies [26, 27]. It is a measure of population differentiation due to genetic structure with values ranging from 0 to 1. A value of 0 indicates no differentiation between subpopulations, whereas a value of 1 indicates complete differentiation. For the purpose of considering a population of microfilariae from a dog, we consider frequencies of over 0.15 at several of the indicator SNPs as likely showing a resistant population in the dog, while frequencies of the alternative allele at several SNPs of > 0.05 to 0.15 may be borderline or suggesting an early stage in the selection for resistance.

## Results

# $F_{ST}$ Genotyping

DNA from the 3 US and 11 European *D. immitis* samples were sent to Génome Québec and underwent MiSeq Illumina Sequencing. The regions surrounding the 9 SNP markers of interest were aligned to the *D. immitis* reference genome nDi.2.2. A 2-SNP pairwise $F_{ST}$ model was used to estimate an ML-susceptibility profile in comparison to the earlier characterized susceptible profile (Table 2). The 2 SNP markers utilized are located on nDi.2.2.scaf00046 at positions 22857 and 76278; herein referred to as SNP 4 and SNP 5, respectively. The highly predictive 2-SNP pairwise $F_{ST}$ model utilized in this study was determined via ROC curve performance as previously described [11].

Table 2
Calculated 2-SNP model pairwise $F_{ST}$ of SNP 4 and SNP 5 previously described (Ballesteros et al. [11]) and predicted profile of the 3 US laboratory-maintained isolates and the 11 European clinical samples.

| Sample ID | $F_{ST}$ (Sample vs SUS profile) | Profile predicted based on Genotype |
|---|---|---|
| **US laboratory-maintained isolates** | | |
| WildCat | 0.33 | Resistant |
| Berkeley | 0.00 | Susceptible |
| Georgia II | 0.07 | Susceptible |
| **European clinical samples** | | |
| T2 | 0.00 | Susceptible |
| T3 | 0.00 | Susceptible |
| T4 | 0.00 | Susceptible |
| T9 | 0.00 | Susceptible |
| T10 | 0.00 | Susceptible |
| T11 | 0.00 | Susceptible |
| C1 | 0.00 | Susceptible |
| C2 | 0.00 | Susceptible |
| C4 | 0.00 | Susceptible |
| C5 | 0.00 | Susceptible |
| M | 0.00 | Susceptible |

The laboratory-maintained putative resistant isolate WildCat had a 2-SNP model pairwise $F_{ST}$ of 0.33 calculated based on SNP 4 and SNP 5, which signals a deviation from the wildtype (susceptible) genome. Georgia II displayed minor deviation from the reference genome with a 2-SNP model pairwise $F_{ST}$ of 0.07. Whereas, Berkeley had a fixation index of 0.00 which denotes no differentiation from the wildtype genotype. The pairwise 2-SNP model $F_{ST}$ values of the 11 European clinical samples were 0.00 (Table 2). Similarly, the 11 European samples from 7 dogs also displayed a pairwise $F_{ST}$ of 0.00 at the other 7 SNP loci. It indicates that the European samples most resemble the US ML-susceptible isolates and have genotypes consistent with susceptibility.

The three adult female worm samples from Lombardy, Italy: T2, T3, and T4, were all collected from the same canine host. The three samples all presented a consistent 2-SNP pairwise $F_{ST}$ genotype between the worms collected from the same host (Dataset S1). The same phenomenon was seen in the three

adult female worms samples T9, T10, and T11, which were collected from one canine host in Hungary. Based on this analysis using a very limited number of worms, a mixed genotype *Dirofilaria* infection does not appear to be occuring in the Lombardy region of Italy, or in Hungary.

Alternate Allele Frequency

The allele frequencies were calculated from the read frequencies using BVAtools in comparision to the reference genome nDi.2.2 (Table S2). The average alternative allele frequency for each of the 9 SNP positions was then plotted for the three US laboratory-maintained isolates and the European sample M (Fig. 2). M acts as a representative for the 11 European samples. Compared to all other samples, WildCat carried higher frequencies of the alternate nucleotide for all SNP markers, with a genotype comparable to other known ML-resistant laboratory-maintained isolates, such as JYD-34 [9].

The Berkeley isolate displayed some variation to the reference genome at SNPs 2, 7 and 9. The Georgia II isolate displayed a low level of variation from 5 to 14% at SNP 9 across 5 of the 9-SNP markers. The M sample and all other European samples displayed no alternate allele frequency and had genotypes consistent with ML-susceptibility and the reference genome. The alternative nucleotide frequencies at SNPs 1 to 7 best differentiated the resistant isolate, WildCat, from the other isolates and putative susceptible isolates. SNP 9 showed the least differentiation between all the USA samples.

# Discussion

Previous research indicates an increase in *D. immitis* infections in the USA [28, 29]. ML-resistant isolates, genetically distinct from the wildtype population, have been confirmed and documented in the southern US during the last decade. This is perhaps not unexpected after long term and widespread use of prophylactic MLs [7–11]. SNP analysis has been conducted on previously established US laboratory-maintained isolates with known phenotypes such as the ML-susceptible Missouri and Kentucky isolates, as well as the ML-resistant JYD-34 and Metairie isolates [9]. The current study is the first to complete genomic level analysis of the new putative susceptible isolates Berkeley and Georgia II, and the putative resistant isolate WildCat.

The research of Ballesteros et al. [11] demonstrated that the most highly predictive SNP pairwise $F_{ST}$ combination was that of SNP 4 and 5; compared to the original 9 SNP markers. The 2-SNP pairwise $F_{ST}$ model correctly differentiated all samples in the Ballesteros et al. study. Using the 9 most effective markers, our data illustrated the same phenomenon. The 2-SNP pairwise $F_{ST}$ better differentiated the US laboratory-maintained samples. The European clinical samples were so closely aligned to the reference genome both predictive models functioned comparably. When reviewing the alternate allele frequency (Fig. 2) it was also apparent that SNPs 1–7 were more reliable compared to SNP 8 and 9; particularly SNPs 1 and 3 through 6. These markers demonstrate the highest level of alternate allele variability between the putative susceptible Berkeley and Georgia II isolates with the putative resistant WildCat isolate.

The Berkeley isolate displays a susceptible genotype consistent with that of the other previously categorized ML-susceptible isolates at the 9 SNP markers in comparison with the *D. immitis* reference genome nDi.2.2. The putative ML-resistant WildCat isolate has a high degree of variability across all 9 SNP markers. It has a 2-SNP pairwise $F_{ST}$ of 0.33. WildCat presents an alternate allele frequency of greater than 20% at all 9 SNP markers, and greater than 39% at SNP markers 1–7. Conversely, Georgia II displays both an alternate allele frequency and 2-SNP pairwise $F_{ST}$ between the genotypically susceptible Berkeley and genotypically resistant WildCat isolates. Georgia II has a 2-SNP pairwise $F_{ST}$ of 0.07. Moreover, the Georgia II isolate has a consistent level of variability at SNPs 2, 4, 5, 6 and 9, compared with other published susceptible isolates and Berkeley and all the European isolates in this study. Additional research is required to confirm whether the Georgia II isolate is genotypically and phenotypically fully ML-susceptible or perhaps in the early stages of being selected towards ML-resistance. Unfortunately, when clinical trials are run *in vivo*, only one dose rate is usually tested, that proposed for the commercial product. Therefore, it is not easy to pick up early evidence of resistance selection. The genomic variability of the Georgia II isolate raises several questions. To date US laboratory-maintained isolates are characterized as ML susceptible due to their elimination at the commercial dose rate of treatment or proposed lack of ML-drug exposure. A lack of history of prophylactics in a particular dog does not necessarily mean that the ancestors of the challenge worms had not been exposed to repeated ML chemoprophylaxis. In fact, the AHS recommendation that all dogs in the USA be treated 12 months of the year means that *D. immitis*, in the USA, whose ancestors are truly naïve to ML prophylaxis are probably becoming rare.

Genomic level testing via the SNP molecular markers provide key background information on the *D. immitis* isolates currently being used in laboratory and pharmaceutical research. However, little information has been documented on the potential development of ML-resistance in European *D. immitis* populations. The European M sample, a representative for the 11 European samples, had the genotype and allele frequencies most closely aligned with the *D. immitis* reference genome nDi.2.2 (Fig. 2). The 11 European heartworm infections could have arisen because of the development of ML-resistance or due to incomplete use of ML-based heartworm preventives. The genotype analysis of the European clinical samples showed that all 11 samples had genotypes consistent with susceptibility as defined by the molecular marker 2-SNP model pairwise $F_{ST}$. While only a small number of *D. immitis* samples were genotyped, from a limited number of countries, the results of the study indicate no evidence for the development of ML-resistance in Europe or its introduction via movement of infected animals. This conclusion is supported by data presented at the 6th Congress of the European Society of Dirofilariosis and Angiostrongylosis in Belgrade, in 2018 [30]. A larger study across more geographical locations should be considered for conclusive evidence.

# Conclusion

Autochthonous transmission of canine *D. immitis* appear to be migrating from the Mediterranean and Iberian Peninsula moving further into Central and Northern Europe [13–15, 19], likely, a result of the

movement of dogs and cats around Europe, and possibly a result of increasing mosquito populations. To date, no case of ML-resistant *D. immitis* infection has been documented in Europe. As the number of *D. immitis* infections continues to rise and spread throughout Europe, the early adoption of genotyping of clinical *D. immitis* samples could provide an early indication of the potential development of ML-resistance and aid to distinguish clinical cases of heartworm infection due to ML resistance from those due to a lack of prevention or inadequate compliance, as has been seen in North America. Epidemiological surveys of *D. immitis* samples collected across Europe from historically endemic regions of the Mediterranean, newly endemic regions such as the Balkans and Austria, and previously non-endemic regions of Northern Europe can provide insight into the genetic makeup and genetic diversity of European clinical samples [18, 20, 21]. The 2-SNP model pairwise $F_{ST}$ genotypic analysis of clinical *D. immitis* samples can be utilized to help monitor the efficacy and susceptibility of ML-based heartworm preventives.

# Abbreviations

ML
Macrocyclic Lactone; mf:Microfilaria; SNP:Single nucleotide polymorphism; PETS:EU Pet Travel Scheme; FDA/CVM:US Food and Drug Administration / Center for Veterinary Medicine; LOE:Loss of efficacy; BAM:Binary alignment map files.

# Declarations

### Ethics approval and consent to participate

All experimental procedures were approved by McGill University in accordance with relevant guidelines and regulations.

### Consent for publication

Elanco consents to release of this information for publication.

### Availability of data and materials

The data supporting the conclusions of this article are included within the article.

### Competing interests

The research was supported by Elanco Animal Health. LY and HS are employees of the sponsor. However, the sponsor of the research exercised no influence over the conduct of the research. The other authors declare no conflicts of interest that could influence the conduct or results of this study.

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### Authors' contributions

Experiments were conceived and designed by LY and RKP. *D. immitis* samples were provided by DT, EC, LK, and HS. Experiments and data analysis were performed by EC*. The manuscript was written by EC* and RKP. All authors read and approved the final manuscript.

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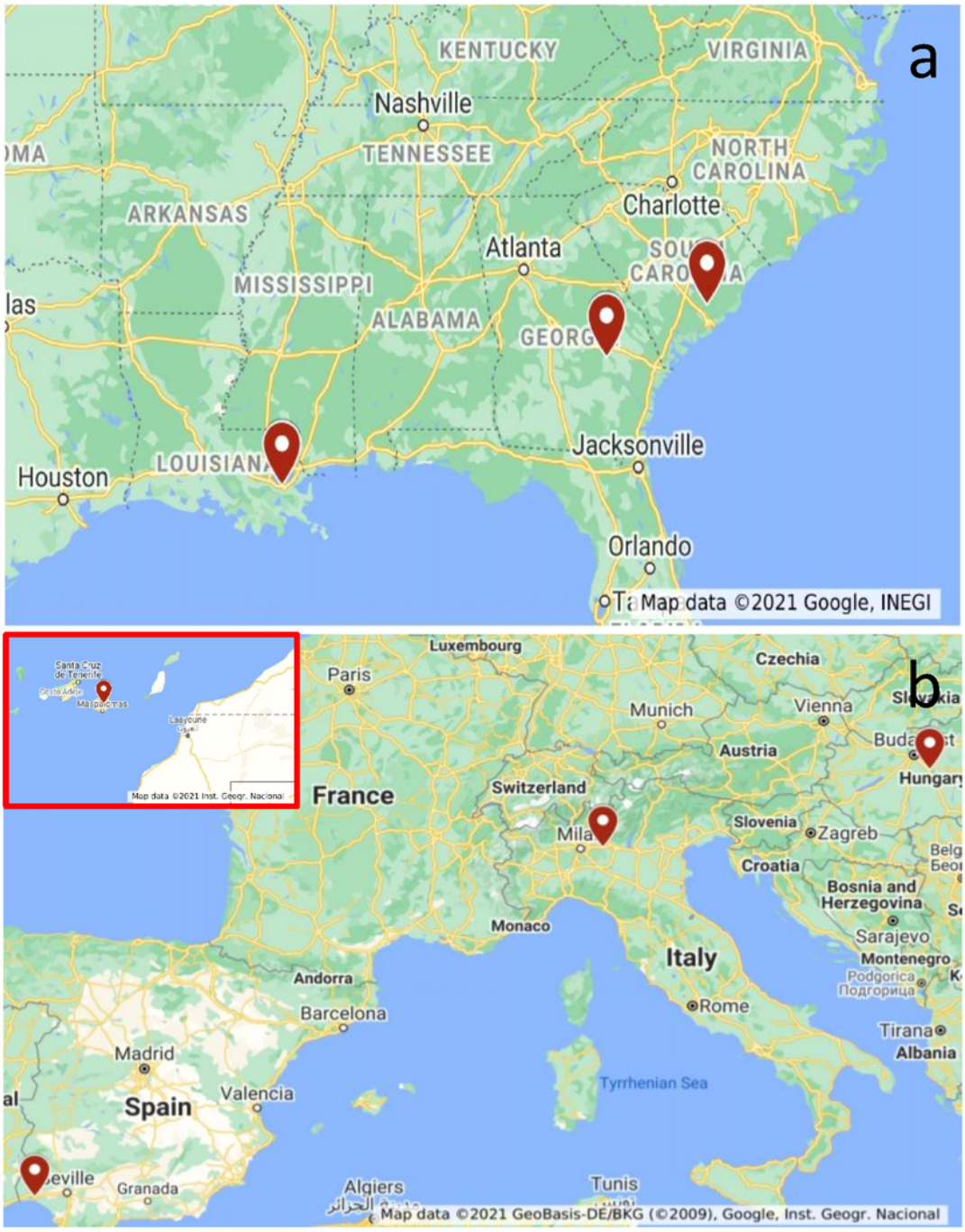
# Figures




## Figure 1

a) Distribution of US laboratory-maintained isolates received from TRS Laboratories b) Distribution of European samples received from Italy, Hungary, Spain, and the Canary Islands. The maps were created using Google Maps, accessed May 2nd and August 26th, 2021.

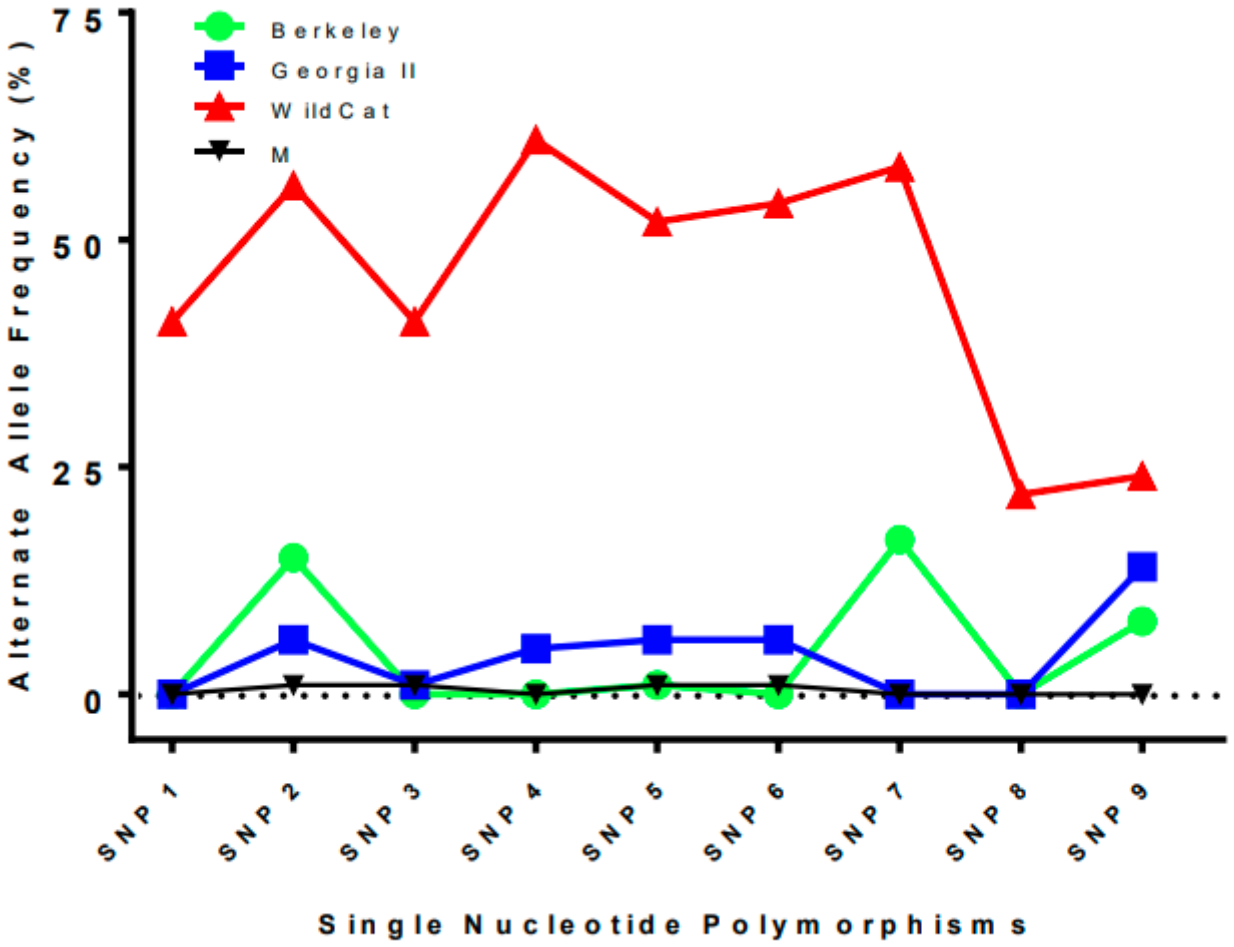



**Figure 2**

The genetic profiles of Berkeley (green circle and line), Georgia II (blue square and line), WildCat (red triangle and line), and M (black inverted triangle and line) as the difference in the percentage of the alternative nucleotide frequencies for the 9 SNPs molecular markers when compared to the D. immitis reference genome nDi.2.2.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CURRYEmilyetalElancoParaVectorTableS1300821.docx
- CURRYEmilyetalElancoParaVectorFileS1270821.docx
- CURRYEmilyetalElancoParaVectorGraphicalAbstract26082021.png