

Drug resistance in canine heartworm (*Dirofilaria immitis*): Surveillance in Australia and global genomic insights

*A thesis with publication submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy (Science)*

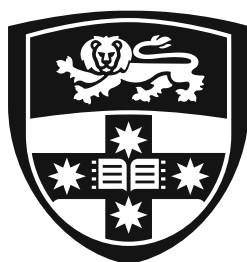
by

Rosemonde Isabella Power

BAnVetBioSc (Hons I)

Primary supervisor: Professor Jan Šlapeta

Secondary supervisor: Dr Nichola Calvani



Sydney School of Veterinary Science, Faculty of Science

The University of Sydney

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scholarship to the PhD Candidate*

Statement of originality

This thesis is submitted to The University of Sydney in order to fulfil the requirements for the degree of Doctor of Philosophy. I, Rosemonde Isabella Power, certify that to the best of my knowledge, the content of this thesis is my own original work, unless acknowledged in the text.

This thesis has not been submitted for any other degree or purposes at The University of Sydney or any other academic institution.

Rosemonde Isabella Power

26th September 2024

Authorship attribution statement

All publications in this thesis are accompanied by an author attribution statement that clarifies the contribution of all authors. This thesis contains one unpublished manuscript, which features a detailed outline of all authors' contributions thus far. Additional co-authored publications are included in the Appendix and are provided solely for reference due to their relevance to this thesis; they are not part of the main text.

In addition to the statements above, in cases where I am not the corresponding author of a published item in the main text, permission to include the published material has been granted by the corresponding author.

Rosemonde Isabella Power

26th September 2024

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements in this thesis are correct.

Professor Jan Šlapeta

26th September 2024

Preface

This thesis contains four main chapters, with three sub-chapters (Chapters 2.1, 2.2, and 3.2) published as peer-reviewed papers, where I am the first author. The chapters are organised based on the overall narrative of the thesis and do not necessarily reflect the order of publication.

Chapter 1 presents an overview of heartworm and drug resistance, identifies relevant knowledge gaps and concerns specific to Australia, and outlines the aims and objectives of this thesis.

Chapter 2 investigates potential drug resistance in Australian heartworm populations. Specifically, Chapter 2.1 examines a cohort of dogs in Queensland and New South Wales, Australia for phenotypic and genotypic evidence of resistance, using approaches adopted from the USA. Chapter 2.2 extends this research by conducting the first microfilarial suppression test (MFST) on Australian heartworms, along with genetic screening for resistance-associated markers.

Chapter 3 employs whole genome sequencing techniques to characterise the global genomic background of heartworms. Specifically, Chapter 3.1 presents a population genomics analysis based on whole genome sequences from 127 adult heartworms collected across four continents. Chapter 3.2 evaluates the use of microfilariae samples as an alternative to adult worms for large-scale genome studies.

Chapter 4 provides a general discussion of the key outcomes and significant findings of this thesis, their implications, and suggests future directions for research.

The work presented in this thesis was in part supported by the Canine Research Foundation and Dogs Victoria, Australia and by the Australian Companion Animal Health Foundation Research Grant. During my candidature, I was a recipient of an Australian Government Research Training Program (RTP) Stipend, RTP Fee Offset, and The Jean Walker Trust Fellowship. My travel to national and international conferences was funded by the Australian Society for Parasitology (ASP) and the University of Sydney Postgraduate Research Support Scheme (PRSS). My research exchange to the Wellcome Sanger Institute and the University of Cambridge, UK was supported by the ASP and the University of Sydney's Eric Horatio Scholarship.

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Publications, presentations and awards arising from candidature

Publications

- **Power, R. I.**, & Šlapeta, J. (2024). Delayed canine heartworm (*Dirofilaria immitis*) microfilarial reduction following Advocate™ for dogs (imidacloprid, moxidectin) treatment. *The Veterinary Journal*, 307, 106209. <https://doi.org/10.1016/j.tvjl.2024.106209> (**Chapter 2.2**).
- **Power, R. I.**, Doyle, S. R., & Šlapeta, J. (2024). Whole genome amplification and sequencing of individual *Dirofilaria immitis* microfilariae. *Experimental Parasitology*, 263-264, 108806. <https://doi.org/10.1016/j.exppara.2024.108806> (**Chapter 3.2**).
- Gandasegui, J., **Power, R. I.**, Curry, E., Lau, D. C.-W., O'Neill, C. M., Wolstenholme, A., Prichard, R., Šlapeta, J., & Doyle, S. R. (2024). Genome structure and population genomics of the canine heartworm *Dirofilaria immitis*. *International Journal for Parasitology*, 54(2), 89-98. <https://doi.org/10.1016/j.ijpara.2023.07.006> (**Appendix 3**).
- Lau, D. C.-W., **Power, R. I.**, & Šlapeta, J. (2024). Exploring multiplex qPCR as a diagnostic tool for detecting microfilarial DNA in dogs infected with *Dirofilaria immitis*: A comparative analysis with the modified Knott's test. *Veterinary Parasitology*, 325, 110097. <https://doi.org/10.1016/j.vetpar.2023.110097> (**Appendix 2**).
- **Power, R. I.**, & Šlapeta, J. (2022). Exploration of the sensitivity to macrocyclic lactones in the canine heartworm (*Dirofilaria immitis*) in Australia using phenotypic and genotypic approaches. *International Journal for Parasitology: Drugs and Drug Resistance*, 20, 145-158. <https://doi.org/10.1016/j.ijpddr.2022.11.003> (**Chapter 2.1**).
- Huang, H. H. H., **Power, R. I.**, Mathews, K. O., Ma, G. C., Bosward, K. L., & Šlapeta, J. (2021). Cat fleas (*Ctenocephalides felis* clade 'Sydney') are dominant fleas on dogs and cats in New South Wales, Australia: Presence of flea-borne *Rickettsia felis*, *Bartonella* spp. but absence of *Coxiella burnetii* DNA. *Current Research in Parasitology & Vector-Borne Diseases*, 1, 100045. <https://doi.org/10.1016/j.crpvbd.2021.100045>

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- **Power, R. I.** (2024). *Drug resistance in canine heartworm (Dirofilaria immitis): Surveillance in Australia and global genomic insights*. Presented an exit seminar to the Sydney School of Veterinary Science (SSVS) at The University of Sydney, Australia (30-min oral presentation).
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- **Power, R. I.** (2022). *Suspect drug resistance cases with the canine heartworm (*Dirofilaria immitis*): mismatch between microfilarial suppression test and SNP results in Australia*. Presented at the Sydney School of Veterinary Science (SSVS) Postgraduate & Staff Research Conference in Sydney, Australia (2-min oral presentation & poster).
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- **Power, R. I.** (2021). *Investigating macrocyclic lactone resistance in canine heartworm (*Dirofilaria immitis*) from Australia*. Presented at the Sydney School of Veterinary Science (SSVS) Postgraduate & Staff Research Conference held online (5-min oral presentation).
- **Power, R. I.** (2021). *Investigating drug resistance in canine heartworms (*Dirofilaria immitis*) in Australia*. Presented at the higher degree by research webinar series for the Australian Association of Animal Science (AAAS) held online (5-min oral presentation).
- **Power, R. I., & Šlapeta, J.** (2021). *Investigating macrocyclic lactone resistance in canine heartworm (*Dirofilaria immitis*) from Australia using Illumina amplicon NGS*. Presented at the Australian Society for Parasitology (ASP) Annual Conference held online (3-min oral presentation).

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- Awarded ‘Runner-up Best 5 Minute Talk presented by a student’ at the Australian Society for Parasitology (ASP) Annual Conference in Cairns, Australia (2022).
- Student Conference Travel Grant. Australian Society for Parasitology (ASP) (2022). Received for travel to the ASP Annual Conference in Cairns, Australia.
- Recipient of the Jean Walker Trust Fellowship. Sydney School of Veterinary Science (SSVS), The University of Sydney (2021-2024).
- Recipient of the Research Training Program (RTP) Scholarship. The University of Sydney (2021-2024).
- Awarded ‘Specially Commended Talk by a student’ at the Australian Society for Parasitology (ASP) Annual Conference held online (2021).

Thesis abstract

Canine cardiopulmonary dirofilariasis caused by heartworms (*Dirofilaria immitis*) is a potentially fatal condition affecting domestic and wild Carnivora species globally. To prevent dirofilariasis, the veterinary industry has heavily relied on the macrocyclic lactone (ML) drug class. However, the widespread and routine use of these drugs has led to the emergence of drug-resistant isolates in the USA. In Australia, heartworm infections are re-emerging and there are growing suspicions of potential drug resistance, particularly in the northern tropical and subtropical regions of Queensland (QLD). Yet, no phenotypic and only limited genotypic testing for resistance have been conducted in these populations. The enigmatic origins, history, and current population structure of this parasite in Australia further hinder our ability to understand the likelihood and rate at which resistance may develop and spread. The aim of this thesis was to use phenotypic and genotypic tools to uncover the potential presence of drug-resistant heartworms in Australia, while elucidating their cryptic origins and genomic background. Sequential modified Knott's tests on a cohort of dogs from QLD and New South Wales (NSW) revealed sub-optimal reductions in microfilaremia ($\leq 90\%$) 30 days post-ML treatment in 16 out of 22 dogs, suggesting potential drug resistance. This was further supported by microfilarial reductions of $<40\%$ in two dogs from Townsville, Australia using the in-vivo 7-day microfilarial suppression test (MFST). Although these dogs initially showed sub-optimal reductions, their microfilaremia eventually cleared after an extended period of time. Genetic screening of microfilariae from all dogs did not reveal any resistance-associated signatures found in the USA, which contradicted the phenotypic test results. To explore heartworm genomics globally, the largest genome-wide study of this parasite to date was conducted using 127 adult specimens from four continents. Population genomics analyses revealed genetically distinct heartworm populations on each continent, indicating that resistance markers developed in the USA are likely non-informative elsewhere. The study also discovered that Australian heartworms are of Asian origin and likely entered the country with dingoes. Finally, attempts to conduct whole genome sequencing on individual microfilariae proved unsuitable due to poor genome coverage. The outcomes of this thesis not only shed light on the intriguing history of heartworms and the potential emergence of drug resistance in Australia, but also have broader global applications that can assist in the future control of this expanding parasite.

List of abbreviations used

µg	Microgram
µL	Microlitre
µm	Micrometre
AHS	American Heartworm Society
ASP	Australian Society for Parasitology
ASV	Amplicon sequence variant
AUS	Australia
BAM	Binary Alignment Map
BEAST2	Bayesian Evolutionary Analysis Sampling Trees 2
BEAUti	Bayesian Evolutionary Analysis Utility
BIC	Bayesian information criterion
BKK	Bangkok
BLAST	Basic Local Alignment Search Tool
BNE	Brisbane
BOC	Boca Chica
bp	Base pairs
BSP	British Society for Parasitology
BUC	Bucharest
CDC	Centers for Disease Control and Prevention
CENAM	Central America
Chr	Chromosome
cm	Centimetres
CNS	Cairns
COM	Comana
<i>CoxI</i>	Cytochrome c oxidase subunit I
CRI	Costa Rica
C _t or C _q	Cycle threshold
CVM	Center for Veterinary Medicine
ddH ₂ O	Double-distilled water
ddPCR	Droplet digital polymerase chain reaction
DEC	Diethylcarbamazine citrate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENA	European Nucleotide Archive

ESS	Effective sample size
EUR	Europe
FDA	U.S. Food and Drug Administration
FLO	Florida
<i>ftsZ</i>	Filamenting temperature-sensitive mutant Z
G or Gb	Gigabase
g/L	Grams per litre
GAPDH	Glyceraldehyde3-phosphate dehydrogenase
GEO	Georgia
GIU	Giurgiu
GRC	Greece
HET	Heterozygous
ILL	Illinois
ITA	Italy
IVM	Ivermectin
kb	kilo base pairs
kg	Kilogram
Kya	Thousand years ago
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Fourth-stage larvae
LD	Linkage disequilibrium
LHR	Lockhart River
LOE	Lack of efficacy
LOU	Louisiana
MCMC	Markov chain Monte Carlo
mf/mL	Microfilariae per millilitre
MFST	Microfilarial suppression test
mg	Milligram
Min	Minute
MIS	Missouri
mL	Millilitre
ML	Macrocyclic lactone
MLR	Macrocyclic lactone resistance
mm	Millimetre
MO	Milbemycin oxime

MOX	Moxidectin
mtDNA	Mitochondrial deoxyribonucleic acid
Mya	Million years ago
MYS	Malaysia
NCBI	National Center for Biotechnology Information
NEA	Northeast
NGS	Next generation sequencing
nm	Nanometre
nM	Nanomolar
NSW	New South Wales
NTC	No template control
OD	Optical density
OUT	Outgroup
p.o.	By mouth
PAN	Panama
PAV	Pavia
PBS	Phosphate-buffered saline
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
<i>Pgp</i>	P-glycoprotein
PUE	Puerto Armuelles
QC	Quality control
QLD	Queensland
qPCR	Real-time polymerase chain reaction
RCF	Relative centrifugal force
RES	Resistant
RFU	Relative fluorescence unit
ROK	Rockhampton
ROU	Romania
RTP	Research Training Program
s	Seconds
s.c.	Subcutaneous
SAM	Sequence Alignment Map
SD	Standard deviation
SEL	Selamectin
SJO	San José

SLO	San Lorenzo
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SRA	Sequence read archive
SSVS	Sydney School of Veterinary Science
SUS	Susceptible
sWGA	Selective whole genome amplification
SYD	Sydney
TEX	Texas
THA	Thailand
TSV	Townsville
UFBoot	Ultrafast bootstrap approximation
UK	United Kingdom
US	United States
USA	United States of America
VCF	Variant call format
VNM	Vietnam
WGA	Whole genome amplification
WGS	Whole genome sequencing
WHO	World Health Organization
XAN	Thessaloniki/Xanthi

Table of contents

Statement of originality	i
Authorship attribution statement.....	ii
Preface.....	iii
Acknowledgements	iv
Publications, presentations and awards arising from candidature	v
Publications.....	v
Conference presentations and invited seminars	v
Awards, scholarships and grants.....	vi
Thesis abstract.....	viii
List of abbreviations used	ix
Table of contents	xiii
Chapter 1 Introduction: Background, knowledge gaps, and thesis aims	1
1.1 <i>Dirofilaria immitis</i> – the agent of canine heartworm disease	2
1.2 Life cycle and clinical signs.....	2
1.3 Worldwide distribution	3
1.4 Diagnosis.....	4
1.5 Heartworm prevention	6
1.6 Drug-resistant <i>Dirofilaria immitis</i> in the United States	7
1.7 Tools to detect drug resistance in <i>Dirofilaria immitis</i>	9
1.8 <i>Dirofilaria immitis</i> in Australia.....	12
1.9 Knowledge gaps and thesis aim	13
References.....	16
Chapter 2 Screening for potential drug resistance in Australian heartworm populations.....	21
2.1 Exploration of the sensitivity to macrocyclic lactones in the canine heartworm (<i>Dirofilaria immitis</i>) in Australia using phenotypic and genotypic approaches	22
Author attribution statement	23
Published manuscript.....	24
Connecting statement 1.....	38

2.2 Delayed canine heartworm (<i>Dirofilaria immitis</i>) microfilarial reduction following Advocate™ for dogs (imidacloprid, moxidectin) treatment	39
Author attribution statement	40
Published manuscript	41
Connecting statement 2	49
Chapter 3 Understanding the genomic background of heartworms in Australia and beyond....	50
3.1 Population genomics reveals ancient origin of heartworms in canids	51
Abstract.....	55
Introduction.....	56
Results and Discussion	58
Concluding remarks	64
Methods	65
Data availability	70
Code availability	70
References.....	71
Acknowledgements.....	77
Figures	78
Extended Data Figures.....	84
Supplementary Figures	94
Connecting statement 3.....	99
3.2 Whole genome amplification and sequencing of individual <i>Dirofilaria immitis</i> microfilariae	100
Author attribution statement	101
Published manuscript.....	102
Chapter 4 General discussion	109
4.1 Thesis overview and significance	110
4.1.1 Confirming the perceived presence of phenotypic drug resistance in Australian heartworms using traditional microscopy-based diagnostic tools	110
4.1.2 Revealing whether Australian heartworms possess genetic signatures associated with drug resistance in the USA	111

4.1.3 Uncovering the ancestral origin and genetic background of Australian heartworms using a whole genome sequencing approach	112
4.2 Future directions	113
4.3 Conclusion	115
References.....	116
Appendix	119
Appendix 1 <i>Dirofilaria immitis</i> isolates from the USA demonstrating <100% macrocyclic lactone efficacy in clinical experimental infection studies.....	120
Appendix 2 Exploring multiplex qPCR as a diagnostic tool for detecting microfilarial DNA in dogs infected with <i>Dirofilaria immitis</i> : A comparative analysis with the modified Knott's test	126
Author attribution statement	127
Published manuscript.....	128
Appendix 3 Genome structure and population genomics of the canine heartworm <i>Dirofilaria immitis</i>	134
Author attribution statement	135
Published manuscript.....	136

———— Chapter 1 ————

Introduction:

Background, knowledge gaps, and thesis aims

1.1 *Dirofilaria immitis* – the agent of canine heartworm disease

Canine heartworm disease is a potentially fatal parasitic disease impacting dogs, cats, and numerous other mammals worldwide (Diakou & Prichard, 2021). The disease is caused by the filarial nematode *Dirofilaria immitis* (Leidy, 1856) which belongs to the family Onchocercidae. It is closely related to human filarial parasites such as *Onchocerca volvulus*, the causative agent of river blindness, as well as *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, which cause lymphatic filariasis (Centers for Disease Control and Prevention (CDC), 2017; World Health Organisation (WHO), 2023). It has been estimated that heartworm costs pet owners a global total of US \$2.47 billion dollars annually, with Australia accounting for \$80 million (Klug & Drake, 2022).

1.2 Life cycle and clinical signs

The life cycle of *D. immitis* requires an intermediate mosquito host and a canine definitive host, with no free-living stages of the parasite. The earliest life stages of *D. immitis* are called microfilariae, which can be found circulating in the bloodstream of an infected animal. Microfilariae are small in size and are ingested by mosquito vectors during a blood meal (Russell & Geary, 1996). Over 60 mosquito species can transmit *D. immitis* including those belonging to the genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, *Coquillettidia*, *Ochlerotatus*, and *Psorophora* (Bowman & Atkins, 2009; Cancrini et al., 2006; Manrique-Saide et al., 2008; Russell & Geary, 1996; Theis et al., 2000). Inside mosquito vectors, microfilariae develop into first-stage larvae (L1) to infective third-stage larvae (L3) before they are transmitted to other mammalian hosts during subsequent blood meals (Noack et al., 2021).

The L3s in newly-infected hosts rapidly develop into fourth-stage larvae (L4) and become adults approximately six months after the initial infection (Bowman & Atkins, 2009; Noack et al., 2021). The large size of adult *D. immitis*, coupled with the potential presence of multiple adult worms in the lobar arteries, pulmonary artery, and in severe cases the right cardiac ventricle, can lead to life-threatening cardiopulmonary complications (Noack et al., 2021). Adult *D. immitis* cause vascular lesions, disturbances in blood flow, and raised blood pressure in the pulmonary artery (Bowman & Atkins, 2009). On a clinical level, this initially manifests as a persistent cough and exercise intolerance in dogs, potentially progressing to right-sided heart failure (Noack et al., 2021). The presence of both adult male and female *D. immitis* within the cardiopulmonary system can lead to the production of microfilariae offspring. These microfilariae circulate in the host's bloodstream, ready to be ingested by a mosquito vector. The complete life cycle of *D. immitis* is illustrated in Fig. 1.

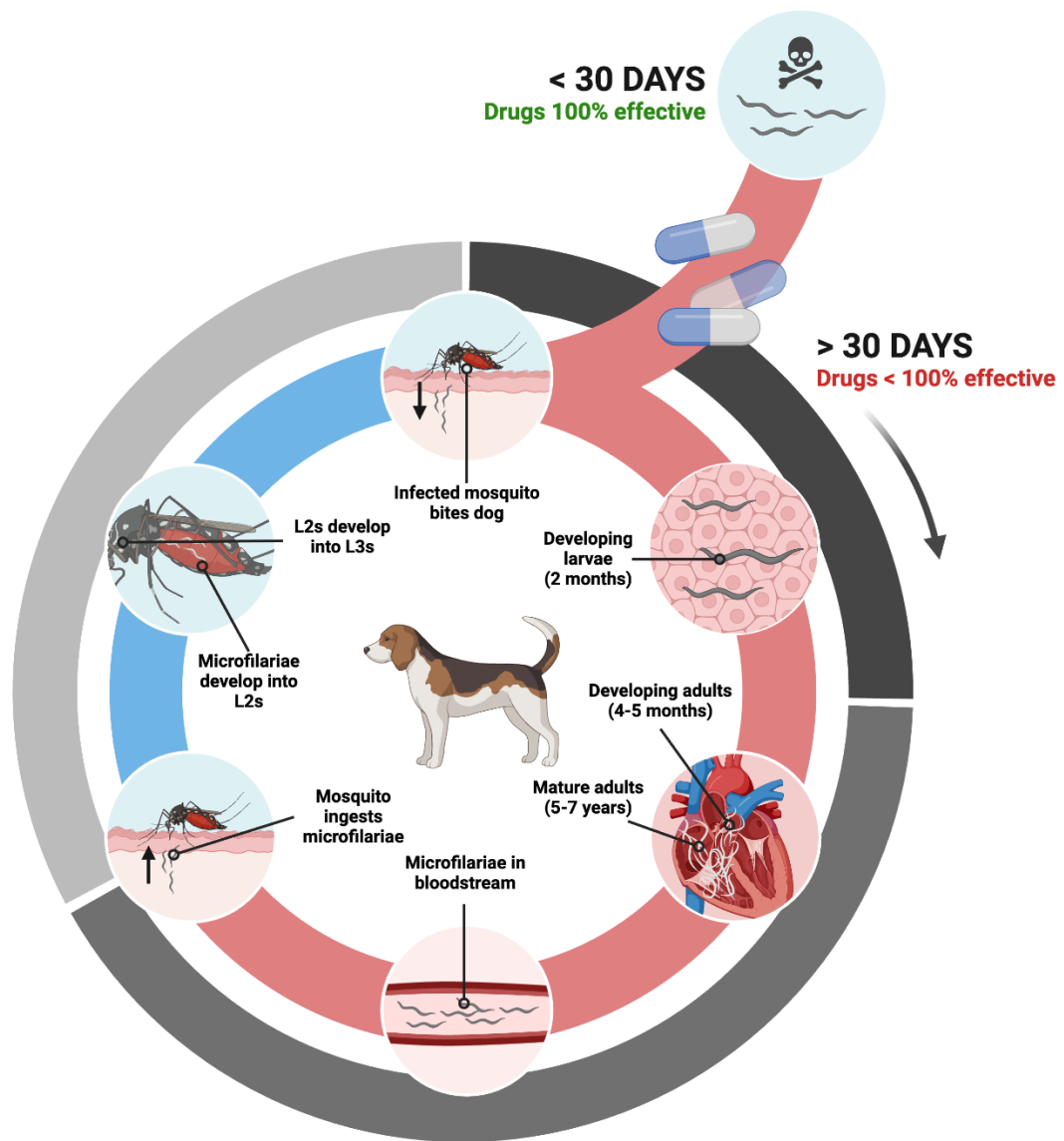


Fig. 1 An overview of the *Dirofilaria immitis* life cycle and the effectiveness of macrocyclic lactone preventives against larval stages of the parasite. Figure adapted from the life cycle diagram by American Heartworm Society (AHS) (2020). Created with BioRender.com.

1.3 Worldwide distribution

The climatic conditions and mosquito vectors required for *D. immitis* development have played a crucial role in its distribution, with *D. immitis* currently found in tropical, subtropical, and temperate regions (Dantas-Torres et al., 2023). *D. immitis* infections have been documented in most continents of the world including North America, South America, Europe, Africa, Australia, and Asia (Dantas-Torres et al., 2023; Genchi & Kramer, 2020; Noack et al., 2021; Simón et al., 2012). A positive correlation exists between *D. immitis* development in mosquitoes and temperature (Genchi et al., 2009). As such, predicted increases in temperature and changes in rainfall and humidity due to climate change are expected to increase the prevalence of *D. immitis* and expand its geographical range (Genchi et al.,

2009; Knight & Lok, 1998; Simón et al., 2012). In addition, the migration of infected dogs remains a constant threat (Knight & Lok, 1998). For example, Levy et al. (2011) surveyed over 400 dogs that were rescued and transferred to other regions of the USA after Hurricane Katrina in 2005. It was revealed that almost 50% of these rescued dogs were infected with *D. immitis*.

1.4 Diagnosis

The American Heartworm Society (AHS) recommends that all dogs over the age of 7 months should be screened for both *D. immitis* antigen and microfilariae each year (Nelson et al., 2024).

Antigen

Commercial *D. immitis* antigen tests detect a protein in the infected host's blood, which is secreted by adult female *D. immitis* (Weil, 1987). Rapid commercial antigen tests are suitable for in-clinic and field use, as only a few drops of blood or plasma are required to obtain a test result (Lu et al., 2017; Panarese et al., 2022). Most antigen tests are almost 100% specific with the ability to detect as few as one adult female *D. immitis* in the host (Nelson et al., 2024). Other tests utilising enzyme-linked immunosorbent assays (ELISAs) are available (Donnett et al., 2018). Due to the long prepatent period of *D. immitis*, antigen testing can only detect *D. immitis* approximately five months after infection (Nelson et al., 2024).

Microfilariae

Detection of *D. immitis* microfilariae is dependent on the presence of both male and female adults in the host. Non-concentration techniques such as blood smears or buffy coat examinations are simple, rapid approaches for identifying microfilariae in the clinic. A blood smear requires a single drop of fresh blood on a microscope slide, whereas a buffy coat examination involves examining microfilariae movement in a microhematocrit tube (Nelson et al., 2024; Trancoso et al., 2020). Despite being more time-consuming and labourous, techniques which concentrate the microfilariae and quantify their numbers are often more accurate (Knott, 1939). The most common approach for microfilariae concentration is the modified Knott's test (Fig. 2a), which can be done either in-clinic or outsourced to veterinary diagnostic laboratories (Culda et al., 2022; Laidoudi et al., 2021).

Adults

Extraction of *D. immitis* adults can only be performed during surgery or necropsy (Fig. 2b) (Alsarraf et al., 2023; Panayotova-Pencheva et al., 2020). Alternatively, adult stages and their impacts can be detected using echocardiography or radiography (McKeever et al., 2021).

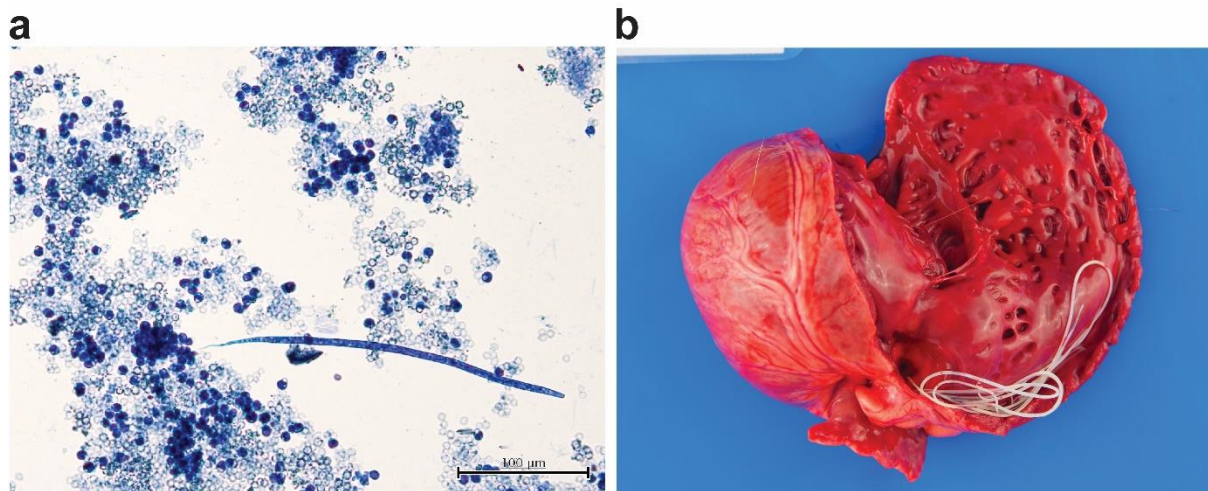


Fig. 2 Detectable life stages of *Dirofilaria immitis*. **a** *D. immitis* microfilaria from dog blood, observed during the modified Knott's test. Image taken by the author (Rosemonde Power). **b** Adult *D. immitis* found in a dog heart during necropsy. Image provided by the Sydney School of Veterinary Science, The University of Sydney.

Molecular

Molecular diagnostic techniques such as polymerase chain reaction (PCR), Sanger sequencing, and next-generation sequencing can be performed to identify *D. immitis* microfilariae (Alsarraf et al., 2023; Bourguinat et al., 2015; Smith et al., 2022). The traditional approach of detecting *D. immitis* microfilariae using the modified Knott's test is time-consuming, subjective, and susceptible to human error. This is particularly an issue when dealing with cases of high microfilaremia, where overlapping microfilariae can be difficult to distinguish. Molecular approaches on the other hand are often more rapid, cost-effective, and accurate, with sequencing providing the added benefit of genotyping *D. immitis* samples (Bourguinat, Keller, Prichard, et al., 2011). The technology and skills required to perform molecular testing are often not available in-clinic and must be outsourced to a diagnostic laboratory.

Wolbachia

Wolbachia are endosymbiotic bacteria found within *D. immitis* (Kozek, 2005). They are located in the lateral chords of adult heartworms, microfilariae, and other larval stages (Kozek, 2005). In female heartworms, *Wolbachia* are also present in the oocytes and in the developing microfilarial stages within the uterus (Kozek, 2005). The reliance of *D. immitis* on *Wolbachia* has made the bacteria a key target in heartworm treatment (Nelson et al., 2024). Administering doxycycline prior to adulticide treatment has been shown to eliminate *Wolbachia*, which in turn kills all L3s and L4s, reduces the number of juvenile worms, decreases microfilaremia levels, and blocks the transmission of the parasite (McCall et al., 2014; McCall et al., 2011). The endosymbiont can also be used to diagnose heartworm and monitor treatment outcomes. For example, measuring *Wolbachia* DNA concentrations in the blood can serve as a surrogate marker for heartworm microfilariae levels.

1.5 Heartworm prevention

Daily administration of diethylcarbamazine citrate (DEC) during the mosquito season was the primary method of heartworm prevention up until the 1980s (Geary, 2023). Despite the effectiveness of DEC, the daily dosing regimen required was laborious (Geary, 2023). A negative microfilariae test was also required before commencing DEC due to its toxicity in previously untreated microfilaremic dogs (Nelson et al., 2005). The discovery of ivermectin (IVM), a type of macrocyclic lactone (ML), and its introduction into both the human and veterinary pharmaceutical markets in the 1980s has revolutionised parasite control worldwide (Ōmura & Crump, 2004). IVM allowed pet owners to protect their dogs against *D. immitis* by administering preventive only once a month (Ohishi et al., 1987). Several other MLs have since been developed for heartworm prevention including milbemycin oxime (MO), moxidectin (MOX), and selamectin (SEL) (Nelson et al., 2024). Oral formulations of IVM, MO, and MOX, and topical formulations of MOX and SEL have been registered to be 100% effective at killing *D. immitis* larvae less than 30 days old (Fig. 1) (Prichard, 2021). By doing so, the infective larvae in the host are prevented from reaching the heart and maturing into pathogenic adult stages (Noack et al., 2021). The AHS and U.S. Food and Drug Administration (FDA) recommend year-round heartworm prevention to not only prevent disease, but to reinforce owner compliance (Nelson et al., 2024). To further improve compliance, a slow-release (SR) formulation of MOX-impregnated lipid microspheres has been developed for subcutaneous injection every six or 12 months (Nelson et al., 2024).

1.6 Drug-resistant *Dirofilaria immitis* in the United States

Drug complaints

ML preventives have successfully protected dogs against *D. immitis* for the past three decades. Despite this success, the Center for Veterinary Medicine (CVM), FDA received the first complaints about lack of efficacy (LOE) of heartworm preventives in 1998 (Hampshire, 2005). The incidence of LOE complaints rose over the next few years, with an almost two-fold (57%) increase in complaints from 2000 to 2002, and again from 2002 to 2003 (Hampshire, 2005). A large portion of these LOE complaints originated from regions experiencing high levels of endemic *D. immitis* infection, including the Mississippi River (Hampshire, 2005). In the same year this FDA report was published, a review by Prichard (2005) concluded that resistance in *D. immitis* was unlikely due to the early life stages of *D. immitis* targeted and the drug dosages administered. In addition, it was suggested that the majority of the heartworm population remained in refugia, i.e., worms not exposed to drug treatment and free from selection pressure for resistance (Prichard, 2005). A later study by Atkins et al. (2014) evaluated over 270 cases of LOE from 2004 to 2011. By doing so, it was revealed that over 80% of LOE cases had either insufficient ML purchase history or other factors which likely prevented the dog from receiving sufficient and timely treatment (Atkins et al., 2014). Although these data indicated that the underlying cause of LOE cases was often due to inadequate compliance, the study did encounter five cases that could not be explained by non-compliance or any other potentially disrupting events (Atkins et al., 2014). Considerable scepticism and disagreement persisted in the USA throughout the early 2000s regarding the topic of ML-resistance (MLR) in *D. immitis*.

First reports of ML-resistance

The first studies claiming the identification of ML-resistant *D. immitis* were published in 2011. In Bourguinat, Keller, Blagburn, et al. (2011), an in-vitro sensitivity assay was deployed on microfilariae collected from client-owned dogs from Arkansas and Louisiana. Exposure to an IVM concentration that is lethal to 95% of susceptible microfilariae resulted in the death of only 8% and 39% of microfilariae from one of the dogs (Bourguinat, Keller, Blagburn, et al., 2011). Doubling the IVM concentration still resulted in sub-optimal (24% and 50%) killing of microfilariae (Bourguinat, Keller, Blagburn, et al., 2011). Alongside the identification of this in-vitro MLR phenotype, a significant correlation was observed between this phenotype and a 'GG' genotype at positions 11 and 618 in a fragment of a *D. immitis* P-glycoprotein (*Pgp*) gene (i.e., a 'GG-GG genotype'). In another study by Bourguinat, Keller, Bhan, et al. (2011), in-vivo MLR was demonstrated in an infected dog from New Orleans following Hurricane Katrina. Despite the persistent administration of MO and IVM at increased dosages, this dog remained microfilaremic throughout the study. Genetic analysis of the *D. immitis* from

this dog revealed a high (45%) frequency of the GG-GG genotype, and it was concluded that the *D. immitis* in this dog were truly ML-resistant. This conclusion was disputed in a comment paper by Clark (2011), which argued that there was insufficient evidence to definitively confirm the presence of MLR in this dog.

Although Bourguinat, Keller, Blagburn, et al. (2011) and Bourguinat, Keller, Bhan, et al. (2011) discovered microfilariae with decreased susceptibility to MLs, it can be argued that the resistance status of microfilariae may not necessarily mirror that of the adult stages (Wolstenholme et al., 2015). Clinical experimental infection studies demonstrating <100% efficacy of MLs were urgently needed to truly confirm the existence of ML-resistant *D. immitis*. In 2011, three clinical studies were conducted using a *D. immitis* isolate from Georgia termed ‘Miss Piggy 3’ (MP3). Single doses of various approved ML preventives (IVM, MO, SEL) were <100% effective against this MP3 isolate (Blagburn et al., 2011; Snyder, Wiseman, Cruthers, et al., 2011). A subsequent study revealed that while a single dose of MO was <100% effective against MP3, this isolate should not be classified as truly MLR, as two or three repeated monthly treatments provided complete protection (Snyder, Wiseman, Bowman, et al., 2011; Wolstenholme et al., 2015). Given the conflicting and generally insufficient evidence obtained thus far, it remained unclear whether MLR in *D. immitis* had indeed emerged in the USA.

Confirmation of MLR

The existence of MLR in *D. immitis* was finally confirmed in the USA in 2014 following a clinical experimental infection study which demonstrated the heritability of MLR (Pulaski et al., 2014). In the study, two field isolates (LSU-10 and LSU-13) from LOE cases in Louisiana were used to experimentally infect laboratory dogs (Pulaski et al., 2014). Monthly administration of IVM (12 µg/kg) at double the label dose rate (6 µg/kg) for six months did not prevent the development of patent *D. immitis* infections for either isolate (Pulaski et al., 2014). Infective L3s originating from the dog infected with the LSU-10 isolate were used to experimentally infect a second dog (Pulaski et al., 2014). This second dog was treated with IVM (24 µg/kg) for 3 months at four times the label dose rate, yet still developed a patent *D. immitis* infection (Pulaski et al., 2014). These findings unequivocally proved, for the first time, the heritability and existence of ML-resistant *D. immitis*.

After the discovery of MLR, research efforts focused on determining how prevalent and widespread the issue was in the USA. Additional clinical experimental infection studies were performed using different isolates and ML treatments. A summary of *D. immitis* isolates demonstrating <100% efficacy after ML treatment is presented in Appendix 1. To date, at least ten *D. immitis* isolates have been clinically classified as ML-resistant. These resistant isolates are centred around the Mississippi Delta region in states such as Louisiana, Mississippi, and Arkansas, although resistant isolates have

been identified further north in Illinois (JYD-34 and ZoeMO) (Blagburn et al., 2016; Bourguinat et al., 2015; Maclean et al., 2017; McTier et al., 2017; Pulaski et al., 2014).

Impact of ML type, dosage, and treatment regimen

MLR in *D. immitis* has been documented for every ML drug, but efficacies can vary depending on the active ingredient (Appendix 1). Overall, moxidectin appears to be more effective against ML-resistant isolates compared to the other MLs (Blagburn et al., 2016; Bowman et al., 2017; McTier et al., 2021; McTier et al., 2019). In addition to the active ingredient used, the dose and treatment regimen can impact the effectiveness of heartworm preventives against resistant isolates. For example, a single dose of oral moxidectin (ProHeart® tablets; 3 µg/kg) resulted in sub-optimal efficacies of 19% (JYD-34 isolate), 44% (ZoeLA), and 82% (ZoeMO) (McTier et al., 2019). When treatments were repeated for three consecutive months and dosages were increased to either 40 µg/kg (JYD-34 and ZoeMO) or 60 µg/kg (ZoeLA), prevention was 100% effective (McTier et al., 2019). While the emergence of MLR in *D. immitis* threatens the usefulness of current heartworm preventives, there is some reassurance in the knowledge that resistant isolates can still be controlled using higher dosages and repeated treatments. However, there is a risk that excessive use of these drugs, such as increasing dosage or frequency of administration, could create high selection pressure for resistance, as seen in livestock parasites (Coles et al., 2005).

1.7 Tools to detect drug resistance in *Dirofilaria immitis*

Experimental infections

The gold standard approach for unequivocally confirming MLR is a clinical experimental infection study. This is a lengthy process requiring a LOE case, laboratory-raised mosquitoes, and naïve dogs. The typical methodology for these experiments is described in detail in Fig. 3. Despite being the most reliable technique for identifying ML-resistant isolates, experimental infection studies are time-consuming, invasive, and have an estimated cost of at least US \$15,000 (Wolstenholme et al., 2015). Tools more suitable for large-scale screening in the field are required to monitor the prevalence and spread of MLR.

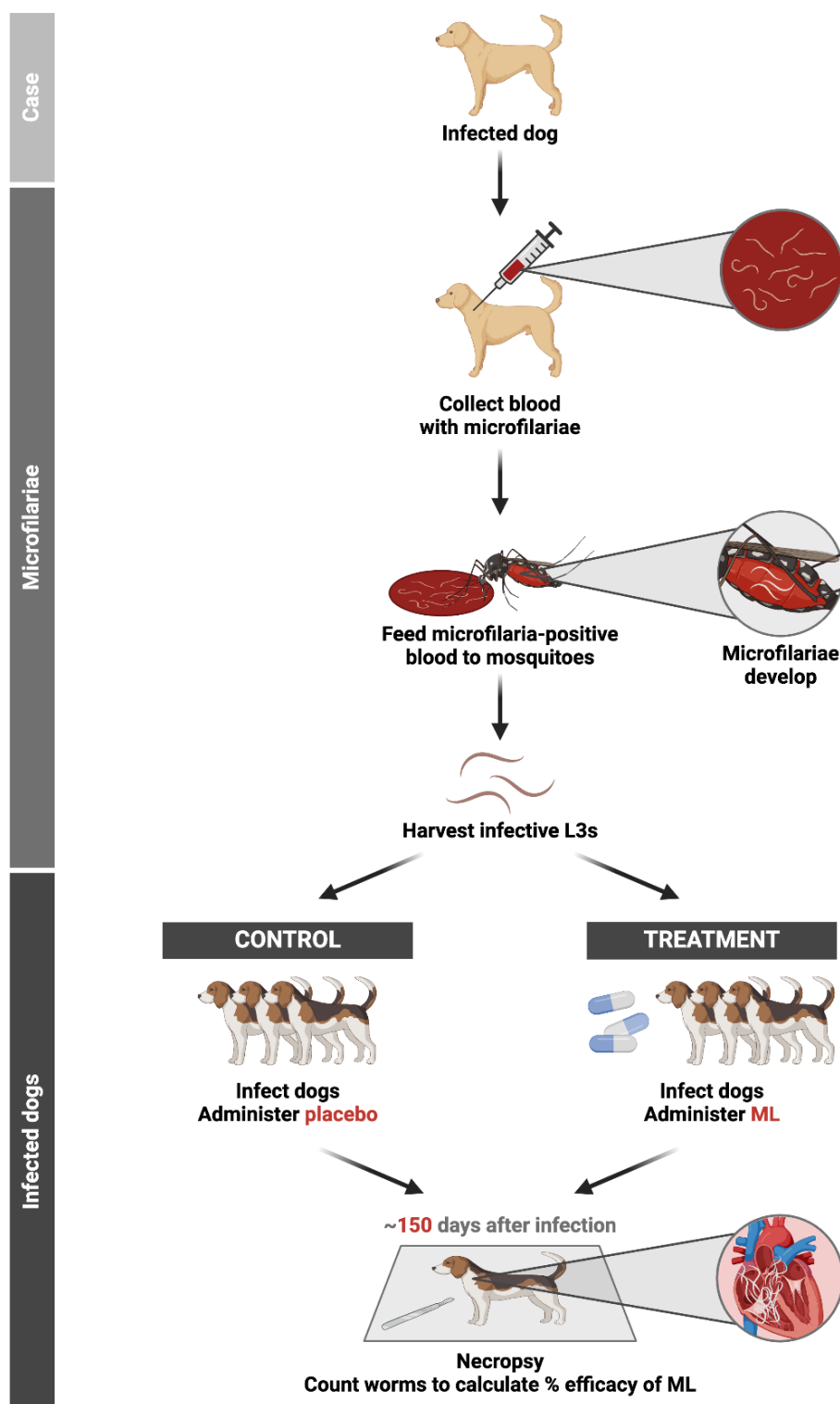


Fig. 3 General methodology of a clinical experimental infection study to determine macrocyclic lactone (ML) efficacy against *Dirofilaria immitis*. Microfilariae-positive blood is obtained from lack of efficacy (LOE) cases, the blood is fed to mosquitoes where the microfilariae will develop into infective L3s, and then the L3s are harvested for inoculation into laboratory dogs (Blagburn et al., 2016). An ML of interest is administered at the labelled dosage one month after infection (Blagburn et al., 2016). The dogs are euthanised and necropsied approximately 150 days later to recover the adult worms (Blagburn et al., 2016). The efficacy (%) of the ML is calculated by comparing the number of adult worms found in treated versus control dogs (Blagburn et al., 2016). Created with BioRender.com.

In-vitro motility tests

The migration of *D. immitis* larvae in the host, coupled with previous findings of MLs causing paralysis in nematodes, has prompted researchers to explore motility as a potential indicator for MLR (Evans et al., 2017; Wolstenholme & Rogers, 2005). Studies have performed in-vitro motility tests, which involve exposure of microfilariae or larvae to MLs. Motility has been assessed using video imaging or by counting the number of larvae passing through a barrier (Evans et al., 2017; Maclean et al., 2017). In both cases, no significant differences in motility were observed between susceptible and resistant/LOE *D. immitis* (Evans et al., 2017; Maclean et al., 2017). The drug concentrations needed to paralyse *D. immitis* in-vitro were much higher than that required in-vivo (Evans et al., 2017; Maclean et al., 2017). Motility tests are therefore considered unsuitable for the identification of MLR.

Microfilarial suppression test

The in-vivo 7-day microfilarial suppression test (MFST) suggested by Geary et al. (2011) involves enumerating microfilariae levels in the blood before and seven days after administration with a microfilaricidal dose of ML. Enumeration of microfilariae is typically performed via a modified Knott's test. While the sensitivity of microfilariae to MLs may not directly reflect that of L3s or L4s, their rapid clearance after ML treatment makes them a potentially useful surrogate marker for MLR (Eisenstein, 2017; Geary et al., 2011). It was originally suggested that a microfilariae reduction of <75% after the seven days was indicative of MLR (Geary et al., 2011). The MFST is faster, cheaper, and less invasive than an experimental infection study. However, when performing the MFST in the field using client-owned dogs, it can be challenging to ensure that the client returns for a second blood sample exactly seven days later. A field study by Ballesteros et al. (2018) extended the time interval between sampling to 2-4 weeks, and successfully categorised the ML response phenotype into animals displaying microfilariae reductions of <10%, ≤90%, and >90%.

Genetic markers

The mechanism of MLR in *D. immitis* remains unknown, but genetic markers associated with MLR have been identified (Wolstenholme et al., 2015). The first genetic marker was identified by Bourguinat, Keller, Blagburn, et al. (2011), where a GG-GG genotype on the gene encoding a P-glycoprotein (*Pgp*) was highly associated with an IVM resistance phenotype. More recently, a collection of 186 single nucleotide polymorphisms (SNPs) associated with MLR have been detected (Bourguinat et al., 2015). These SNPs were identified via whole genome comparisons of susceptible and LOE populations (Bourguinat et al., 2015). By calculating the allele frequencies of each SNP in

phenotypically-characterised parasites, the number of SNPs was narrowed down to 42 (Bourguinat et al., 2015).

For large-scale field surveillance studies, an even smaller subset of markers was needed. Hence, a series of 2, 3, 5, and 10 SNP predictive models were tested using various SNP combinations (Bourguinat et al., 2017). Although all models performed well, the 3 and 5 SNP models were further assessed and appeared to be most suitable for field application (Bourguinat et al., 2017). A similar approach was described in Ballesteros et al. (2018) where 2, 3, 5 and 10 SNP predictive models were clinically validated in dogs from Southeastern USA. The 2 SNP model using the top 2 genetic markers provided the best outcome with 100% sensitivity and specificity (Ballesteros et al., 2018). Since MLR has only been confirmed in the USA, it is important to note that these genotypic assays have been developed and validated in the USA using local *D. immitis* populations. The utility of these genetic markers for detecting MLR outside the USA has not yet been evaluated.

1.8 *Dirofilaria immitis* in Australia

Historical prevalence

The prevalence of canine heartworm infection was high in Australia in the 1970s and 1980s. During this time, *D. immitis* prevalences of 28%, 11%, and 1% were documented in Victoria, New South Wales (NSW), and South Australia, respectively (Blake & Overend, 1982; Copland et al., 1992; Martin & Collins, 1985). Higher prevalence rates were found further north in the tropics and subtropics, with maximum infection rates of 90% (Welch et al., 1979). Once ML preventives were introduced into the veterinary market in the 1980s, the prevalence of *D. immitis* infection in Australia drastically declined (Orr et al., 2020).

Current prevalence

Despite an overall decline in the prevalence of *D. immitis* over the past three decades, an apparent re-emergence of heartworm was discovered in Central Queensland (QLD) in 2016 (Nguyen et al., 2016). Serology of blood samples collected from shelter dogs revealed *D. immitis* prevalence rates of 20% in Northern QLD and 5% in Southern QLD (Panetta et al., 2021). In addition, pig hunting dogs were identified as an at-risk population (prevalence: 21%) in Central QLD, and dingoes represent important reservoir hosts in far North QLD (prevalence: 73%) (Orr et al., 2020; Smout et al., 2016). Cases of *D. immitis* infection have recently been reported in NSW but are generally considered to be a result of ‘baggage canine heartworm’ (McKeever et al., 2021). This involves the importation of infected mosquitoes from QLD to NSW, leading to the infection of unprotected local dogs. Alternatively, dogs

from NSW can become infected with *D. immitis* whilst accompanying their owners on holiday to QLD. It is essential to obtain detailed prevention and travel history for all cases of heartworm infection. This allows veterinarians and researchers to monitor the potential emergence and spread of *D. immitis* throughout Australia.

Preliminary investigations of MLR

Environments with intensive ML treatment and limited refugia, such as kennels, can create strong selection pressure for MLR in high heartworm transmission areas. In Australia, these conditions are more likely to occur in Northern and Central QLD, where prevalence rates are highest (Orr et al., 2020; Panetta et al., 2021). However, the presence of refugia in shelter dogs, pig-hunting dogs, dingoes, and other untreated dogs in Australia could potentially delay the emergence of MLR (Orr et al., 2020; Panetta et al., 2021; Smout et al., 2016). The only studies that have explored the MLR status of *D. immitis* in Australia are those by Nguyen et al. (2016) and Lau et al. (2021). In Nguyen et al. (2016), a large proportion of the QLD dogs were positive for *D. immitis* infection despite allegedly receiving ‘rigorous’ heartworm prevention. Sequencing the *Pgp* region from three of these cases produced only the wild-type AA-GG genotype, rather than the GG-GG genotype previously associated with resistance in the USA (Nguyen et al., 2016). The study by Lau et al. (2021) conducted further genetic analyses on five adult heartworm specimens obtained from a dog in Sydney, NSW. Whole genome sequencing and implementation of the 5, 3 and 2 SNP predictive models developed by Bourguinat et al. (2017) identified all specimens as ML-susceptible (Lau et al., 2021). To date, no evidence of ML-resistant *D. immitis* has been found in Australia.

1.9 Knowledge gaps and thesis aim

For the past three decades, MLs have played a pivotal role in protecting domestic dogs and cats from canine heartworm disease. In 2014, after years of speculation, the presence of ML-resistant *D. immitis* was confirmed in the USA through experimental infection studies (Pulaski et al., 2014). The MLR status of *D. immitis* in Australia and other regions of the world remains unknown, primarily due to a lack of surveillance efforts and the limited availability of suitable diagnostic tools.

The experimental infection studies that were conducted to confirm MLR in the USA may not be feasible in other countries due to the considerable costs, labour, and time involved (Eisenstein, 2017). Recently, the USA has developed faster and more cost-effective surrogate markers for identifying the MLR phenotype and genotype (Ballesteros et al., 2018; Geary et al., 2011). These markers have only been analysed in a handful of adult *D. immitis* in Australia, for which no phenotypic information was available (Lau et al., 2021). Although these surrogate markers offer an alternative means of evaluating

MLR, they were developed based on USA isolates and may not be universally applicable. To determine the suitability of these markers and evaluate the genetic likelihood of MLR emerging within Australia and other parts of the world, population genomics studies are required to better understand genetic variation and patterns associated with resistance.

Aim

The overarching aim of this thesis is to discover the origin of Australian heartworms and the potential reality of macrocyclic lactone resistance in these parasites using phenotypic and genotypic approaches.

Objective 1. To confirm the perceived presence of phenotypic drug resistance in Australian heartworms using traditional microscopy-based diagnostic tools.

The first study aims to define the phenotypic response of Australian heartworms to MLs in a cohort of dogs from QLD and NSW (Chapter 2.1, published in *International Journal for Parasitology: Drugs and Drug Resistance* 2022). To do so, reductions in microfilaremia levels are assessed at various time points after ML treatment using the traditional modified Knott's test. In the second study, the MFST is applied to Australian heartworms for the first time to assess phenotypic drug resistance in two case studies (Chapter 2.2, published in *The Veterinary Journal* 2024). This involves evaluating microfilarial reductions precisely seven days after treatment with a microfilaricidal dose of ML. Limitations of the traditional modified Knott's test are recognised in both studies, leading to the validation of a rapid real-time polymerase chain reaction (qPCR) assay as an equivalent method for measuring microfilaremia and monitoring the phenotype (Chapter 2.1).

Objective 2. To reveal whether Australian heartworms possess genetic signatures associated with drug resistance in the USA.

The first and second studies aim to investigate the drug resistance genotypes of Australian heartworms (Chapter 2.1, published in *International Journal for Parasitology: Drugs and Drug Resistance* 2022; Chapter 2.2, published in *The Veterinary Journal* 2024). To do so, Australian heartworm populations are examined for SNPs previously linked to drug resistance in USA heartworms using an Illumina amplicon metabarcoding approach. To streamline the genetic SNP testing, a rapid and convenient in-vitro rhAmp™ SNP qPCR assay targeting the top two markers is developed and validated (Chapter 2.2). This SNP qPCR assay is compared to the original Illumina amplicon metabarcoding approach to evaluate its ability to accurately genotype susceptible and resistant populations.

Objective 3. To uncover the ancestral origin and genetic background of Australian heartworms using a whole genome sequencing approach.

A major outcome of this thesis is the curation of the world's largest collection of heartworm genomes, spanning the continents of Australia, North America, Europe, and Asia (Chapter 3.1). First, whole genome sequencing is conducted on genomic DNA derived from adult heartworm specimens. The sequencing data is processed using a standard genomics workflow, which entails mapping to a heartworm reference genome and identifying variants across the global cohort. Population genomics analyses are then performed to investigate the population structure, ancestral background, and demographic history of Australian heartworms in relation to other continents, simultaneously revealing their geographical and host origins. To assess whether whole genomes can be obtained from more accessible immature life stages, whole-genome amplification and sequencing are performed on individual microfilariae collected from the bloodstream (Chapter 3.2, published in *Experimental Parasitology* 2024).

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———— **Chapter 2** ————

Screening for potential drug resistance in
Australian heartworm populations

———— Chapter 2.1 ————

Exploration of the sensitivity to macrocyclic lactones in the canine heartworm (*Dirofilaria immitis*) in Australia using phenotypic and genotypic approaches

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Rosemonde Isabella Power in the preparation and submission of the following manuscript:

Power, R. I., & Šlapeta, J. (2022). Exploration of the sensitivity to macrocyclic lactones in the canine heartworm (*Dirofilaria immitis*) in Australia using phenotypic and genotypic approaches. *International Journal for Parasitology: Drugs and Drug Resistance*, 20, 145-158.
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Contribution to the manuscript of all authors is as follows:

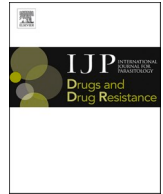
Task	Contributing co-authors
Conceptualisation	RIP, JŠ
Data collection and curation	RIP, JŠ
Formal analysis	RIP
Funding acquisition	JŠ
Investigation	RIP
Methodology	RIP, JŠ
Supervision, discussion and consultation	JŠ
Preparation of figures used in the manuscript	RIP, JŠ
Writing of the original draft	RIP
Critical review and editing of the manuscript	RIP, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct.

Kind regards,

Professor Jan Šlapeta

26th September 2024



Exploration of the sensitivity to macrocyclic lactones in the canine heartworm (*Dirofilaria immitis*) in Australia using phenotypic and genotypic approaches

Rosemonde Isabella Power^a, Jan Šlapeta^{a,b,*}

^a Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales, 2006, Australia

^b The University of Sydney Institute for Infectious Diseases, New South Wales, 2006, Australia

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ABSTRACT

Canine heartworm disease is a potentially deadly cardiopulmonary disease caused by the mosquito-borne filarial nematode *Dirofilaria immitis*. In Australia, the administration of macrocyclic lactone (ML) drugs has successfully reduced the prevalence of *D. immitis* infection. However, the recent re-emergence of *D. immitis* in dogs in Queensland, Australia and the identification of ML-resistant isolates in the USA poses an important question of whether ML-resistance has emerged in this parasite in Australia. The aim of this study was to utilise phenotypic and genotypic approaches to examine the sensitivity to ML drugs in *D. immitis* in Australia. To do this, we surveyed 45 dogs from Queensland and New South Wales across 3 years (2019–2022) for the presence of *D. immitis* infection using an antigen test, quantitative Modified Knott's test, and qPCR targeting both *D. immitis* and the *D. immitis* symbiont *Wolbachia*. A phenotype observed by utilising sequential quantification of microfilariae for 23/45 dogs was coupled with genetic testing of filtered microfilariae for SNPs previously associated with ML-resistance in isolates from the USA. Sixteen (16/45) dogs tested positive for *D. immitis* infection despite reportedly receiving 'rigorous' heartworm prevention for 12 months prior to the study, according to the owners' assessment. The phenotype and genotypic assays in this study did not unequivocally demonstrate the presence of ML-resistant *D. immitis* in Australia. Although the failure of 16 dogs to reduce microfilaremia by >90% after ML treatment was considered a suspect phenotype of ML-resistance, no genotypic evidence was discovered using the genetic SNP analysis. The traditional quantitative Modified Knott's test can be substituted by qPCR targeting *D. immitis* or associated *Wolbachia* endosymbiont DNA for a more rapid measurement of microfilariae levels. More definitive phenotypic evidence of resistance is critically needed before the usefulness of SNPs for the detection of ML-resistance in Australia can be properly assessed.

1. Introduction

Canine heartworm disease is a potentially deadly cardiopulmonary disease caused by the mosquito-borne filarial nematode *Dirofilaria immitis*, which is distributed across many parts of the world (Simón et al., 2012; Genchi and Kramer, 2020; Lau et al., 2021; Noack et al., 2021). Although the prevalence of *D. immitis* in Australia is low, the parasite is endemic to the country and has recently re-emerged in dogs in Queensland (Nguyen et al., 2016; Orr et al., 2020; Panetta et al., 2021). For the past 30 years in Australia and the USA, the main strategy for canine heartworm prevention has been the administration of macrocyclic lactone (ML) anthelmintics (Orr et al., 2020; Diakou and Prichard,

2021; Lau et al., 2021; Noack et al., 2021; Prichard, 2021). These ML preventatives include monthly oral or topical formulations of ivermectin, milbemycin oxime, moxidectin and selamectin, or extended-release injections of moxidectin microspheres (Nguyen et al., 2016; McTier et al., 2019a). When used according to product instructions, these preventatives are approved to be 100% effective against less than 30-day old *D. immitis* (Hampshire, 2005). Over the past 20 years, lack of efficacy (LOE) reports for all heartworm product categories have occurred in the Lower Mississippi River Valley region of the USA (Hampshire, 2005). Most of these LOE reports were associated with compliance issues and extenuating circumstances, which prevented dogs from receiving sufficient product every 30 days (Atkins et al.,

* Corresponding author. Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales, 2006, Australia.

E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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2014). A small number of LOE cases were not linked to noncompliance, indicating the potential development of ML-resistance in canine heartworms (Atkins et al., 2014). Laboratory studies have now identified several ML-resistant *D. immitis* isolates circulating in the Lower Mississippi Valley region of the USA (Blagburn et al., 2011, 2016; Pulaski et al., 2014; Bourguinat et al., 2015; Maclean et al., 2017; McTier et al., 2017b; Diakou and Prichard, 2021). These studies involved experimentally infecting ML-naïve dogs with infective L3 obtained from field LOE cases (Pulaski et al., 2014). In addition, <100% efficacy has been experimentally demonstrated for all currently marketed MLs (Bourguinat et al., 2015; Blagburn et al., 2016; McTier et al., 2019b).

MLs are essentially the only class of drugs currently available for canine heartworm prevention in Australia, in addition to the daily diethylcarbamazine citrate (Noack et al., 2021). Hence, the emergence of ML-resistance in *D. immitis* in the USA presents a significant concern for the veterinary industry by limiting the usefulness of ML products and jeopardising canine health. Prolonging the efficacy of MLs for canine heartworm prevention is warranted. With the absence of rapid biological tests that would demonstrate LOE of the target population (<30-day old *D. immitis*), discovery of heritable DNA molecular markers is needed for monitoring ML-resistance in adult *D. immitis* populations or their microfilariae within the dog bloodstream. Previous studies from the USA have compared the genotypes of different groups of *D. immitis* and identified single nucleotide polymorphisms (SNPs) potentially linked to ML-resistance (Bourguinat et al., 2011, 2015). A whole genome approach was used in Bourguinat et al. (2015) to identify 186 SNP loci associated with the inability of dogs to remove circulating microfilariae after ML administration. From these 186 SNPs, a subset of 42 SNPs was initially validated as being highly associated with the ML-resistance phenotype in *D. immitis* (Bourguinat et al., 2015). The 42 SNPs were further evaluated in 10 additional ML-susceptible and ML-resistant isolates to generate 2, 3, 5 and 10-SNP predictive models for ML-resistance (Bourguinat et al., 2017). The 10 SNP approach that most effectively separated ML-susceptible and ML-resistant isolates was validated in an extensive study from south-east USA with field-collected *D. immitis* microfilariae, demonstrating high levels of sensitivity and specificity (Ballesteros et al., 2018). The SNP-based genotype predicted the phenotype which was defined by the inability of MLs to clear microfilariae using a suppression test and thus implied the presence of ML-resistant *D. immitis* infection (Ballesteros et al., 2018).

In contrast to the USA, it is currently unknown whether ML-resistance has emerged in *D. immitis* in Australia. A recent study revealed a large proportion of Australian dogs that were microfilaria-positive despite 'rigorous' administration of MLs in far-north Queensland (Nguyen et al., 2016). However, evidence of ML purchase history or ML administration was not provided by clients (Nguyen et al., 2016). A study that adopted the predictive SNP models developed in the USA to determine ML-resistance status did not find evidence of SNPs associated with ML-resistance in adult *D. immitis* from Sydney, Australia (Lau et al., 2021). As *D. immitis* prevention remains critical and prevalent across tropical Australia, further inquiry into ML-resistance in Australian *D. immitis* populations is necessary (Orr et al., 2020; Panetta et al., 2021). New screening tools and surveillance are required that apply or adapt the microfilarial suppression test originally proposed by Geary et al. (2011) and subsequently utilised by Ballesteros et al. (2018).

The aim of this study was to utilise phenotypic and genotypic approaches to determine the presence of ML-resistant *D. immitis* in Australia. To do so, we surveyed dogs across 3 years (2019–2022) for the presence of *D. immitis* using an antigen test, quantitative Modified Knott's test, and qPCR targeting both *D. immitis* as well as the *D. immitis* symbiont *Wolbachia*. Material was collected prior to ML application and post-ML application at varying intervals. A Modified Knott's test was coupled with filtration of microfilariae for genetic testing of SNPs previously associated with ML-resistance in isolates from the USA.

2. Material & methods

2.1. Blood collection from dogs in Queensland and New South Wales

Blood samples were collected opportunistically from dogs in Queensland and New South Wales, Australia between September 2019 and January 2022. The dogs selected for this study were privately-owned animals from participating veterinary practices where the practitioner had either confirmed or suspected the presence of *D. immitis* infections. Blood samples (≤4 mL) were collected by registered veterinarians and shipped in EDTA tubes to the Sydney School of Veterinary Science, University of Sydney, Australia for diagnostic *D. immitis* testing. The following details were obtained for each dog: sex, age, breed, desexing status, locality, heartworm prevention history for the previous 12 months based on the owner's assessment, and whether the animal had lived or visited other states or territories. Dog owners were required to complete a consent form prior to blood collection.

2.2. Sequential Modified Knott's test

To determine the effect of ML administration on *D. immitis* microfilariae counts from infected dogs, we utilised a Modified Knott's test. This was used to enumerate the number of *D. immitis* microfilariae in dog blood before ML treatment and then at a second point following the ML treatment. The time interval between drug treatment and the follow-up count varied across the study as necessitated by the need to follow timelines within the veterinary practices that were providing blood samples for the study, but was generally in the period 30–40 days after treatment. In some cases, an additional post-treatment microfilariae count was made at a later timepoint. The type of ML product administered was at the veterinarian's discretion, according to their best practice in heartworm disease management. We calculated the percentage (%) reduction of microfilariae between the pre-treatment and post-treatment samples. In cases in which dogs showed <90% reductions in microfilariae, additional blood samples were collected where possible to observe whether microfilariae levels eventually declined.

To perform the Modified Knott's test, 1 mL of whole blood was mixed with 10 mL of 2% formalin in a 15-mL tube (Knott, 1939; Mylonakis et al., 2004). The mixture was centrifuged at 2,300 relative centrifugal force (RCF) for 5 min using a benchtop centrifuge (POCD Scientific, Australia). The pellet was mixed with 1% methylene blue stain and enumerated microscopically. The slide was examined under a microscope (BX40, Olympus, Australia) using 10 × objective and the number of microfilariae observed in the entire slide was counted.

2.3. Detection of *D. immitis* antigen using *DiroCHEK*® ELISA

Prior to performing *DiroCHEK*® ELISA (Zoetis, Australia) testing, the stored plasma samples were thawed at room temperature and vortexed for 3 s. Both heated and unheated (neat) aliquots of each plasma sample were tested. To heat the plasma samples, a 100 µL aliquot of plasma was diluted with 100 µL of phosphate buffered saline (PBS; pH = 7.4) in a plain 1.5-mL tube. For samples with insufficient plasma volume, only 50 µL of plasma was diluted with 50 µL of PBS. The diluted plasma samples were heated at 103 °C for 10 min using a dry heat block (Major Science). Heated samples were immediately centrifuged at 16,100 RCF for 5 min using a benchtop centrifuge (Eppendorf, Australia) set at 4 °C. The supernatant was transferred into a separate 1.5-mL tube for use in *DiroCHEK*® ELISA testing. To load the *DiroCHEK*® ELISA plate, 50 µL of heated and unheated plasma for each sample were transferred into separate wells. Positive and negative controls provided by the manufacturer were included, and samples with leftover heated plasma were included as repeats. The *DiroCHEK*® ELISA test was then performed according to manufacturer's instructions. The optical density (OD) of each sample in the *DiroCHEK*® ELISA plate was measured at 620 nm in a Halo LED 96 microplate reader (Dynamica). To distinguish between

negative and positive samples, the following equation was applied as a threshold:

$$\text{Threshold} = 3 \times \text{SD} (\text{Mean}[\text{OD of negative controls}])$$

2.4. Microfilariae extraction

To isolate microfilariae from dog blood, a filtration procedure was adopted and modified (Bourguinat et al., 2015). Prior to filtration, 1 mL of whole blood was diluted with 35 mL of Red Blood Cell lysis buffer (8 g/L sodium carbonate, 5 mL/L Triton X-100) in a 50-mL tube. For one sample (HW52), only 100 µL of blood was diluted. The diluted blood was then pushed through a syringe containing a polycarbonate membrane filter (pore size 20 µm, filter diameter 25 mm; Sterlitech® Corporation, WA). This allowed the microfilariae to be collected on the filter membrane whilst other blood components passed through the syringe. The syringe was then flushed with 50 mL of phosphate buffered saline (PBS). The filter membrane was removed from the syringe, placed into a 1.5-mL tube, and stored at –20 °C until further use.

2.5. DNA isolation

DNA was isolated from 100 µL of whole blood using the blood protocol from the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia). In addition, DNA was isolated from microfilariae on filter membranes using the same kit. DNA isolation from filter membranes followed the animal tissue sample protocol and used Proteinase K. Each DNA isolation batch contained an extraction blank. The eluted DNA was stored at –20 °C until further use in the qPCR and next generation sequencing (NGS) assays.

2.6. Quantitative PCR targeting dog, *D. immitis* and *Wolbachia* sp. of *D. immitis*

A qPCR targeting the partial canine glyceraldehyde3-phosphate dehydrogenase (*GAPDH*) region was performed to verify the presence of dog DNA using primers with a probe, as previously described (Orr et al., 2020; Panetta et al., 2021) (Supplementary Table S1). A diagnostic TaqMan probe qPCR targeting the *cox1* gene was performed to detect *D. immitis* DNA using primers with a probe, as previously described (Panetta et al., 2021) (Supplementary Table S1). A qPCR targeting the *ftsZ* gene of *D. immitis*-associated *Wolbachia* was performed using primers and probe described in Laidoudi et al. (2020) (Supplementary Table S1).

The dog, *D. immitis* and *Wolbachia* qPCR assays were carried out in 10 µL final volumes of Luna® Universal Probe qPCR Master Mix (New England Biolabs, Australia) containing 1 µL of template DNA isolated from whole dog blood and primers and probe at a final concentration of 400 nM and 100 nM, respectively. To confirm the presence of *D. immitis*, qPCRs targeting dog and *D. immitis* DNA were repeated using DNA from extracted microfilariae. For these reactions, the volumes of primer, probe, PCR-grade water, and template DNA were doubled to reach final volumes of 20 µL. All qPCRs were carried out on a CFX96 Real-Time PCR system with the corresponding Bio-Rad CFX Maestro Software (BioRad, Australia). Cycling conditions for all three assays consisted of an initial denaturation at 95 °C for 1 min followed by 40 two-step cycles at 95 °C for 15 s and 60 °C for 30 s. Each qPCR run included DNA extraction blanks and a no template control (NTC) of PCR-grade water. The arbitrary qPCR baseline threshold was set to 100 RFU and C_t -values were reported for all samples. A qPCR targeting the *GAPDH* region of the dog genome confirmed the presence of dog DNA in all samples. All No Template Control (NTC) reactions remained negative.

2.7. Statistical analysis of phenotypic data

A paired *t*-test was performed to compare the OD between neat and

heated plasma samples in the DiroCHEK® ELISA. Additional paired *t*-tests were performed to observe differences between pre- and post-ML treatment samples in the qPCRs. To evaluate linear relationships between phenotypic datasets, Pearson's correlation coefficients were generated. Prior to performing correlation analyses with the qPCR data, 'N/A' results were replaced with a C_t -value of '40'. All statistical analyses were performed using GraphPad Prism (version 9.3.1).

2.8. Selection of SNP markers

Studies from the USA identified 10 SNPs that best differentiated ML-susceptible *D. immitis* from ML-resistant isolates (Bourguinat et al., 2017). Seven of these SNPs were selected for analysis in this study as they covered both 5-SNP models generated by Ballesteros et al. (2018) and Bourguinat et al. (2017). The 2 SNP markers (L15709_A, L30575) reported as giving the best prediction of ML-resistance in Ballesteros et al. (2018) were included in these 7 SNPs. Additional information about the 7 SNPs selected for this study including their position in the genome and various names used in other studies are available in Supplementary Table S2.

2.9. Illumina PCR amplicon next generation sequencing targeting 7 SNPs associated with ML-resistance in USA

Illumina overhang adapters were added to all 7 primer sets which targeted regions encompassing the SNPs of interest (L42411, L21554, L45689, L9400, L20587, L15709_A, and L30575) according to Bourguinat et al. (2015). Their reference sequences were mapped to *D. immitis* genome nDi.2.2 scaffolds (Godel et al., 2012). The first stage PCRs for Illumina Next Generation Sequencing (NGS) were performed in-house according to the '16 S Metagenomic Sequencing Library Preparation' instructions for the Illumina MiSeq system (Illumina, Australia). Conventional PCR assays were carried out with Q5 Hot Start High-Fidelity 2 × Master Mix (New England Biolabs, Australia). For some samples, dilutions of 1:10 or 1:100 were used due to low C_t -values obtained from the *D. immitis*-specific qPCR. Conventional PCRs were performed on a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Australia). The cycling conditions included an initial denaturation step at 98 °C for 30 s followed by 27–35 cycles of 98 °C for 5 s, 55 °C for 10 s and 72 °C for 20 s with a final extension at 72 °C for 2 min. The number of cycles varied so that each sample reached the exponential phase of amplification, as previously described (Power et al., 2021). To increase efficiency of the first stage PCRs, conventional PCR assays were replaced with qPCR assays with SensiFAST™ SYBR® No-ROX Kit (Bioline, Australia). The cycling conditions included an initial denaturation at 95 °C for 3 min followed by 28–40 cycles of 95 °C for 5 s, 55 °C for 10 s and 72 °C for 20 s with a final melt curve. The inclusion of a melt curve allowed us to confirm the presence of the desired PCR product. DNA of an adult *D. immitis* collected from a dog in Sydney (P6/20D), Australia was used as a positive control (Lau et al., 2021). A no template control (NTC) of PCR-grade water was included for each SNP assay. Presence of expected PCR products was confirmed by visualisation on agarose gel for conventional PCR and qPCR products with ambiguous melt profiles. To determine whether comparable results were obtained using the two PCR approaches, three samples (HW19, HW20, HW32) were run as both conventional PCR and qPCR reactions for 5 loci (L42411, 21,554, 45,689, 9400, 20,587), along with additional samples for L42411 (HW33) and L20587 (HW63). To investigate the impact of DNA concentration on the NGS results, four samples in the L45689 assay (HW20, HW41, HW45, HW66) and four samples in the L9400 assay (HW20, HW31, HW66, P6/20D) were repeated using alternative dilutions or cycle numbers. As one sample (HW52) was analysed using filtered microfilariae from 100 µL of blood, repeats for three loci (L20587, L15709_A, L30575) were included using filtered microfilariae from 1 mL of blood. There was no difference that would compromised SNP calls between the approaches irrespective of the dilution, blood volume, cycle number or

PCR approach used.

After the first-stage PCR, products were submitted to the Ramaciotti Centre for Genomics, University of New South Wales, Australia for indexing (384), library preparation and next generation sequencing (NGS) using Illumina MiSeq v2 250 PE sequencing run.

2.10. SNP analysis of NGS samples

Data as FastQ files were analysed in R 4.0.5 within RStudio 2022.07.1 Build 554 (RStudio Team, 2022). For each SNP locus, primers were identified and removed from all sequencing reads using Cutadapt 4.0 (Martin, 2011) followed by DADA2, an open-source R package (<https://github.com/benjjneb/dada2>). DADA2 pipeline (1.18.0; Callahan et al. (2016)) was used to infer the *D. immitis* SNP amplicon sequence variants (ASVs) present in the filtered microfilariae DNA samples. The quality profiles of the cut sequencing reads were visualised and inspected using the “plotQualityProfile” tool. The cut forward and reverse reads (FastQ) were filtered, denoised and trimmed to 180 and 150 nucleotides, respectively, using the “filterAndTrim” tool. Overall, >92%, >75%, >97%, >71%, >96%, >91%, and >40% of input reads were retained and 0.03%, 0.01%, 0.58%, 0.01%, 0.01%, 0.00%, and 0.00% of merged paired reads were removed as chimeras for L42411, L21554, L45689, L9400, L20587, L15709_A, and L30575, respectively. For data cleaning purposes, the proportion (%) of each ASV in each sample was calculated. In an effort to remove misassigned reads caused by sample bleeding or barcode hopping, a threshold of <2.5% was applied (Mitra et al., 2015). This involved removing the reads of ASVs whose proportions represented <2.5% in a sample and replacing them with ‘0’. Alternative thresholds of <1% and <0.5% were tested but not selected for further downstream analysis. A reference table containing frequencies of sequence fragments of all 7 SNP loci was created, with options for each possible SNP polymorphism. This reference table was imported into DADA2 and the “AssignSpecies” tool was used to assign the remaining ASVs to a reference sequence if they were a perfect match. ASVs were imported into CLC Main Workbench (version 22.0) (CLC bio, Qiagen, Australia) and “BLAST at NCBI” was performed. Based on the BLAST results, ASVs that were not identified as *D. immitis* were excluded and ASV proportions for each sample were re-calculated. The remaining

D. immitis ASVs were aligned to their respective SNP reference sequences and visually inspected using CLC Main Workbench.

2.11. Animal ethics

Research performed at the University of Sydney was approved by the Animal Research Ethics Committee, which follows the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8th edition (2013).

2.12. Data accessibility

Raw FastQ sequences were deposited at SRA NCBI BioProject: PRJNA889241. Additional data for this study is available at LabArchives (<https://dx.doi.org/10.25833/ygs4-ha97>).

3. Results

3.1. Blood collection pre- and post-ML treatment

A total of 45 dogs (Dog 1 to Dog 45) and their 78 blood samples (HW01 to HW63, HW65 to HW79) were collected and followed up over 2.5 years (2019–2022) as suspect to be infected with *D. immitis* based on the veterinarians’ judgement (Supplementary Table S3). At the beginning of the study, infection with *D. immitis* was confirmed in 44/45 dogs using at least one diagnostic test. Dogs testing positive for *D. immitis* were domiciled at eleven locations across eastern Australia, with thirty-eight dogs from Queensland and six dogs from New South Wales (Fig. 1). All dog bloods were processed using an exhaustive diagnostic laboratory workflow (Fig. 2). Twenty-three (n = 23) dogs were sampled at the time of diagnosis (pre-ML treatment) and at least once post-diagnosis; 14 dogs once after treatment (post-ML), eight dogs twice after treatment, and one dog (Dog 40) three times after treatment (Figs. 2 and 3). In addition, twenty-two dogs were only sampled pre-ML treatment and following samples were not possible to obtain.



Fig. 1. Location of dogs infected with *Dirofilaria immitis* included in this study from Eastern Australia. These dogs (n = 44) were sampled between 2019 and 2022 for presence of *D. immitis* antigen, *D. immitis* DNA and associated *Wolbachia* DNA. The red circles indicate a location that is associated with the town name and the number indicates the number of *D. immitis*-positive dogs using any test. On the right, two close-ups of Townsville, Queensland and Greater Sydney, New South Wales are shown. Image from Google Earth; data from SIO, NOAA, U.S Navy, NGA, GEBCO (Image Landsat/Copernicus, CNES/Airbus, Maxar Technologies). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

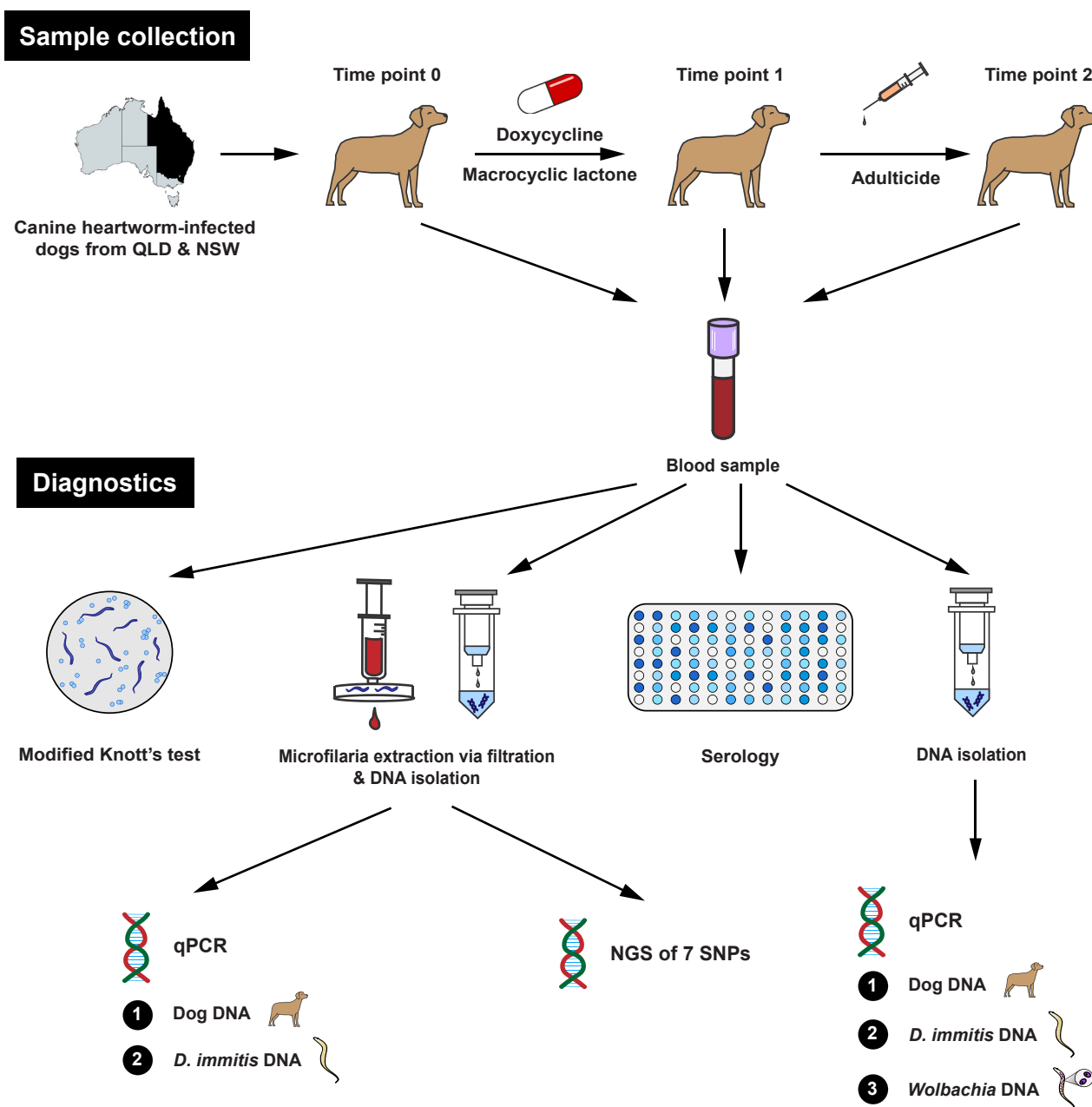


Fig. 2. Diagnostic methods for the diagnosis of *Dirofilaria immitis* infection and response to macrocytic lactone administration in dogs. Blood samples were collected at the time of diagnosis, followed by the administration of doxycycline antibiotic and macrocytic lactone, and then the administration of adulticide injections. Each blood sample was assayed using a Modified Knott's test to quantify microfilariae, microfilariae were extracted from blood via filtration for DNA isolation, plasma samples were assayed for the presence of *D. immitis* antigen and DNA isolated directly from blood was assayed quantitatively for the presence of *D. immitis* DNA and endosymbiotic *Wolbachia* DNA. DNA isolated from filtered microfilariae were tested for the presence of *D. immitis* DNA and endosymbiotic *Wolbachia* DNA in addition to single nucleotide polymorphisms (SNPs) associated with macrocytic lactone resistance from the USA. SNPs were assayed using Illumina amplicon metabarcoding next generation sequencing (NGS).

3.2. Effects of ML administration on *Dirofilaria immitis* microfilariae counts

A total of 37/44 (84.1%) *D. immitis*-positive dogs were microfilaraemic at the beginning of the study, with a mean initial microfilariae count of 7689 microfilariae/mL. Sequential Modified Knott's tests were performed to demonstrate the suspect ML-resistance phenotypes of *D. immitis* (Fig. 2). The percentage (%) reduction of microfilariae counts after ML administration was obtained for 22 dogs that had sequential blood samples. The mean initial microfilariae count for 21 dogs was 6,617 microfilariae/mL (min. 38 to max. 38,250 microfilariae/mL) plus one had 1 microfilaria/mL (Dog 1) (Fig. 3, Table 1). In addition, one dog

(Dog 19) had 0 microfilariae/mL, but tested positive on PCR and serology so a post-ML sample was also obtained and processed. Sixteen dogs (73%, 16/22) had $\leq 90\%$ reduction in microfilariae count, including three dogs (Dog 25, Dog 2, Dog 7) which had an increase in microfilariae (Fig. 3, Table 1).

Using seven dogs sampled pre-ML which were then administered moxidectin (ProHeart SR-12, s.c.) and sampled again at least twice post-ML, we show a 47% (14%–67%, Fig. 3A) reduction of microfilariae by day 27–49. This was reduced to 95% (73%–100%) by 60–136 days. Four of these dogs were subjectively considered to be on 'rigorous' heartworm prevention by their owners (Fig. 3A). All remaining dogs but three were administered moxidectin (ProHeart SR-12, s.c.) at the time of

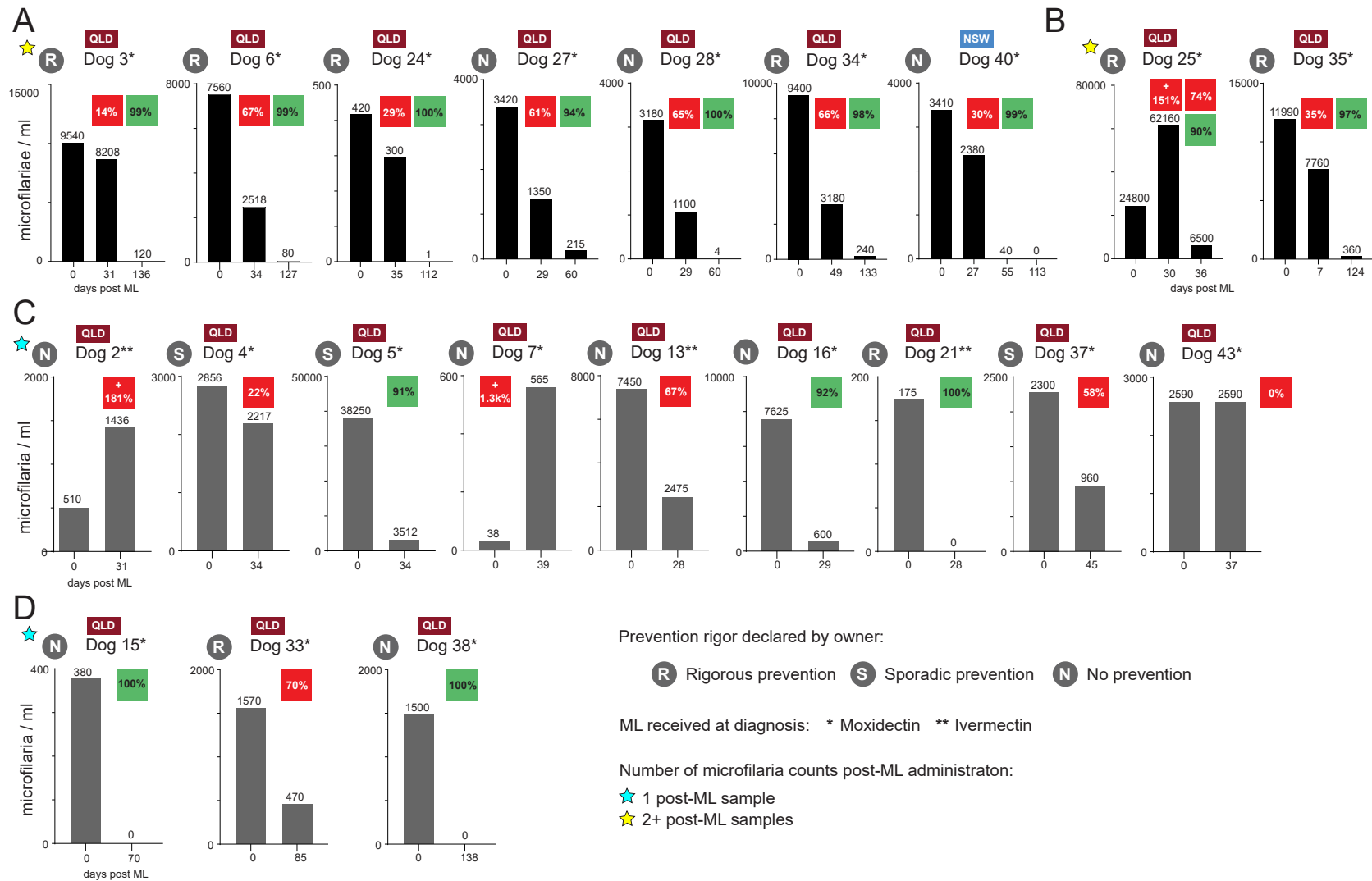


Fig. 3. Sequential Modified Knott's tests performed on 21 dogs positive for *Dirofilaria immitis*. (A) Dogs ($n = 7$) that exhibited $<90\%$ (red square %) microfilarial reduction at the first post-ML sampling, but consecutive second post-ML sampling demonstrated $>90\%$ microfilarial reduction (green square %). (B) Dog 25 at the first post-ML sampling had an increased number of microfilariae, but one week later the microfilariae were reduced by $<90\%$ (red squares); however, if comparing the 1st and 2nd post-ML samples, the reduction was $>90\%$. Dog 35 has had its 1st post-ML sample collected on day 7. (C) Dogs ($n = 9$) that were sampled only once post-ML with variable reduction of microfilariae. (D) Dogs ($n = 3$) that were sampled once, yet this sampling was delayed to 70 or more days post-ML. QLD – Queensland, Australia, NSW – New South Wales, Australia, yellow star – dogs with at least two consecutive post-ML blood samples tested, ML – macrocyclic lactone. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1Microfilariae quantification, antigen and qPCR diagnostics on *Dirofilaria immitis*-infected dogs from Australia.

Dog	Location (Australia)	<i>D. immitis</i> prevention		Microfilariae reduction			DiroCHEK® ELISA		qPCR C _t	
		Compliance (past 12 months)	ML at diagnosis	pre-ML/post-ML		% mf change	pre-ML/post-ML		pre-ML/post-ML	
				ID	mf/ml		Neat	Heat	<i>D. immitis</i>	<i>Wolbachia</i>
>90% microfilariae (mf) reduction										
1	Cairns, QLD	Sporadic	HeartGard Plus	HW1/HW2	1/0	−100	+/−	+/+	37.7/-	−/−
15	Deeragun, QLD	No	ProHeart SR-12	HW25/ HW36	380/0	−100	+/−	+/+	26.0/-	−/−
21	Tugun, QLD	Yes	HeartGard Plus	HW31/ HW34	175/0	−100	−/+	+/+	32.2/ 32.1	−/−
38	Deeragun, QLD	n/a	ProHeart SR-12	HW63/ HW75	1,500/0	−100	+/+	+/+	23.4/-	29.5/-
16	Deeragun, QLD	n/a	ProHeart SR-12	HW26/ HW33	7,625/600	−92	+/+	+/+	23.1/ 25.3	28.1/34.7
5	Deeragun, QLD	Sporadic	ProHeart SR-12	HW7/HW13	38,250/3,512	−91	+/+	+/+	20.0/ 22.4	25.1/32.3
<90% microfilariae (mf) reduction										
33	Deeragun, QLD	Yes	ProHeart SR-12	HW55/ HW59	1,570/470	−70	+/+	+/+	24.6/ 25.2	30.3/-
13	Cairns, QLD	No	HeartGard Plus	HW20/ HW22	7,450/2,475	−67	+/+	+/+	20.5/ 21.8	25.2/32.8
6	Deeragun, QLD	Yes	ProHeart SR-12	HW8/HW15	7,560/2,518	−67	+/+	+/+	21.3/ 22.3	26.7/32.0
34	Deeragun, QLD	Yes	ProHeart SR-12	HW56/ HW58	9,400/3,180	−66	+/+	+/+	21.2/ 22.9	27.3/35.5
28	Westbrook, QLD	No	ProHeart SR-12	HW44/ HW49	3,180/1,100	−65	+/+	+/+	25.1/ 24.9	28.8/33.7
27	Westbrook, QLD	No	ProHeart SR-12	HW43/ HW48	3,420/1,350	−61	+/+	+/+	25.8/ 23.9	29.8/33.8
37	Deeragun, QLD	Sporadic	ProHeart SR-12	HW61/ HW65	2,300/960	−58	+/+	+/+	22.6/ 25.4	29.9/-
35	Deeragun, QLD	Yes	ProHeart SR-12	HW57/ HW62	11,990/7,760	−35	+/+	+/+	19.5/ 22.7	27.3/32.6
40	Nana Glen, NSW	No	ProHeart SR-12	HW68/ HW71	3,410/2,380	−30	+/−	+/+	22.7/ 27.0	28.2/-
24	Deeragun, QLD	Yes	ProHeart SR-12	HW38/ HW40	420/300	−29	+/+	+/+	26.6/ 26.0	31.7/33.5
4	Deeragun, QLD	Sporadic	ProHeart SR-12	HW6/HW12	2,856/2,217	−22	+/+	+/+	22.0/ 22.9	26.6/32.3
3	Deeragun, QLD	Yes	ProHeart SR-12	HW5/HW14	9,540/8,208	−14	+/+	+/+	22.7/ 24.4	26.1/35.1
43	Deeragun, QLD	n/a	ProHeart SR-12	HW74/ HW78	2,590/2,590	0	+/+	+/+	24.7/ 22.7	30.6/33.1
19	Tugun, QLD	Yes	HeartGard Plus	HW29/ HW35	0/0	n/a	+/−	+/+	33.1/-	−/−
Increase in microfilariae (mf)										
7	Townsville, QLD	n/a	ProHeart SR-12	HW9/HW16	38/565	+1386	+/+	+/+	28.7/ 24.0	31.8/34.3
2	Thursday Is, QLD	No	HeartGard Plus	HW3/HW4	510/1,436	+182	+/+	+/+	21.8/ 22.9	27.0/31.9
25	Deeragun, QLD	Yes	ProHeart SR-12	HW39/ HW41	24,800/ 62,160	+151	+/+	+/+	21.3/ 24.1	26.1/28.8

Note: n/a – not available, + (DiroCHEK) – positive, - (DiroCHEK) – negative, - (qPCR) – negative (>40), ML – macrocyclic lactone, mf – microfilariae, HeartGard Plus (ivermectin/pyrantel; Boehringer-Ingelheim), ProHeart SR-12 (moxidectin; Zoetis).

diagnosis (Fig. 3B,C,D). Three dogs were administered ivermectin (HeartGard Plus, p.o.). Dog 21 on ivermectin (HeartGard Plus) completely reduced their microfilariae by day 28 from a relatively low initial microfilaremia of 175 microfilariae/mL (Fig. 3C). The remaining two dogs (Dog 2, 13) on ivermectin (HeartGard Plus) experienced <90% reductions in microfilaremia post-ML, with Dog 2 experiencing an increase in microfilariae by 181% (Fig. 3C).

3.3. Presence of heartworm antigen using DiroCHEK® ELISA

Out of all dogs with confirmed *D. immitis* infection at the start of this study, 41/44 (93.2%) dogs initially tested positive for *D. immitis* antigen using DiroCHEK® ELISA with unheated (neat) plasma samples and 43/44 (97.7%) initially tested positive using DiroCHEK® ELISA with heated plasma samples. Out of all blood samples collected in this study, 88.2%

(67/76) of neat plasma samples and 98.7% (75/76) of heated plasma samples tested positive for *D. immitis* antigen using DiroCHEK® ELISA, respectively (Fig. 4A, Table 1). A paired *t*-test was performed on the raw DiroCHEK® ELISA data, revealing a significant difference in OD₆₂₀ between neat and heated plasma samples (Fig. 4B). Moderate positive correlations were found between raw microfilariae counts and the OD₆₂₀ of neat and heated dog plasma in DiroCHEK® ELISA (Fig. 5A). When performing the modified Knott's test on samples with microfilariae counts >20,000 mf/mL, we observed the overlap and clumping of microfilariae, which made counting these samples difficult. To determine whether similar statistical results were still obtained for samples with microfilariae counts <20,000 mf/mL, the correlation analyses between microfilariae counts and the DiroCHEK® ELISA were repeated. By doing so, moderate positive correlations were maintained (Fig. 5B).

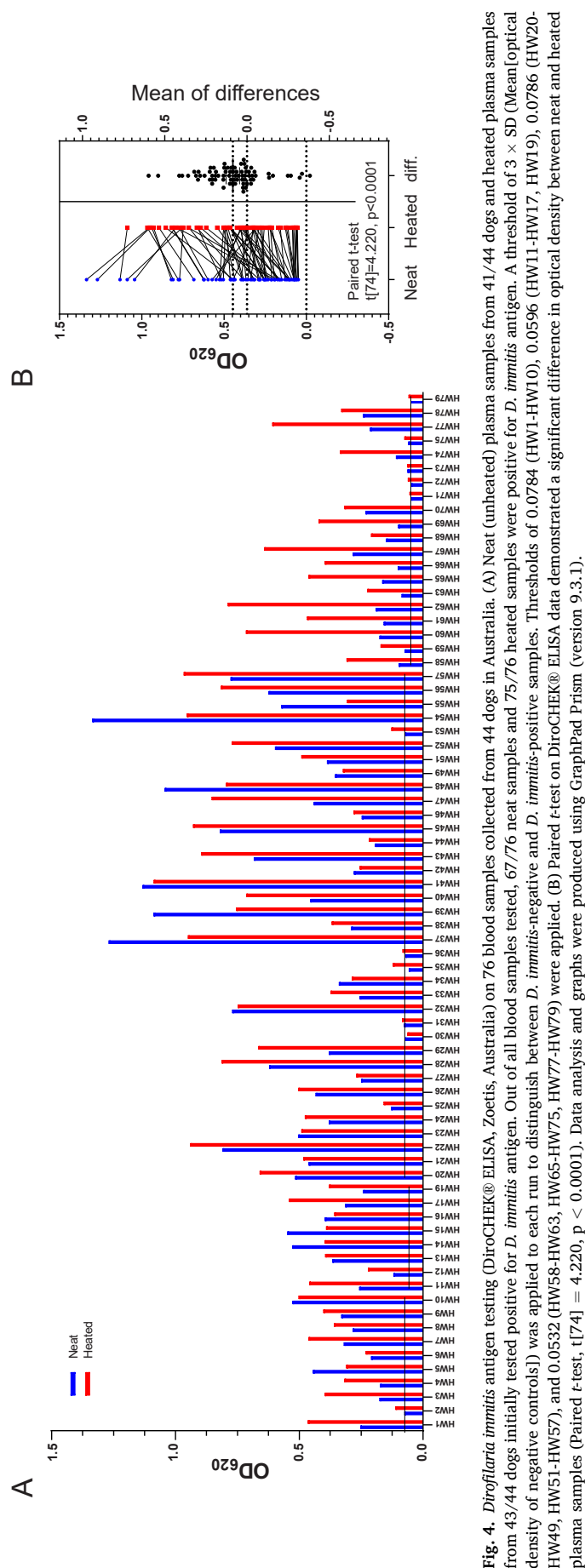


Fig. 4. *Dirofilaria immitis* antigen testing (DiroCHEK® ELISA, Zoetis, Australia) on 76 blood samples collected from 44 dogs in Australia. (A) Neat (unheated) plasma samples from 41/44 dogs and heated plasma samples from 43/44 dogs initially tested positive for *D. immitis* antigen. Out of all blood samples tested, 67/76 neat samples and 75/76 heated samples were positive for *D. immitis* antigen. A threshold of $3 \times \text{SD}$ (Mean [optical density of negative controls]) was applied to each run to distinguish between *D. immitis*-negative and *D. immitis*-positive samples. Thresholds of 0.0784 (HW11-HW10), 0.0596 (HW11-HW17, HW19), 0.0786 (HW20-HW49, HW51-HW57), and 0.0532 (HW58-HW63, HW65-HW75, HW77-HW79) were applied. (B) Paired t-test on DiroCHEK® ELISA data demonstrated a significant difference in optical density between neat and heated plasma samples (Paired t-test, t[74] = 4.220, p < 0.0001). Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

3.4. Presence of *Dirofilaria immitis* and *Wolbachia* sp. of *D. immitis* using qPCR

DNA samples obtained from dog blood were subjected to qPCR targeting the *cox1* gene of *D. immitis* and the *ftsZ* gene of *D. immitis*-associated *Wolbachia*. *D. immitis* and *Wolbachia* DNA were initially detected in 39/44 (88.6%) and 34/44 (77.3%) dogs with confirmed *D. immitis* infection, respectively. At Day 0 (pre-ML), *D. immitis* and *Wolbachia* were detected in 100% (23/23, C_t mean: 24.63, C_t range: 19.48–37.68) and 82.61% (19/23, C_t mean: 28.21, C_t range: 25.07–31.75) of dogs, respectively. At ~ Day 30 (post-ML), *D. immitis* was detected in 82.61% (19/23, C_t mean: 24.37, C_t range: 21.80–32.13) of dogs and *Wolbachia* was detected in 65.22% (15/23, C_t mean: 33.10, C_t range: 28.78–35.48) of dogs. By Day 30+, 77.8% (7/9, C_t mean: 29.00, C_t range: 25.89–36.45) of dogs were positive for *D. immitis* and 33.33% (3/9, C_t mean: 30.27, C_t range: 21.01–35.78) of dogs were positive for *Wolbachia*. The *D. immitis*-specific qPCR on extracted microfilariae DNA found one additional *D. immitis* DNA positive dog (Dog 31: 0 microfilariae/mL) that tested negative for *D. immitis* DNA on a sample from whole blood. In addition, two dogs (Dog 1: 1 microfilariae/mL, Dog 20: 0 microfilariae/mL) were *D. immitis* DNA-positive only on whole blood but *D. immitis* DNA-negative using extracted microfilariae (Supplementary Table S3).

A strong negative correlation was demonstrated between the *D. immitis* qPCR C_t -values of whole blood DNA and microfilariae counts that were <20,000 mf/mL (Fig. 5C). Samples with microfilariae counts >20,000 mf/mL were excluded from this analysis due to difficulties with counting clumped and overlapping microfilariae. Similarly, a strong negative correlation was observed between the *Wolbachia* qPCR C_t -values of whole blood DNA and microfilariae counts <20,000 mf/mL (Fig. 5D). These statistical analyses were repeated to determine whether similar correlations were still obtained when including samples with microfilariae counts >20,000 mf/mL. By doing so, a moderate negative correlation was shown between the *D. immitis* qPCR C_t -values of whole blood DNA and microfilariae counts (Fig. 5E). For all *Wolbachia* qPCR of whole blood DNA, a moderate negative correlation was observed with microfilariae counts (Fig. 5F). A strong positive correlation was found between the *D. immitis* and *Wolbachia* qPCR C_t -values of whole blood DNA samples (Fig. 5G). Samples with C_t -values of 40 were considered negative and excluded from the analysis, resulting in a moderate positive correlation (Fig. 5H).

Paired t-tests of the raw *D. immitis* and *Wolbachia* qPCR data revealed statistically significant increases in C_t -value from pre-ML to first post-ML sampling (Fig. 6A and B). To determine whether similar results were obtained for samples with pre-ML C_t -values <30 in the *D. immitis* qPCR, the analyses were repeated. By doing so, we confirmed a significant increase in C_t -value. (*D. immitis* paired t-test, t[19] = 2.159, p = 0.0439). To determine if we would obtain similar results for samples with pre-ML C_t -values <35 in the *Wolbachia* qPCR, the analyses were repeated. This confirmed a significant increase in C_t -value. (*Wolbachia* qPCR paired t-test, t[18] = 9.290, p < 0.0001). Similarly, paired t-tests of the raw *D. immitis* and *Wolbachia* qPCR data showed statistically significant increases in C_t -values from pre-ML to second post-ML sampling (Fig. 6C and D).

3.5. Illumina PCR amplicon NGS identification of ML-susceptible *D. immitis* in Australian dogs

Illumina NGS amplicon metabarcoding from 64 extracted microfilariae DNA samples from 37 dogs and one DNA sample from an adult *D. immitis* (P6/20D, positive control) was performed to determine the nucleotide proportions at 7 SNPs of interest (65 DNA samples $\times$ 7 loci = 455 PCRs) (Fig. 7). Successful amplification of the target region was achieved for 96.5% (439/455) of PCR samples. Three DNA samples had loci which failed the first-stage PCR amplification and were not submitted for Illumina NGS amplicon sequencing (HW34: 1/7 SNPs submitted, 0 microfilariae/mL; HW50: 5/7 SNPs submitted, 1

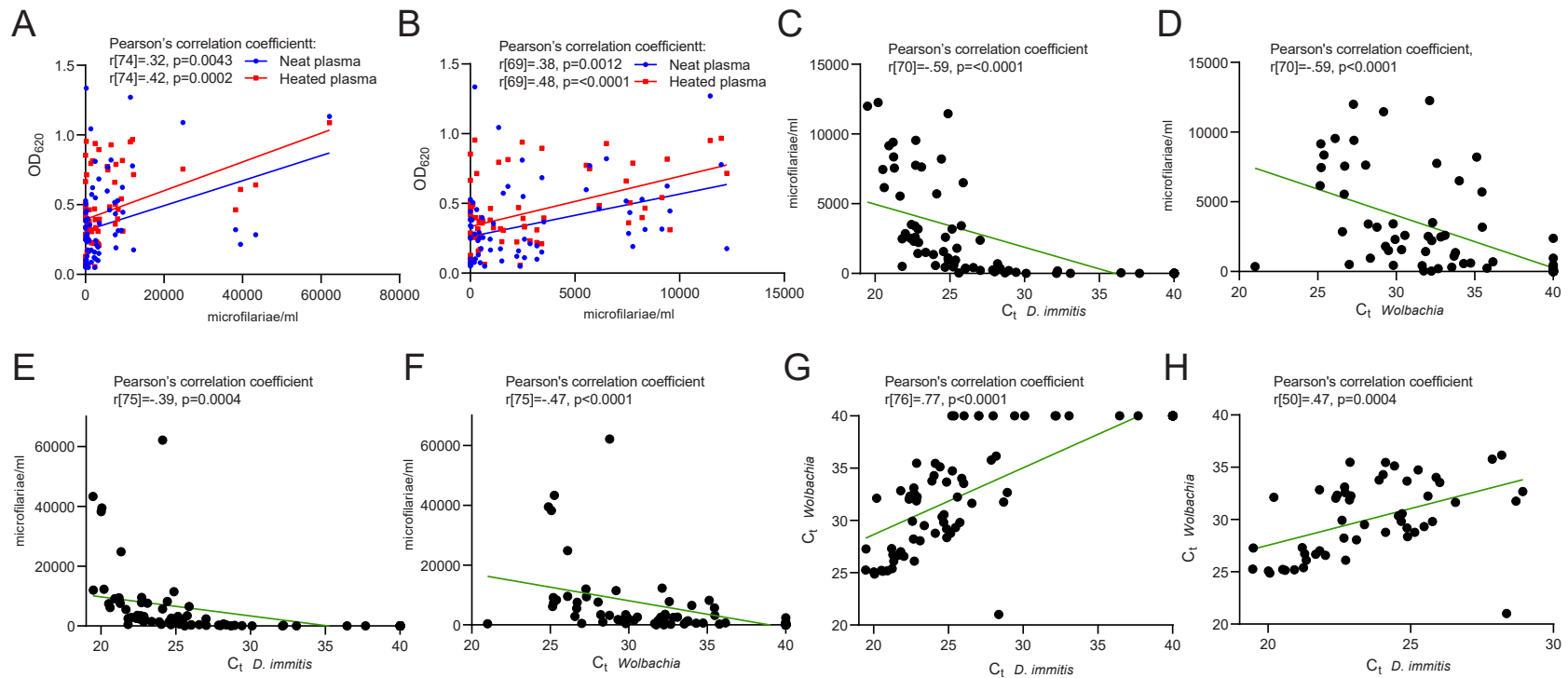


Fig. 5. Correlations between microfilariae count (mf/mL), DiroCHEK® ELISA (Zoetis, Australia) of neat (unheated) and heated plasma, and qPCR of whole blood DNA targeting *D. immitis* and associated *Wolbachia*. (A) Moderate positive correlations between microfilariae counts and OD₆₂₀ of neat ($n = 76$) and heated ($n = 76$) plasma. (B) Moderate positive correlations between microfilariae counts and OD₆₂₀ of neat ($n = 71$) and heated ($n = 71$) plasma for samples with microfilariae counts $< 20,000$ mf/mL. (C) Strong negative correlation between *D. immitis* qPCR C_t -values of whole blood DNA and microfilariae counts ($n = 72$) for samples with $< 20,000$ mf/mL. (D) Strong negative correlation between *Wolbachia* qPCR C_t -values of whole blood DNA and microfilariae counts ($n = 72$) for samples with $< 20,000$ mf/mL. (E) Moderate negative correlation between *D. immitis* qPCR C_t -values of whole blood DNA and microfilariae counts ($n = 77$). (F) Moderate negative correlation between *Wolbachia* qPCR of whole blood DNA and microfilariae counts ($n = 77$). (G) Strong positive correlation between *D. immitis* and *Wolbachia* qPCR C_t -values of whole blood DNA ($n = 78$). (H) Moderate positive correlation between *D. immitis* and *Wolbachia* qPCR C_t -values of whole blood DNA ($n = 52$) for samples with C_t -values < 40 . Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

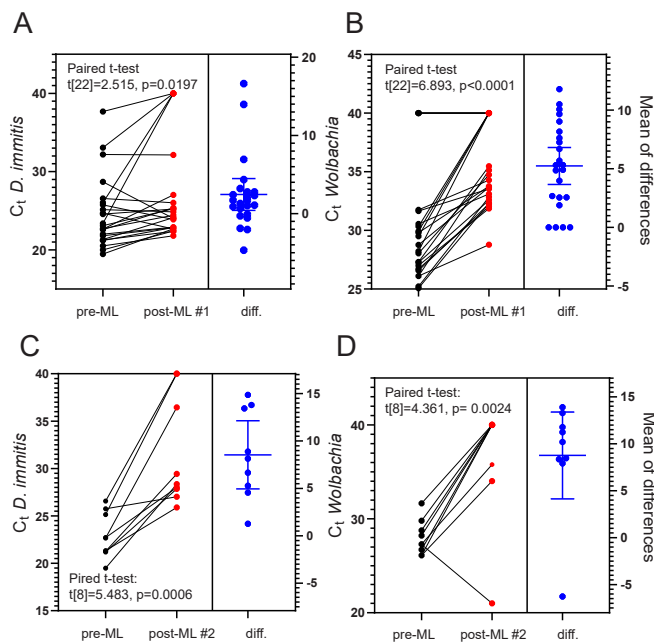


Fig. 6. Paired *t*-test analyses on *D. immitis* and *Wolbachia* qPCR C_t -values pre and post-ML treatment. (A) *D. immitis* qPCR demonstrated statistically significant increase in C_t -value between pre-ML and first post-ML sampling ($n = 23$). (B) *Wolbachia* qPCR demonstrated statistically significant increase in C_t -value between pre-ML and first post-ML sampling ($n = 23$). (C) *D. immitis* qPCR demonstrated statistically significant increase in C_t -value between pre-ML and second post-ML sampling ($n = 9$). (D) *Wolbachia* qPCR demonstrated statistically significant increase in C_t -value between pre-ML and second post-ML sampling ($n = 9$). Data analysis and graphs were produced using GraphPad Prism (version 9.3.1).

microfilariae/mL; HW51: 1/7 SNPs submitted, 0 microfilariae/mL). Only 5/7 SNPs were submitted for P6/20D. FastQ files were obtained, and SNP calling was successful for 98.2% (431/439) of submitted PCR samples. Four DNA samples had loci which failed to produce reads for SNP calling (HW29: 6/7 SNPs successful; HW34: 0/1 SNPs successful; HW50: 0/5 SNPs successful; HW51: 0/1 SNPs successful). For the purpose of reproducibility, an additional 28 PCR samples were submitted as 'repeats'. These repeat samples demonstrated identical outcomes in terms of SNP calling. Overall, a total of 8,742,348 merged non-chimeric paired reads were obtained to acquire amplicon sequence variants (ASVs) which were matched to the reference ML 'susceptible' and ML 'resistant' allele for their respective SNP (Fig. 7).

3.5.1. L42411 (Di.2.2.scaf00001:466,197)

A total of 1,368,512 merged non-chimeric paired reads were obtained for L42411 with an average of 20,426 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 62 samples had 100% of their reads assigned to a single ASV (L42411_ASV1) which was a perfect match to the ML 'susceptible' reference sequence.

3.5.2. L21554 (nDi.2.2.scaf00046:222,254)

A total of 1,167,570 merged, non-chimeric paired reads for 362 ASVs were obtained for L21554 with an average of 17,690 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 14 had 100% of their reads assigned to a single ASV (L21554_ASV1) which was a perfect match to the 'susceptible' reference sequence. The remaining 48 samples contained L21554_ASV1 along with low levels of other ASVs (L21554_ASV2, L21554_ASV3) which possessed a SNP at another position in the sequence.

3.5.3. L45689 (nDi.2.2.scaf00185:10,639)

A total of 1,664,418 merged, non-chimeric paired reads for 101 ASVs were obtained for L45689 with an average of 23,777 paired reads per sample. Overall, 61/65 DNA samples had 100% of the susceptible nucleotide at the suspect position. Of these 61 samples, 15 had 100% of their reads assigned to a single ASV (L45689_ASV1) which was a perfect match to the 'susceptible' reference sequence. The remaining 46 samples contained multiple ASVs (L45689_ASV1, L45689_ASV2, L45689_ASV3, L45689_ASV4, L45689_ASV8, L45689_ASV10, L45689_ASV11) which possessed 1–2 SNPs at other positions in the sequence. One sample (Dog 25 – HW39) contained low levels (3.8%) of L45689_ASV6 which possessed a nucleotide of unknown ML association at the suspect position (Fig. 8).

3.5.4. L9400 (nDi.2.2.scaf00005:662,854)

A total of 908,148 merged, non-chimeric paired reads for 543 ASVs were obtained for L9400 with an average of 12,613 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 57 had 100% of their reads assigned to a single ASV (L9400_ASV1) which was a perfect match to the 'susceptible' reference sequence. The remaining 5 samples contained L9400_ASV1 along with other ASVs (L9400_ASV2, L9400_ASV30) which possessed a SNP at another position in the sequence.

3.5.5. L20587 (nDi.2.2.scaf00185:62,174)

A total of 1,600,533 merged, non-chimeric paired reads for 90 ASVs were obtained for L20587 with an average of 23,537 paired reads per sample. Overall, 62/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. Of these 62 samples, 57 had 100% of their reads assigned to a single ASV (L20587_ASV1) which was a perfect match to the 'susceptible' reference sequence. The remaining five samples contained L20587_ASV1 along with other ASVs (L20587_ASV2, L20587_ASV4, L20587_ASV5, L20587_ASV6, L20587_ASV8, L20587_ASV11) which possessed a SNP at another position in the sequence.

3.5.6. L15709_A (nDi.2.2.scaf00046:76,278)

A total of 1,526,468 merged, non-chimeric paired reads for 78 ASVs were obtained for L15709_A with an average of 24,620 paired reads per sample. Overall, 61/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 61 samples had 100% of their reads assigned to a single ASV. Thirty samples had all reads assigned to L15709A_ASV1 which was a perfect match to the 'susceptible' reference sequence. The other 31 samples had all reads assigned to L15709A_ASV2 which possessed a deletion elsewhere in the sequence.

3.5.7. L30575 (nDi.2.2.scaf00046:22,857)

A total of 506,699 merged, non-chimeric paired reads for 542 ASVs were obtained for L30575 with an average of 8,173 paired reads per sample. Overall, 60/65 DNA samples displayed 100% of the susceptible nucleotide at the suspect position. All 60 samples had 100% of their reads assigned to a single ASV (L30575_ASV1) which was a perfect match to the 'susceptible' reference sequence.

4. Discussion

The use of MLs over the past 30 years has successfully reduced the prevalence of canine heartworm infection (Orr et al., 2020; Lau et al., 2021). However, the re-emergence of *D. immitis* infection in Queensland, Australia and the confirmation of ML-resistant isolates in the USA poses an important question of whether ML-resistance exists in Australia (Blagburn et al., 2011, 2016; Pulaski et al., 2014; Bourguinat et al., 2015; Nguyen et al., 2016; Maclean et al., 2017; McTier et al., 2017b; Orr et al., 2020; Panetta et al., 2021). By considering microfilariae counts as the phenotype and genotypic tests adopted from the USA, this

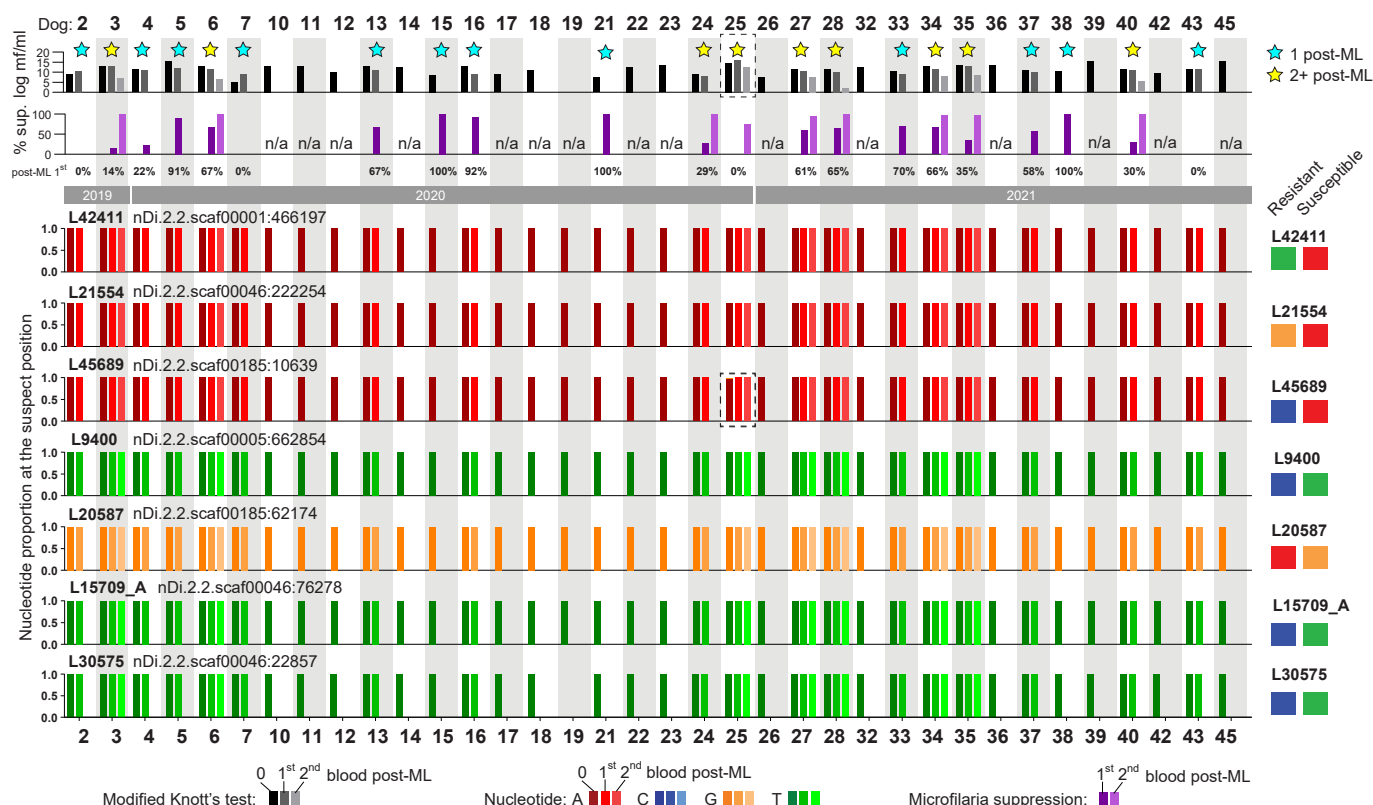


Fig. 7. Australian dogs infected with *Dirofilaria immitis* and response to ML treatment. Dog identification number is indicated at the top. Upper-most bar chart represents log transformed microfilariae (mf) counts pre-ML and post-ML (black to grey; Modified Knott's test). Yellow stars – dogs with at least two consecutive post-ML blood samples tested (2+ post-ML), blue stars – dogs with one post-ML blood sample tested (1 post-ML). Percent (%) reduction of microfilariae at the first and second post-ML time point is plotted (purple bars, n/a – not applicable due to absence of sequential sample). Percentage (%) reduction at 1st post-ML timepoint is written along the year these samples were collected. Nucleotide proportions at SNP regions (identified according to nDi2.2. Genome assembly contigs) are shown for all samples analysed. The blue, red, and orange represent the nucleotides, with the reference nucleotides associated with ML-resistance indicated for each SNP on the right. Dog 25 has microfilariae count and L45689 highlighted by dotted line rectangles. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

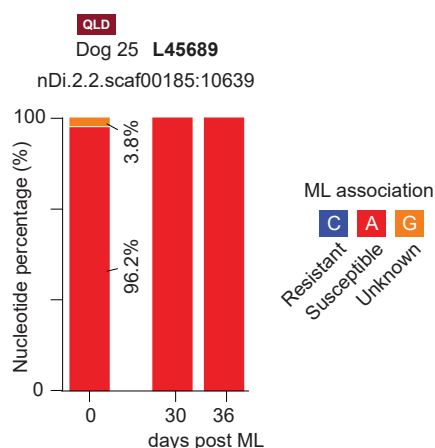


Fig. 8. Nucleotide percentages at a SNP region (L45689) previously associated with ML-resistance in the USA for Dog 25. Blood samples were collected at 0-, 30- and 36-days post-ML treatment and DNA was isolated from filtered *Dirofilaria immitis* microfilariae. The blue, red, and orange boxes in the key represent the nucleotides and their associated ML-resistance status. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

study did not unequivocally demonstrate the presence of ML-resistant *D. immitis* in Australia. Although the sequential Modified Knott's tests showed delayed clearance of circulating microfilariae compared to the

>90% reduction at 28 days described by [McTier et al. \(2017a\)](#), suggestive of suspect ML-resistance, no genotypic evidence was discovered. Our approach was in part modelled to that of [Ballesteros et al. \(2018\)](#), where 27 dogs underwent 'microfilaricidal response phenotyping' *sensu* [Geary et al. \(2011\)](#) and SNP analysis with moxidectin (Elanco, Advantage Multi for dogs, spot on). By doing so, they validated the association between ML microfilaricidal response phenotype and SNP loci genotype ([Ballesteros et al., 2018](#)). The current study obtained a comparable sample size to [Ballesteros et al. \(2018\)](#), and adopted their 5-SNP predictive model which had 94.1% sensitivity and 100% specificity. We utilised Illumina amplicon metabarcoding coupled with DADA2 analysis to detect the susceptible and resistant SNPs, which provided us with reproducible results directly comparable to [Ballesteros et al. \(2018\)](#).

To treat canine heartworm disease, infected dogs are administered doxycycline and ML at the time of diagnosis (Day 0) to target the *Wolbachia* endosymbiont of *D. immitis*, prevent further infection by eliminating *D. immitis* L3/L4 and if possible (depending on the specific ML used) decrease circulating microfilariae ([Nelson et al., 2020](#)). To target the adult stages of *D. immitis*, clinicians in Australia typically follow the three-dose protocol recommended by the American Heartworm Society ([Nelson et al., 2020](#)). Importantly, for over 10 years, Australian veterinarians had access to a yearly injectable moxidectin (ProHeart SR-12, Zoetis) preventative ([Mwacalimba et al., 2021](#)). ProHeart SR-12 does not have a claim for circulating microfilariae in Australia, but it has been shown to reduce circulating microfilariae by >90% 28 days post-administration ([McTier et al., 2017a](#)).

The utilised sequential Modified Knott's test to calculate the

reduction is not identical to the ‘microfilarial suppression testing’ *sensu* Geary et al. (2011) with spot-on moxidectin in the USA based on the label claim for microfilariae and 99% clearance within 14 days in an experimental trial (Geary et al., 2011; Bowman et al., 2015). We extended the post-ML interval to ~30 days for two reasons. Firstly, to suit the veterinary practice when owners brought their dog back for a health check-up, and secondly to utilise the published microfilaricidal effect of ProHeart SR-12 (McTier et al., 2017a). While the choice of ML-preventative was at the veterinarian’s discretion, most were injected with ProHeart SR-12 in our study. The failure of 16 dogs (ML at diagnosis; moxidectin microspheres: 14 dogs, ivermectin: 2 dogs) to reduce microfilaremia by >90% was considered phenotypic evidence of suspect ML-resistance. This finding was not corroborated by genetic SNP analysis or phenotypic testing beyond 30 days, as dogs that were re-tested eventually reached a >90% reduction in microfilaremia by Day 55 to Day 136. Delays in reaching >90% microfilarial reduction following ML treatment have been previously observed. In McTier et al. (2017a), single doses of ProHeart 6 (Zoetis, moxidectin microspheres, 170 µg/kg) and oral moxidectin (250 µg/kg) on Day 0 resulted in ≤90% reductions in mean microfilariae counts for the ML-resistant isolate ZoeMO-2012 at Day 28 (ProHeart 6: 86.5%, oral moxidectin: 83.6%), whereas ProHeart 12 (Zoetis, moxidectin microspheres, equivalent to ProHeart SR-12 registered in Australia, 500 µg/kg) led to a 91.2% reduction in microfilariae. At Day 84, all three MLs reached >90% reductions in microfilariae (ProHeart 6: 96.8%, ProHeart 12: 96.9%, oral moxidectin: 92.6%) (McTier et al., 2017a). In contrast to the experimental trial by McTier et al. (2017a), the current study sampled heterogeneous privately-owned dogs which most likely carried a diverse population of *D. immitis*.

The variability of reduction in microfilariae counts post-ML administration in the present study can be attributed to the varying effectiveness of MLs to clear circulating microfilariae (Prichard, 2021). In Australia, no product has a label claim for microfilariae, unlike in the USA where Advantage Multi for dogs (10% imidacloprid + 2.5% moxidectin topical solution) has such a claim and was therefore used by Ballesteros et al. (2018) in their study (Bowman et al., 2015). A microfilaricidal effect was noted early on for ivermectin in dogs at a dose of 50 µg/kg, but due to toxicity in dolichocephalic breeds, this high dose is not on any label (Blair and Campbell, 1979). The current preventative dose against <30-day old *D. immitis* with ivermectin is only 6 µg/kg, which is considered insufficient to clear microfilariae efficiently. Milbemycin oxime at 500 µg/kg is considered microfilaricidal, but no registered product currently has such a claim (Prichard, 2021). Moxidectin in the spot-on formulation (Elanco, Advantage Multi for dogs/Advocate for dogs at a minimum dose of 2500 µg/kg) was demonstrated to be effective against microfilariae, which has been claimed as such in the Advantage Multi for dogs (Elanco) marketed in the USA (McCall et al., 2014; Bowman et al., 2015).

Sequential quantitative Modified Knott’s tests to enumerate the number of circulating *D. immitis* microfilariae pre- and post-ML treatment enables clinicians to determine whether moxidectin treatment is successfully clearing microfilaremia in the infected dog and whether the dog remains a threat to other dogs. However, performing a non-quantitative Knott’s test is considered impractical for clinicians as it can be time-consuming, labour-intensive, and prone to human error. Alternative tools that are more rapid and accurate are required to measure changes in post-treatment microfilaremia levels. The blood samples in this study were screened for *D. immitis* using DiroCHEK® and qPCR targeting *D. immitis* and its associated *Wolbachia* endosymbiont. Based on our data, performing DiroCHEK® on neat or heated plasma was not suitable for the assessment of ML-treatment efficacy due to its lower correlation with the Modified Knott’s test. The *D. immitis* and *Wolbachia* qPCRs produced higher correlations with the Modified Knott’s test and were able to demonstrate the success of ML-treatment for reducing microfilariae levels. We therefore suggest clinicians to use these faster molecular tools as a substitute for the Modified Knott’s

test when monitoring the success of MLs in canine heartworm cases. The *D. immitis*-specific qPCR detected a larger proportion of infected dogs in this study compared to the *Wolbachia*-specific qPCR and may therefore be a better tool for measuring microfilariae levels. Future studies will benefit from a triplex qPCR targeting *D. immitis*, its associated *Wolbachia*, and dog DNA to reduce time and cost. The same *Wolbachia* qPCR assay was recently used to monitor the *Wolbachia* levels in dogs from Italy following doxycycline and Advocate (moxidectin) treatment (Laidoudi et al., 2020; Louzada-Flores et al., 2022). By doing so, they found that all dogs tested negative for *Wolbachia* after one month. Contrastingly, the current study saw only 8/28 dogs test negative for *Wolbachia* approximately 30 days after ML-treatment, with 4 of these dogs already being negative on Day 0. When infected dogs were re-sampled on Day 30+, only 6/9 tested negative for *Wolbachia*.

The SNPs analysed in Ballesteros et al. (2018) were able to distinguish between ML-susceptible and ML-resistant *D. immitis* in the USA. However, in the current study and Lau et al. (2021), no evidence of ML-resistance in Australian *D. immitis* was found using these same ML resistance-associated SNPs. In Lau et al. (2021), the genome data of five adult *D. immitis* exhibited marked genetic differences compared to those previous phenotypically characterised *D. immitis* isolates from the USA. Genomics data from ML-susceptible and ML-resistant *D. immitis* are required to associate and specifically predict the ML-resistance in clinical cases in Australia. This will enable the early detection of ML resistance in Australia, allowing clinicians, researchers and the animal pharmaceutical industry to develop effective control measures to limit and manage the spread of resistant parasites.

Successful prevention of *D. immitis* heartworm can only be achieved with rigor in prevention due to the inability to successfully remove older than 30-day *D. immitis* with current ML-based preventatives (Prichard, 2021). For veterinarians, however, the inquiry into rigor with ML-prevention will always be difficult with products administered by owners as they can unintentionally skip dosing if dogs regurgitate the dose (Atkins et al., 2014). An unintentionally missed ML-dose may lead to infection – apparent lack of efficacy (LOE) reports – despite owners’ assumed rigor with prevention. In our study, despite owners’ claims that dogs received ‘rigorous’ heartworm prevention with either moxidectin, milbemycin oxime or ivermectin for 12 months prior to the veterinarian’s diagnosis, 16 dogs tested positive for canine heartworm infection. Similar findings were encountered in Nguyen et al. (2016) where microfilaraemic dogs in Queensland previously received ‘rigorous’ prevention. In this study, owner compliance could not be confirmed and was solely based on the owner’s reports.

5. Conclusion

To confirm the presence of drug resistance in a parasite population, one must demonstrate that resistance is genetically inherited (Kohler, 2001). Since 2005, lack of efficacy complaints for heartworm preventatives led to speculation and controversy on the potential emergence of ML-resistant *D. immitis* in the USA (Hampshire, 2005; Atkins et al., 2014). It was not until 2014 that the first ML-resistant strain (LSU 10) was isolated in the USA and resistance to MLs was unequivocally confirmed (Pulaski et al., 2014). To do this, a laboratory dog was experimentally infected with L3s obtained from a field case, and then treated with ivermectin (Pulaski et al., 2014). Infective larvae from this dog were then successfully passaged into a second laboratory dog, which confirmed the genetic heritability of drug resistance (Pulaski et al., 2014). In Australia, we currently lack these types of specialised facilities where laboratory dogs can be experimentally infected with canine heartworm. Due to this limitation, it remains unknown whether ML-resistance is present in Australia, much like the 2005–2014 time period in the USA. Until we can demonstrate the heritability of ML-resistance in Australian *D. immitis* isolates via experimental infection studies, we can only speculate and test for resistance using the available tools as a proxy.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2022.11.003>.

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Connecting statement 1

In Chapter 2.1, a cohort of heartworm-infected dogs in Australia were screened for phenotypic and genotypic evidence of drug resistance. Samples in that study were obtained opportunistically from participating veterinary clinics in Queensland (QLD) and New South Wales (NSW). Clinicians performed their own testing and diagnosis of heartworm disease on Day 0, and left-over blood samples were shipped to me at the University of Sydney for additional diagnostic testing. In particular, I conducted the modified Knott's test to measure pre-treatment microfilaremia levels in the animal. During the appointment, clinicians then administered macrocyclic lactone (ML) and doxycycline to the dogs as per the American Heartworm Society guidelines. The specific ML administered was at the clinician's discretion, although the majority of dogs in the study received ProHeart SR-12.

Approximately 30 days later, the animals returned to their local clinics to begin adulticide treatment. Here, clinicians collected post-ML blood samples for shipment to the University of Sydney, where I conducted a follow-up modified Knott's test to assess reductions in microfilaremia after treatment. This hands-off approach allowed clinicians to follow their usual protocols and routines, thereby maximising the study's sample size. Although this study provided valuable insights into the sensitivity of Australian heartworms to MLs for the first time, a limitation of this approach was that it did not meet the criteria for a microfilarial suppression test (MFST). Specifically, the post-ML samples were collected 30 days after treatment instead of the required seven days, and adequate doses of registered microfilaricidal MLs were not necessarily used. Additionally, the administration of doxycycline could be a confounding factor.

Upon submission for publication, reviewers recognised these limitations and expressed that the phenotypic assay was inadequately performed and did not provide concrete evidence of resistance. I address these limitations in Chapter 2.2, where I performed a MFST on two dogs from Townsville, Australia. This involved administration of a registered microfilaricide (Advocate™ for Dogs, marketed as Advantage Multi® for Dogs in the US), no doxycycline treatment, and re-sampling precisely on Day 7.

———— Chapter 2.2 ————

Delayed canine heartworm (*Dirofilaria immitis*) microfilarial
reduction following Advocate™ for dogs (imidacloprid,
moxidectin) treatment

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Rosemonde Isabella Power in the preparation and submission of the following manuscript:

Power, R. I., & Šlapeta, J. (2024). Delayed canine heartworm (*Dirofilaria immitis*) microfilarial reduction following Advocate™ for dogs (imidacloprid, moxidectin) treatment. *The Veterinary Journal*, 307, 106209. <https://doi.org/10.1016/j.tvjl.2024.106209>

Contribution to the manuscript of all authors is as follows:

Task	Contributing co-authors
Conceptualisation	RIP, JŠ
Data collection and curation	RIP
Formal analysis	RIP
Funding acquisition	JŠ
Investigation	RIP
Methodology	RIP
Supervision, discussion and consultation	JŠ
Preparation of figures used in the manuscript	RIP
Writing of the original draft	RIP
Critical review and editing of the manuscript	RIP, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct.

Kind regards,

Professor Jan Šlapeta

26th September 2024



Delayed canine heartworm (*Dirofilaria immitis*) microfilarial reduction following Advocate™ for dogs (imidacloprid, moxidectin) treatment

Rosemonde Isabella Power^a, Jan Šlapeta^{a,b,*}

^a Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales 2006, Australia

^b Sydney Infectious Diseases Institute, The University of Sydney, New South Wales 2006, Australia

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ABSTRACT

Macrocytic lactone (ML) anthelmintics are currently the only class of drugs available for canine heartworm prevention. Recent reports of *Dirofilaria immitis* infection occurring in dogs reportedly receiving 'rigorous' prevention in Queensland, Australia, coupled with the confirmation of ML-resistant isolates in the USA, has led to speculation about the potential emergence of ML-resistance in Australia. In this study, we describe two cases (Dog 1 and 2) of asymptomatic canine heartworm disease in Townsville, Australia, that were reportedly receiving 'rigorous' heartworm prevention according to the owners' claims. We aimed to deploy currently available tools to assess the phenotypic and genotypic ML-resistance status of these two dogs. For phenotypic testing, we performed an in-vivo 7-day microfilariae suppression test using a dose of spot-on moxidectin (Advocate™ for Dogs, 100 g/L imidacloprid + 25 g/L moxidectin). This formulation is marketed as Advantage Multi® for Dogs in the USA, which claims a *D. immitis* microfilaricidal effect. For genetic testing, an Illumina amplicon metabarcoding approach was used to target single nucleotide polymorphisms (SNPs) previously associated with ML-resistance in *D. immitis* from the USA. Dog 1 and Dog 2 demonstrated <10 % and <40 % reductions in circulating microfilariae seven days after moxidectin treatment, respectively. These phenotypes were not corroborated by genetic SNP testing, as both dogs were classified as susceptible across all examined markers. To streamline testing of *D. immitis* SNPs, we developed a rhAmp™ SNP qPCR approach for rapidly genotyping suspect cases of ML-resistant infections at the two major loci (L15709 A and L30575). These findings illustrate a phenomenon shown in some heartworm cases outside the USA, whereby infected dogs are failing to see marked reductions in microfilaraemia after ML treatment but possess an ML-susceptible genotype.

Introduction

Dirofilaria immitis is a mosquito-borne filarial nematode that causes the potentially life-threatening canine heartworm disease. Macrocytic lactone (ML) anthelmintics are the primary class of drugs available for preventing canine heartworm disease (Hampshire, 2005). In Australia, a 2016 study found that a large proportion of dogs in Queensland were microfilaraemic despite reportedly receiving 'rigorous' ML prevention (Nguyen et al., 2016). This finding, coupled with the confirmation of ML-resistant isolates in the United States of America (USA) in 2014, has led to speculation regarding the potential emergence of ML-resistance in Australia (Pulaski et al., 2014; Nguyen et al., 2016). Although experimental infection studies were conducted in the USA to confirm the resistant phenotype and the genetic heritability of resistance, this approach has not been feasible in Australia due to the high costs and

labour involved (Pulaski et al., 2014). In addition, the genetic changes responsible for causing ML-resistance in *D. immitis* remain unknown (Prichard, 2021). Consequently, the only tools currently at our disposal are those which act as a proxy for detecting ML-resistance. This includes the microfilariae suppression test and the detection of single nucleotide polymorphisms (SNPs) associated with ML-resistance, both of which were developed based on isolates from the USA (Geary et al., 2011; Bourguinat et al., 2015).

The currently accepted gold standard approach to test for resistance in the field is the in-vivo microfilariae suppression test using a microfilaricidal dose of ML (i.e., 50 or 200 µg/kg ivermectin [IVM], depending on the breed, or 2.5 mg/kg moxidectin [MOX]) (Geary et al., 2011; Ballesteros et al., 2018). In Geary et al. (2011), where the microfilariae suppression test was originally proposed, it was suggested that a modified Knott's test may be performed immediately before and seven

* Corresponding author at: Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales 2006, Australia.

E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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days after an animal receives an off-label microfilaricidal dose of IVM (50 or 200 µg/kg IVM). Microfilariae reductions of <75 % were suggestive of ML-resistance (Geary et al., 2011). Alternatively, for microfilariae suppression testing, Ballesteros et al. (2018) validated the use of a spot-on MOX product (Advantage Multi® for Dogs, 10 % imidacloprid + 2.5 % MOX) that has been registered as a microfilaricidal in the USA. Ballesteros et al. (2018) postulated that high doses of IVM or Advantage Multi® for Dogs should lead to microfilariae reductions of >90 % after 2–4 weeks. Experimentally, Bowman et al. (2015) evaluated the efficacy of such topical solutions (Advantage® Multi, Advocate®) for the treatment of *D. immitis* circulating microfilariae in dogs and demonstrated 99.4 % efficacy at seven days post-treatment. In Australia, Advantage Multi® for Dogs is marketed under the name Advocate™ and has the same topical formulation, but unlike in the USA, the Australian label does not claim a *D. immitis* microfilaricidal effect (Australian Pesticides and Veterinary Medicines Authority, Australian Government; Elanco Australia).

In our previous study, we screened 45 dogs from veterinary practices in Queensland and New South Wales, Australia for phenotypic and genotypic evidence suggestive of ML-resistant canine heartworm infections (Power and Šlapeta, 2022). We observed an unusual phenomenon where 16 dogs failed to see a reduction in microfilaraemia by >90 % after ML treatment, yet all possessed susceptible alleles at SNP positions associated with ML-resistance in USA isolates (Power and Šlapeta, 2022). Since we followed procedures and timelines that best suited the veterinary practices in our study, we encountered variations in the types of ML products administered (i.e., Heartgard® Plus or ProHeart® SR-12, not registered as microfilaricidal) and the time interval between ML treatment and the post-ML microfilariae count (typically 30–40 days). In addition, the phenotypic approach used in our study was potentially ambiguous as the dogs were on doxycycline (Power and Šlapeta, 2022). Further investigations into cases of suspect ML-resistance in Australia are urgently required using the recommended microfilariae suppression test method with the 7-day timeframe suggested by Geary et al. (2011) and application of MOX according to Ballesteros et al. (2018).

In this study, we present two cases (Dog 1 and 2) of asymptomatic canine heartworm disease from Townsville, Australia that were reportedly on ‘rigorous’ heartworm prevention according to the owners’ claims. We aimed to deploy currently available tools to assess the phenotypic and genotypic ML-resistance status of these two dogs. To do so, we performed the in-vivo 7-day microfilariae suppression test after application of Advocate™ for Dogs (equivalent to Advantage Multi® for Dogs) topical solution with MOX (25 g/L). This microfilariae suppression test was coupled with genetic testing of SNPs associated with ML-resistance in *D. immitis* isolates from the USA using an Illumina amplicon metabarcoding approach. To circumvent the long waiting times typically experienced when outsourcing next-generation sequencing services, we developed a more rapid and convenient in-vitro rhAmp™ SNP qPCR assay and compared this to the Illumina amplicon metabarcoding approach.

Material and methods

Dog histories

Dog 1, a 5-year-old Labrador crossbred neutered male, and Dog 2, a 4-year-old Staffy crossbred neutered male, presented to Bohle Veterinary Surgery, Townsville, Australia, in November 2022 and February 2023, respectively, for a C5 vaccination. During the two visits, both animals underwent a routine in-house WITNESS Dirofilaria (Zoetis Australia) test for the detection of canine heartworm antigen. The animals were found to be positive for canine heartworm antigen and were microfilaraemic. No clinical signs of canine heartworm disease were observed and neither animal lived in or visited any other states or territories. Based on the owners’ assessments, both animals received

apparent rigorous heartworm prevention since they were young. For the past 12 months, they reportedly received NexGard SPECTRA® (minimum 2.5 mg/kg afoxolaner + 0.5 mg/kg milbemycin oxime, Boehringer Ingelheim Animal Health Australia) with the most recent dose administered two weeks prior to diagnosis. For Dog 1, the owner claimed that the heartworm preventative was consistently administered at the start of each month, and they ensured that the tablet was ingested. To confirm whether “suspect resistance” cases are attributed to lack of compliance or inherited resistance, it is essential to conduct a retrospective analysis of the veterinary records. This allows us to determine whether sufficient heartworm preventative for the dog was purchased. Locating and reviewing veterinary records can be challenging and complete information may not always be available. In the current study, inspection of veterinary records for weight and dose sizes purchased revealed that Dog 1 was not tested for *D. immitis* before starting NexGard SPECTRA®. Dog 2 tested negative for *D. immitis* antigen and microfilariae approximately 28 months prior to diagnosis, but no further information on purchase history and dosing could be obtained.

Microfilariae suppression testing

To test for the potential presence of ML-resistant parasites, we performed an in-vivo 7-day microfilariae suppression test *sensu* Geary et al. (2011). Blood samples (≤ 4 mL) were collected in EDTA tubes by a registered veterinarian immediately before the administration of spot-on Advocate™ for dogs (Elanco, Australia). Another blood sample was collected seven days later. In Australia, Advocate™ does not have a microfilaricidal label claim. In the US, Advocate™ is marketed under the name Advantage Multi®, which does have a label claim against microfilariae and has demonstrated a 99 % microfilariae clearance seven days post-treatment (Bowman et al., 2015). In the current study, the pre-ML and post-ML blood samples were shipped to the Laboratory of Veterinary Parasitology at the University of Sydney, Australia for diagnostic *D. immitis* testing. The owners completed a consent form prior to participation in the study; the animals were enrolled under the study’s Animal Ethics guidelines. At the University of Sydney, a quantitative modified Knott’s test was performed as previously described in Power and Šlapeta (2022) to enumerate the number of microfilariae in the dog blood before and seven days after ML treatment. An Anigen Rapid *Dir-ofilaria immitis* (Heartworm) Ag Test Kit (BioNote, Korea) was used according to the manufacturer’s instructions for the qualitative detection of *D. immitis* antigen. After the seven-day period, the dogs were treated according to the American Heartworm Society guidelines (Nelson et al., 2020). This treatment regime included administration of a macrocyclic lactone, doxycycline, and melarsomine. Additional blood collections and treatment details are outlined in Table 1.

DNA isolation and real-time PCR

Microfilariae present in whole dog blood were extracted using a filtration procedure and DNA was isolated as described previously (Power and Šlapeta, 2022). We performed a duplex real-time PCR (qPCR) targeting the cytochrome c oxidase subunit I (*cox1*) gene of *D. immitis* and the filamenting temperature-sensitive mutant Z (*ftsZ*) gene of *D. immitis*-associated *Wolbachia* (Laidoudi et al., 2020; Panetta et al., 2021). As a positive DNA extraction control, we included a simplex qPCR targeting the partial canine glyceraldehyde3-phosphate dehydrogenase (*GAPDH*) region (Orr et al., 2020; Panetta et al., 2021). Details and procedures for the duplex and simplex qPCRs were adopted from Lau et al. (2024) and Power and Šlapeta (2022). Baseline thresholds for all qPCR runs were auto calculated using Bio-Rad CFX Maestro Software (BioRad, Australia).

Table 1
Microfilariae suppression testing on two cases of canine heartworm disease from Townsville, Australia using spot-on Advocate™ for Dogs (25 g/L moxidectin + 100 g/L imidacloprid).

Dog	HW compliance (past 12 m)	HW preventive	HW treatments	Sample ID	Day	Microfilariae/ ML	Microfilariae reduction (%)	Rapid antigen test	<i>D. immitis</i> qPCR (Ct-value)	<i>Wolbachia</i> qPCR (Ct-value)
Dog 1	Inconsistent*	NexGard SPECTRA®	Advocate™ for Dogs	HW88/22	0	28,540	N/A	+	21.12	24.64
			ProHeart® SR-12, doxycycline	HW89/22	7	26,790	-6.13	+	22.35	26.13
			Immiticide injection	HW90/23	76	870	-96.95	+	27.83	-
			Immiticide injection	HW92/23	104	80	-99.72	+	29.21	-
			N/A	HW110/23	140	0	-100	-	33.52	-
Dog 2	Rigorous**	NexGard SPECTRA®	Advocate™ for Dogs	HW93/23	0	22,200	N/A	+	20.83	24.43
			ProHeart® SR-12, doxycycline	HW94/23	7	13,600	-38.74	+	21.15	25.7
			Immiticide injection	HW111/23	70	1185	-94.66	+	26.96	-
			Immiticide injection	HW112/23	105	50	-99.77	+	29.38	-
			N/A	HW113/23	136	10	-99.95	+	30.69	-

* Dog was initially reported to be on 'rigorous' prevention, but inspection of veterinary records revealed underdosing.
** Dog was reported to be on 'rigorous' prevention, but this could not be confirmed by the veterinary records.

Illumina amplicon next-generation sequencing (NGS) of ML-resistance-associated SNPs

Following the confirmation of ML-resistant *D. immitis* in the USA, studies have identified 10 SNPs that best separate susceptible and resistant isolates. We performed Illumina PCR amplicon next-generation sequencing (NGS) targeting 7 of these 10 SNPs (L42411, L21554, L45689, L9400, L20587, L15709_A and L30575), which cover all the SNPs in the 5-SNP models from Ballesteros et al. (2018) and Bourguinat et al. (2017). The first-stage PCRs were performed in-house on the first three filtered microfilariae DNA samples obtained from Dog 1 (HW88, HW89, HW90) and Dog 2 (HW93, HW94, HW111). The PCR products for all 7 SNPs were pooled per sample before submission to the Ramaciotti Centre for Genomics, University of New South Wales, Australia, as previously described (Power and Šlapeta, 2022). The NGS data were analysed using the DADA2 pipeline (1.18.0; Callahan et al. (2016)) in R (version 4.3.0) within RStudio (2023.06.1 Build 524) (RStudio Team, 2023), as previously described (Power and Šlapeta, 2022). Amplicon sequence variants (ASVs) were imported into CLC Main Workbench (version 23) (CLC bio, Qiagen, Australia) where they were aligned to their respective reference sequence.

Validation of rhAmp™ SNP qPCR for ML-resistance-associated SNPs

Since the Illumina amplicon NGS in this study was outsourced, the time taken between sample submission and reception of data can be unpredictable. Long waiting times are not ideal when encountering suspect ML-resistance cases, where rapid resistance genotyping results are required. Thus, we developed a rapid, qualitative rhAmp™ SNP

qPCR tool to distinguish between *D. immitis* samples that are ML-resistant, ML-susceptible, and heterozygous. We selected the top two SNPs that gave the best prediction of ML-resistance in Ballesteros et al. (2018), i.e., L15709_A and L30575. Assays were designed using the rhAmp™ SNP Genotyping System (Integrated DNA Technologies, Australia). Primers designed for this study are presented in Table 2. To confirm that the assay could correctly identify the SNPs correlated with resistance, we designed 'susceptible' and 'resistant' synthetic control templates (gBlocks Gene Fragments 125–500 bp, Integrated DNA Technologies, Australia) (Table 3). These gBlocks were diluted to 10⁵, 10⁴, 10³ and 10² copies/μL. To evaluate the assay's ability to identify equal proportions of the alleles in heterozygous samples, we initially mixed the susceptible and resistant gBlocks at a ratio of 50:50. Additional heterozygous controls containing the 'susceptible' and 'resistant' gBlocks at ratios of 25:75 and 75:25 were subsequently included. The rhAmp™ SNP qPCR assays were carried out in 5 μL final volumes of rhAmp™ Genotyping Master Mix and rhAmp™ Reporter Mix (without reference dye) containing 0.25 μL of the respective rhAmp™ SNP assay (20× concentration) and 1 μL of template DNA. Heterozygous controls contained 1 μL of each gBlock at concentrations of 10⁵, 10⁴, 10³ and 10² copies/μL. The gBlock reactions were run in duplicate and no template controls (NTCs) were included in each run. The rhAmp™ SNP qPCR was carried out on a CFX96 Real-Time PCR system with the corresponding Bio-Rad CFX Maestro Software (BioRad, Australia). Cycling conditions consisted of 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 68°C for 20 s. FAM was assigned to the reference allele and VIC dye (Applied Biosystems) was assigned to the alternate allele. After the rhAmp™ SNP qPCRs were performed, the resulting allelic discrimination plate-read files were visually inspected, and automatic allele

Table 2
Primers to target single nucleotide polymorphisms (SNPs) at L15709_A and L30575 using the rhAmp™ SNP Genotyping System (Integrated DNA Technologies). These two SNPs were reported to give the best prediction of ML-resistance in *D. immitis* from the USA.

	L15709_A	L30575
Allele Specific Primer 1	AATTCTTACTTGTATACAGTCCCT	TGGTAGCGCTGTTTCGTAT
Allele Specific Primer 2	ATTCTTACTTGTATACAGTCCCG	GGTAGCGCTGTTCGTAC
Locus Specific Primer	GCCAACTCACTTATCGAAATGTTTTT	GCCAGCCGCAAAAGTATTTAGGAAT

Table 3
gBlocks Gene Fragments used in the rhAmp™ SNP Genotyping assay targeting resistance-associated SNPs in *D. immitis*.

	gBlock
Susceptible	CGTGTGTAATTCTTACTTGTATACAGTCCCTAGTTTTCGACGATAAAAAACATTTTCGATAAGTGAGTTTGAATTAATTG- CCAGTATTTGAAACTGGTAGCGCTGTTCTGATTTGCTGTTGCCATGTTATGTATATGATACATTCTAAATACTTTTTCGGCTGTGGTTTCCAGTGTTC
Resistant	CGTGTGTAATTCTTACTTGTATACAGTCCCTAGTTTTCGACGATAAAAAACATTTTCGATAAGTGAGTTTGAATTAATTG- CCAGTATTTGAAACTGGTAGCGCTGTTCTGATTTGCTGTTGCCATGTTATGTATATGATACATTCTAAATACTTTTTCGGCTGTGGTTTCCAGTGTTC

calls were converted to their corresponding genotype. We deployed the rhAmp™ SNP qPCR assay to rapidly determine the genotype of the *D. immitis* from Dog 1 before (HW88), 7 days after (HW89) and 76 days after (HW90) ML treatment. To further validate the assay and provide additional context on the genotype of Dog 1, we included an additional eighteen *D. immitis* DNA samples that were previously reported in Power and Šlapeta (2022). By doing so, we were able to compare the performance of our rhAmp™ SNP qPCR assay to Illumina amplicon NGS. Only samples with microfilariae counts >2000 mf/ML either before or after ML application were included in this study. During the development of the rhAmp™ SNP qPCR, Dog 2 had not yet been diagnosed with canine heartworm disease and was therefore not included.

Results

Application of Advocate™ for Dogs failed to reduce microfilaraemia by >40 % seven days post-treatment

To examine the ML-resistance phenotype of the *D. immitis* infecting the dogs, we performed microfilariae suppression testing using the modified Knott’s test on blood collected before and seven days after treatment with Advocate® for Dogs spot-on moxidectin *sensu* Geary et al. (2011). Dog 1 had a high level of microfilaraemia (28,540 microfilariae/ML) on Day 0, which decreased by only 6.13 % (26,790 microfilariae/ML) seven days after ML treatment (Table 1). Reductions in circulating microfilariae >90 % were seen in Dog 1 by Day 76 with 870 microfilariae/ML (96.95 % reduction), followed by 80 microfilariae/ML on Day 104 (99.72 % reduction), and 0 microfilariae/ML on Day 140 (100 % reduction). Dog 1 tested positive for *D. immitis* antigen from Day 0–104 but was negative on Day 140. Dog 2 displayed high numbers of microfilariae (22,200 microfilariae/ML) on Day 0, which decreased by only 38.74 % (13,600 microfilariae/ML) after seven days (Table 1). Greater than 90 % reductions in microfilariae were seen in this animal by Day 70 with 1185 microfilariae/ML (94.66 % reduction), followed by 50 microfilariae/ML on Day 105 (99.77 % reduction), and 10 microfilariae/ML on Day 136 (99.95 % reduction). Dog 2 was positive for *D. immitis* antigen throughout the study. Six months after immiticide treatment (Day 351), in-clinic follow-up tests revealed that Dog 2 was negative for *D. immitis* antigen and negative for microfilariae using a thick blood smear. Additional blood samples were not collected and sent for microfilariae enumeration as they fell outside the specified time frame of the study.

DNA samples obtained from filtered microfilariae were subjected to a duplex qPCR targeting the *cox1* gene of *D. immitis* and the *ftsZ* gene of the *D. immitis*-associated *Wolbachia* endosymbiont. Targeting the *Wolbachia* endosymbiont offers an alternative approach for indirectly detecting *D. immitis*. The *Wolbachia* qPCR assay utilised in this study has previously demonstrated its ability to identify occult infections and allows clinicians to monitor *Wolbachia* clearance after doxycycline treatment (Laidoudi et al., 2020). To confirm the successful extraction of DNA from all samples, a simplex qPCR targeting the GAPDH region was performed to verify the presence of the dog host DNA. We found that all DNA samples from both dogs tested positive for *D. immitis* (Table 1) and dog DNA. In the *Wolbachia* qPCR, the first two samples from both animals (2/5, 40 %) tested positive, whereas the remaining samples did not amplify (Table 1).

Detection of SNPs correlated to ML-susceptibility using Illumina amplicon NGS

To genotypically assess the ML-resistance profile of the study animals, we conducted Illumina amplicon next-generation sequencing which targeted seven SNPs linked to ML-resistance in USA *D. immitis*. Microfilarial DNA obtained from the first three blood samples of each dog were included in the NGS run. Overall, >97 %, >93 %, >98 %, >77 %, >98 %, >95 %, and >70 % of input reads were retained and 0 %, 0 %, 0.7 %, 0 %, 0 %, 0 %, and 0.7 % of merged paired reads were removed as chimeras for L42411, L21554, L45689, L9400, L20587, L15709_A, and L30575, respectively. A total of 143,886 merged non-chimeric paired reads were obtained across all samples after data cleaning, with an average of 23,981 reads per sample (minimum: 11,068 reads; maximum: 41,933 reads). Once the sample reads were separated into their respective SNP loci and non-target amplicon sequence variants (ASVs) were removed, we found that 100 % of reads for all samples possessed the susceptible nucleotide at all seven SNP positions (Fig. 1). Single ASVs were produced for L42411, L21554, L9400, L20587 and L30575, and these were perfect matches to their respective susceptible reference sequences. For L45689, an average of 31 % of reads were assigned to an ASV possessing another SNP elsewhere in the sequence. For L15709A, an average of 50 % of reads were assigned to an ASV containing a single deletion elsewhere in the sequence.

A novel rhAmp™ SNP qPCR assay allows rapid identification of SNPs correlated with genetic ML-resistance and ML-susceptibility

We developed a rapid rhAmp™ SNP qPCR assay targeting two SNPs (L15709_A and L30575) that have best predicted ML-resistance in previous studies (Ballesteros et al., 2018). Upon inspection of the allelic discrimination plate-read files, we decided to set the number of cycles to 37 for allele calling as it appeared to best separate the gBlock Gene Fragments. In Fig. 2A, the rhAmp™ SNP qPCR targeting L15709A made correct calls of either ‘Allele 1’ (susceptible), ‘Allele 2’ (resistant) or ‘heterozygous’ for 100 % (6/6) of the gBlock controls tested at a concentration of 10⁵ copies/μL. For lower concentrations of 10⁴, 10³, and 10² copies/μL, we found that 67 % (4/6), 17 % (1/6) and 0 % (0/6) of the gBlock controls were correctly called, respectively. Similarly, the L30575 SNP assay correctly called 100 % (6/6) of the gBlock controls at a concentration of 10⁵ copies/μL. For 10⁴, 10³, and 10² copies/μL concentrations, the L30575 SNP assay correctly called 83 % (5/6), 0 % (0/6) and 0 % (0/6) of the gBlock controls, respectively. We therefore selected 10⁵ as the ideal concentration for the gBlocks in this rhAmp™ SNP qPCR assay. All no template controls (NTC) displayed no amplification and no allele call.

Due to wait times concomitant with outsourcing the Illumina amplicon NGS, we decided to deploy our rhAmp™ SNP qPCR on the microfilarial DNA samples collected from Dog 1 before (HW88) and seven days after (HW89) treatment with spot-on moxidectin (Advocate™ for Dogs). We included an additional 18 clinical heartworm samples from our previous study to further validate the rhAmp™ SNP qPCR assay and better determine where the Dog 1 samples clustered (Power and Šlapeta, 2022). By doing so, we found that both samples from Dog 1 were classified as ‘susceptible’ at L15709_A and L30575, and they closely clustered with the clinical samples (Fig. 2B). The L15709_A assay classified 94 % (17/18) of the clinical samples as ‘susceptible’,

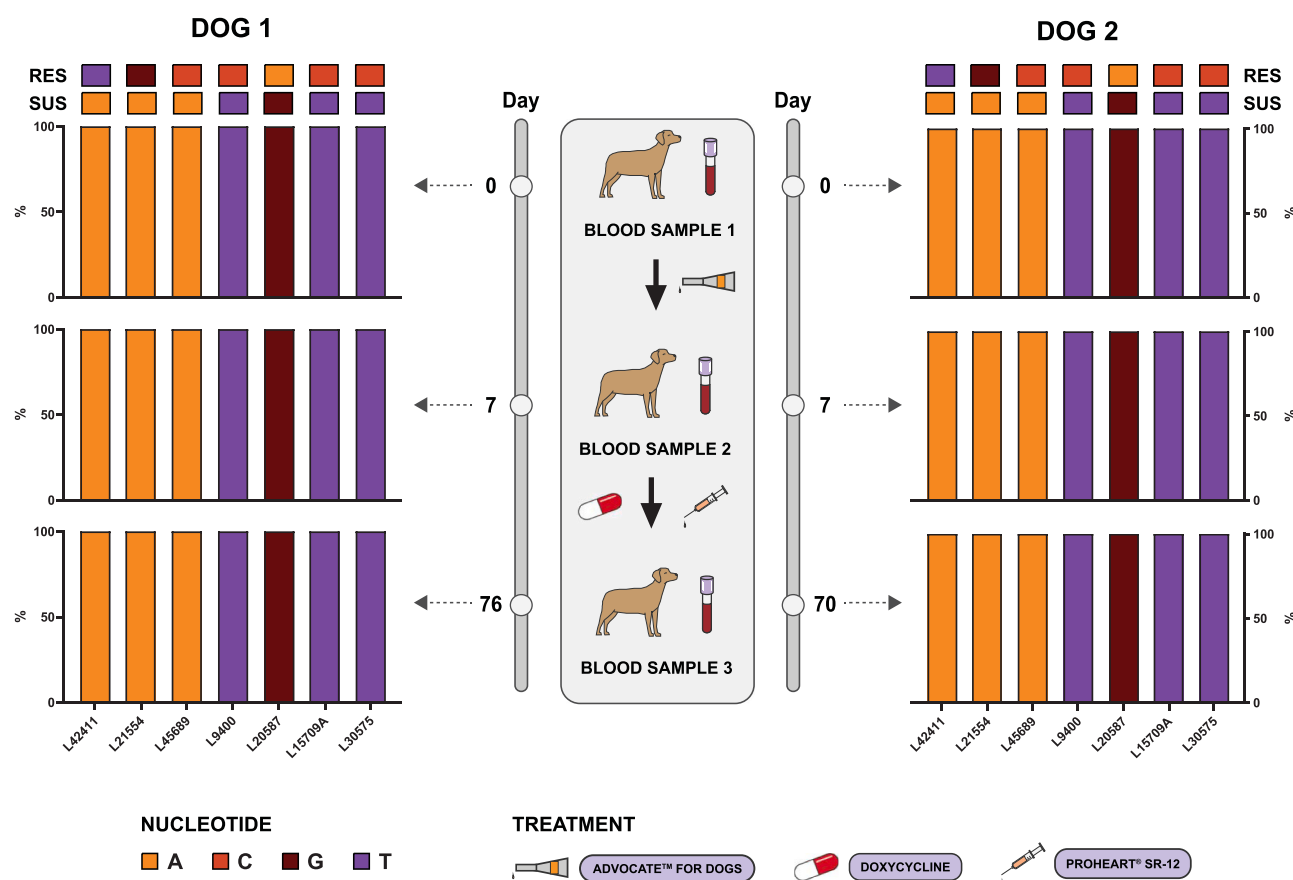


Fig. 1. Drug resistance-associated single nucleotide polymorphisms (SNPs) in two asymptomatic cases of canine heartworm disease from Townsville, Australia. Day 0 blood samples were obtained from Dogs 1 and 2 immediately before the administration of spot-on Advocate™ for Dogs (Elanco, Australia). Both dogs were re-sampled on Day 7 and were treated according to the American Heartworm Society guidelines (Nelson et al., 2020). Additional samples were collected from Dogs 1 and 2 on Days 76 and 70, respectively. DNA samples used for genetic testing originated from microfilariae filtered from 1 mL of blood. Quantitative Illumina PCR amplicon next-generation sequencing (NGS) targeting seven macrocyclic lactone- (ML) resistance-associated SNPs was performed. The selected SNPs (L42411, L21554, L45689, L9400, L20587, L15709A and L30575) were previously shown to be correlated with ML-resistant *D. immitis* populations from the Lower Mississippi Valley region of the USA. These SNPs cover both 5-SNP models described in Ballesteros et al. (2018) and Bourguinat et al. (2017). For each sample, a bar chart illustrates the percentage (%) of sample reads containing various nucleotides at all seven SNP positions. The nucleotide colour key is shown on the bottom left. A guide to the reference 'susceptible' and 'resistant' nucleotides at each SNP position are included above the bar charts. Treatments administered to the dogs throughout the study are indicated in the flowchart, with the key at the bottom.

with the remaining sample unable to be classified (Supplementary Table 1, Fig. 2B). For the L30575 assay, 100 % (18/18) of the clinical samples were classified as 'susceptible' (Supplementary Table 1, Fig. 2B). The classification of these clinical heartworm samples directly corresponded to the Illumina amplicon NGS in Power and Šlapeta (2022) (Supplementary Table 1). We included additional heterozygous controls at various ratios to further assess the allelic composition of Dog 1. A clear separation was observed between the susceptible, resistant, and heterozygous (susceptible:resistant at 25:75, 50:50, 75:25) gBlock controls (Fig. 2C). One replicate for the 75:25 gBlock control was not called in the L30575 assay, but the other replicate was successfully classified as 'heterozygous'. After obtaining the third sample (HW90) from Dog 1 at Day 76, all three samples were classified as 'susceptible' at both SNP positions, which corresponded to the Illumina NGS results (Fig. 1), and they clustered amongst the susceptible gBlocks (Fig. 2C).

Discussion

This study utilised an in-vivo 7-day microfilariae suppression test to investigate two cases of asymptomatic canine heartworm disease from Townsville, Australia (Geary et al., 2011). Initially, both cases reportedly received rigorous heartworm prevention for at least 12 months prior to diagnosis. Despite these claims, veterinary records revealed

underdosing in Dog 1, thus highlighting the challenges associated with distinguishing between cases of non-compliance and potential resistance. The microfilariae suppressing testing implemented in this study was adopted from Geary et al. (2011) and met the required criteria, i.e., a normally microfilaricidal dose of ML (25 g/L moxidectin) and a 7-day interval between the pre- and post-ML microfilariae counts. By doing so, we found that both dogs failed to see a marked reduction in their microfilaraemia after seven days, with Dog 1 and Dog 2 displaying <10 % and <40 % reductions, respectively. These reductions were considerably lower than the expected >75 % reduction suggested by Geary et al. (2011). Although it would have been beneficial to conduct additional microfilariae counts in regular intervals for all animals, this was not possible in the study. In particular, Ballesteros et al. (2018) conducted their follow-up microfilariae count 2–4 weeks after dosing.

The reductions observed in our study differed from those reported by Bowman et al. (2015) who tested the microfilaricidal activity of Advantage® Multi for Dogs (10 % imidacloprid + 2.5 % moxidectin) on naturally infected dogs (n=11) exhibiting 550–42,950 microfilaria/mL. Using either an average microfilariae count obtained over 8 days prior to administration, any microfilariae count obtained over 8 days prior to administration, or a single pre-administration count on Day -6, all dogs on Day 7 had reduced their microfilariae counts by >75 % (88–100 %, 76–100 % and 86–100 %, respectively) (Bowman et al., 2015).

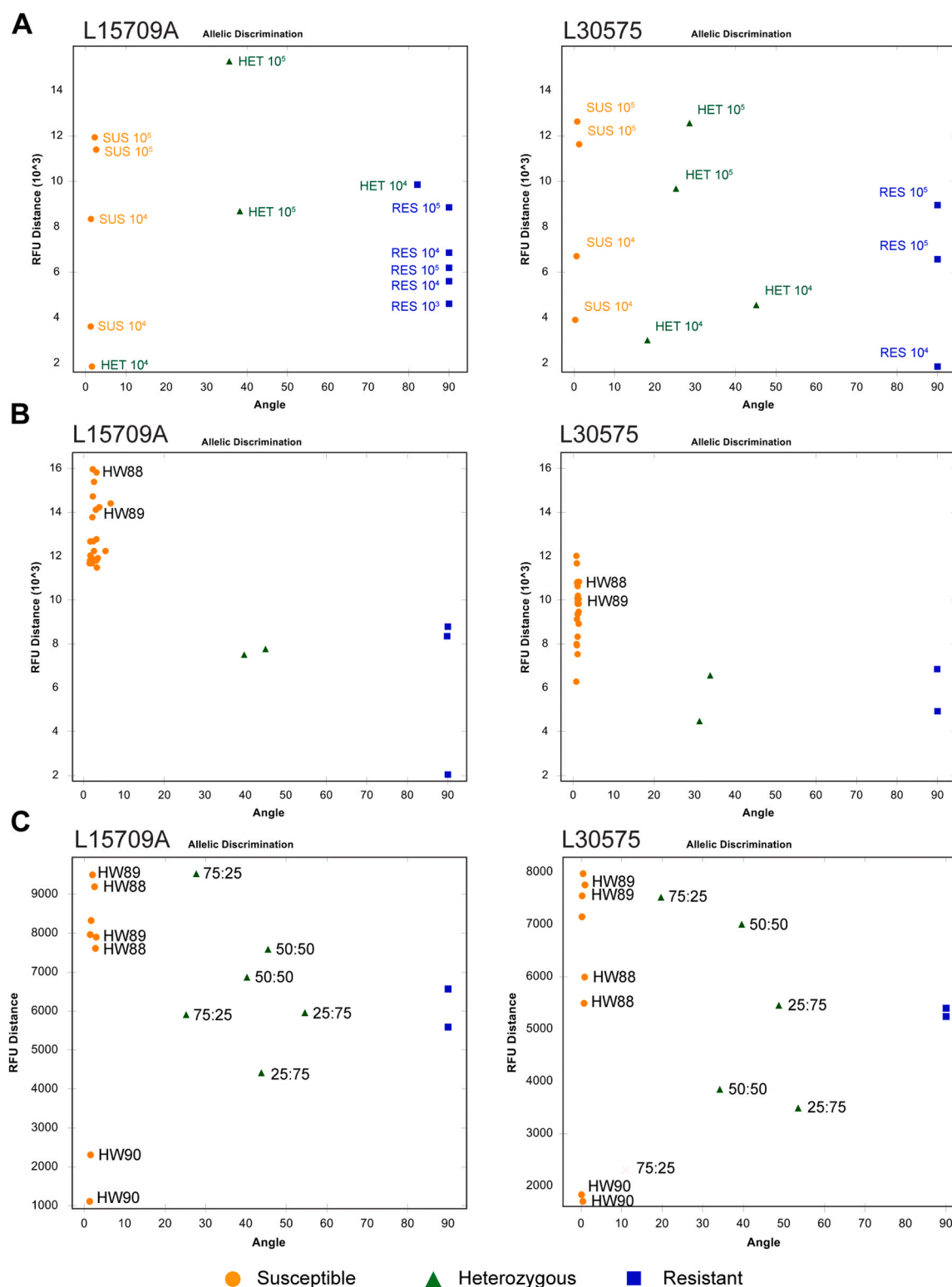


Fig. 2. Validation of a rhAmp™ SNP genotyping assay for L15709 and L30575, which are correlated with macrocyclic lactone- (ML) resistance in the USA. (A) Assay validation using rhAmp™ gBlocks Gene Fragments at concentrations of 10^5 , 10^4 , 10^3 , and 10^2 copies/ μ L. (B) *D. immitis* microfilariae samples obtained from clinical heartworm cases in Australia. Samples from Dog 1 before (HW88) and seven days after (HW89) treatment using Advocate™ for Dogs spot-on moxidectin are included, along with eighteen additional samples from a previous study (Power and Ślapeta, 2022) and rhAmp™ gBlocks Gene Fragments as controls. (C) Inclusion of an additional sample (HW90) from Dog 1 on Day 76. rhAmp™ gBlock Gene Fragments containing SNPs correlated with ML-susceptibility, ML-resistance and heterozygosity (susceptible: resistant — 25:75, 50:50, 75:25) were used as controls.

Although the two dogs in our study did not markedly reduce their microfilaraemia by Day 7, a delayed reduction of >90 % was seen after 70–76 days. Delayed clearance of *D. immitis* microfilariae has been documented historically. One such instance is in Jackson et al. (1986), where 71 % of dogs receiving 50 µg/kg of oral ivermectin cleared their microfilariae within ten days. The remaining dogs required 11 to >30 days to clear their microfilariae, with some dogs requiring multiple doses (Jackson et al., 1986). Thus, in cases where microfilaraemia does not markedly decrease seven days after microfilaricidal treatment, it must be considered whether this is attributable to ML-resistance or if it is a natural response observed in some individuals within the population.

To assess the ML-resistance genotype of the animals in the study, we performed Illumina amplicon next-generation sequencing (NGS) of seven SNPs highly predictive of ML-resistance in *D. immitis* isolates from the USA. Despite phenotypic evidence suggestive of potential ML-resistance, all samples possessed 100 % of the susceptible nucleotide at all seven suspect positions. The delayed clearance of microfilaraemia coupled with a susceptible genotype is echoed in a study from Greece and our previous Australian study. In Diakou et al. (2018), three military dogs from Northern Greece underwent microfilariae suppression testing involving monthly administration of ivermectin (200 µg/kg) for 9 months. Despite frequently observing <75 % reductions in microfilaraemia seven days after ivermectin treatment, MiSeq sequencing of 10 resistance-associated SNPs revealed ML-susceptible profiles for all examined isolates. Similarly, in Power and Šlapeta (2022), 76 % (16/21) of *D. immitis*-infected dogs that underwent a sequential modified Knott's test failed to reduce their microfilaraemia by >90 % after ML treatment, yet remained genotypically susceptible. Despite targeting only seven SNPs in the current study, a larger set of 42 SNPs were originally identified to be correlated to resistance in USA isolates (Bourguinat et al., 2015). Using whole-genome sequencing data, Gandasegui et al., 2024 successfully mapped 40/42 of these SNPs on a new genome assembly of *D. immitis* ("dimmitis_WSI_2.2"). By doing so, discrete clusters of these resistance-associated SNPs were observed (Gandasegui et al., 2024). Two clusters on chromosome 3 appeared to contain the majority of SNPs from the two- and ten-SNP predictive models (Ballesteros et al., 2018; Bourguinat et al., 2015, 2017; Gandasegui et al., 2024). Upon examining the allele frequencies of the *D. immitis* populations, the resistance-associated SNPs were present at a moderate frequency in samples from the USA (Gandasegui et al., 2024). In *D. immitis* populations from other countries (i.e., Australia and Italy), the SNPs were either absent or potentially not predictive of resistance. The latter is corroborated in the current study, as ML treatment failed to markedly reduce microfilaraemia in two canine *D. immitis* infections, but no evidence of genotypic resistance was demonstrated using these SNPs. Since both the SNPs and the microfilariae suppression test were developed based on USA isolates, their suitability for the diagnosis of ML-resistance outside the USA must be investigated.

Rapid and convenient genotyping tools are required to circumvent the long waiting times commonly experienced when outsourcing next-generation sequencing services. In a recent study by Kumar et al. (2023), a droplet digital PCR (ddPCR) technique was developed as a faster and cheaper alternative to the MiSeq platform for detecting ML-resistant *D. immitis*. This ddPCR accurately detected the presence of SNPs at four positions (L15709, L30575, L21554 and L9400) in a range of *D. immitis* isolates from the USA with known or putative phenotypes, including the MO (ML-susceptible) and JYD-34 (ML-resistant) isolates (Kumar et al., 2023). They found that two SNPs, L15709 and L30575, appeared to be better predictors of ML-susceptibility and resistance (Kumar et al., 2023). The rhAmp™ SNP qPCR in our study targeted these same two SNPs, which have been previously described as the top two SNPs associated with ML-resistance in USA *D. immitis* isolates (Ballesteros et al., 2018). Our rhAmp™ SNP qPCR assay clearly identified the SNPs correlated with ML-resistance and ML-susceptibility using rhAmp™ gBlock controls (Fig. 2A). We observed clear separations between heterozygous gBlocks containing different ratios of the

susceptible and resistance-associated SNPs (Fig. 2C). We tested the rhAmp™ SNP qPCR on clinical heartworm samples from Power and Šlapeta (2022), which were previously identified as genotypically susceptible using Illumina amplicon NGS. Using our rhAmp™ SNP qPCR assay, we classified 94 % (17/18) and 100 % (18/18) of the samples as 'susceptible' for L15709_A and L30575, respectively (Supplementary Table 1, Fig. 2B). The congruent results obtained from the rhAmp™ SNP qPCR assay in this study and Illumina amplicon NGS in Power and Šlapeta (2022) demonstrate the feasibility of this rhAmp™ SNP qPCR approach for rapidly genotyping suspect cases of ML-resistant infections. This rhAmp™ SNP qPCR may therefore be a useful rapid diagnostic tool for detecting genotypic resistance in the USA, where the genetic markers L15709_A and L30575 were identified.

Conclusion

This study confirms an unusual phenomenon in some canine heartworm infections, where dogs are failing to see a marked reduction in their microfilaraemia seven days after receiving a microfilaricidal dose of moxidectin (Advocate™ for Dogs, 100 g/L imidacloprid + 25 g/L moxidectin). This phenotype did not correlate to the ML-susceptible genotype that we observed. Since this genotype was determined using SNPs previously associated with resistance in USA isolates, it is possible that these markers are not suitable for predicting resistance in other regions of the world.

CRedit authorship contribution statement

Rosemonde Isabella Power: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jan Šlapeta:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

None of the authors have any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

Data Availability

Data from this study can be accessed via LabArchives (<https://dx.doi.org/10.25833/49c0-f817>). Raw FastQ sequences obtained from Illumina amplicon NGS were deposited at SRA NCBI BioProject: PRJNA1006450.

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Animal ethics

All research conducted in the study has been approved by the Animal Research Ethics Committee at the University of Sydney (Protocol Number: 2020/1746). Animal owners were informed about the research project and a consent form was completed prior to sample collection by a registered veterinarian.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.tvjl.2024.106209](https://doi.org/10.1016/j.tvjl.2024.106209).

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Connecting Statement 2

The microfilarial suppression test (MFST) conducted in Chapter 2.2 uncovered a phenomenon in Australian heartworms, where delayed reductions in microfilaremia were observed after administering normally microfilaricidal doses of macrocyclic lactone (ML). According to the guidelines of the MFST, such delays beyond 7 days would be indicative of phenotypic drug resistance. However, my phenotypic findings were not corroborated by my genotypic testing of resistance-associated markers from the USA. This discrepancy made it difficult to discern whether drug resistance was genuinely present in Australia.

The next chapter (Chapters 3.1 and 3.2) was motivated by the need to better understand the global genetic background of heartworms, particularly to assess the genetic similarities between heartworms from Australia and the USA. This genetic comparison would help determine whether USA-based tools for detecting resistance are applicable in Australia, thereby offering insights into how to best interpret the ambiguous findings in Chapter 2.

Chapter 3.1 is the outcome of several years of collaboration and sample collection. It presents a global population genetics study of heartworms using whole genome sequences from 127 adult worms, including 48 from Australia and 38 from the USA. I examine their genetic diversity, population structure, demography, and phylogeny, while also exploring their global origins and historical dispersal. Notably, Chapter 3.1 provides key findings which directly address the aims of this thesis, particularly in defining the origins and genomic landscape of Australian heartworms and their relationship with those from the USA.

Chapter 3

Understanding the genomic background of
heartworms in Australia and beyond

———— Chapter 3.1 ————

Population genomics reveals ancient origin of heartworms in
canids

Note

Formatting

Chapter 3.1 is an unpublished manuscript and follows the formatting guidelines of Nature Ecology & Evolution.

All figures, extended data figures, and supplementary figures are placed at the end of the manuscript. Supplementary tables in Excel (xlsx) format are available from GitHub: https://github.com/rosemondepower/heartworm_genome/tree/3befc0e95159828224c672c52f904a9d0a55fce8/01_SupplementaryTables.

Authors' contributions

Task	Contributing co-authors
Conceptualisation	RIP, SRD, JŠ
Data collection and curation	RIP, SA, AND, SC, PD, GD, DTB, MTD, JMF, GGV, JLL, MLM, AM, ARM, EP, AR, RS, TS, PT, HSW, SRD, JŠ
Formal analysis	RIP, SRD
Funding acquisition	RIP, JŠ
Investigation	RIP, SRD, JŠ
Methodology	RIP, SRD
Supervision, discussion and consultation	SRD, JŠ
Preparation of figures used in the manuscript	RIP
Writing of the original draft	RIP
Critical review and editing of the manuscript	RIP, GGV, AR, SRD, JŠ

Population genomics reveals ancient origin of heartworms in canids

Rosemonde I. Power^a, Swaid Abdullah^b, Anh Do Ngoc^c, Samuel Candanedo^d, Patrizia Danesi^e, Georgiana Deak^f, Dung Thi Bui^{g,h}, Michael T. Dzimianskiⁱ, Jeremy M. Foster^j, Guilherme G. Verocai^k, Joe L Luksovsky^k, Michelle L. Michalski^l, Andi Mihalca^f, Andrew R. Moorheadⁱ, Elias Papadopoulos^m, Alicia Rojas^{n,o}, Reuben Sharma^p, Tiana Sanders^k, Piyanan Taweethavonsawat^{q,r}, Heather S. Walden^s, Stephen R. Doyle^{t,*} and Jan Šlapeta^{a,u,*}

^a Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales 2006, Australia

^b School of Veterinary Science, The University of Queensland, Gatton, Queensland 4343, Australia

^c Department of Medical Parasitology, Vietnam Military Medical University, Hanoi, Vietnam

^d Department of Veterinary Parasitology, Veterinary Sciences, Healthy Pet Veterinary Hospital SC, David, Chiriquí, Panamá

^e Laboratory of Parasitology, National reference centre/OIE collaborating centre for diseases at the animal-human interface, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy

^f Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Calea Mănăştur 3-5, Cluj-Napoca, 400372, Romania

^g Department of Parasitology, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam

^h Faculty of Ecology and Biological Resources, Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam

ⁱ College of Veterinary Medicine, University of Georgia, Athens, GA, USA

^j New England Biolabs Inc., Ipswich, Massachusetts, 01938, USA

^k Department of Veterinary Pathobiology, School of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, TX, United States

^l University of Wisconsin Oshkosh, Oshkosh, Wisconsin 54902

^m Laboratory of Parasitology and Parasitic Diseases, Faculty of Veterinary Sciences, Aristotle University of Thessaloniki, Thessaloniki, Greece

ⁿ Laboratory of Helminthology, Faculty of Microbiology, University of Costa Rica, San José, Costa Rica

^o Centro de Investigación en Enfermedades Tropicales, University of Costa Rica, San José, Costa Rica

^p Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, UPM, Serdang, Selangor, Malaysia

^q Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

^r Biomarkers in Animals Parasitology Research Unit, Chulalongkorn University, Bangkok 10330, Thailand

^s Department of Comparative, Diagnostic, and Population Medicine, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA.

^t Wellcome Sanger Institute, Cambridgeshire CB10 1SA, United Kingdom

^u The University of Sydney Institute for Infectious Diseases, New South Wales 2006, Australia

* Corresponding author.

E-mail address: sd21@sanger.ac.uk (S. Doyle); jan.slapeta@sydney.edu.au (J. Šlapeta)

Abstract

Heartworms (*Dirofilaria immitis*) are parasitic nematodes that cause significant cardiopulmonary-associated morbidity and mortality in Carnivora species worldwide, with dogs being the most affected. The global dissemination of heartworms is believed to have occurred alongside the dispersal of domesticated dogs; but this theory remains untested. Here we present the largest population genetics study on heartworms to date, with whole genome sequencing of 127 modern adult individuals collected from Carnivora hosts across four continents. Population structure and demographic analyses of the nuclear genome reveal distinct genetic differences between continents, indicating a deeper ancient origin and dispersal in canid hosts than previously recognised. However, this population structure is absent in the mitochondrial genome. Using admixture analyses, we find an Asian origin for Australian heartworms that is consistent with the arrival of dingoes thousands of years ago. Finally, recent divergence of European and Central American heartworms suggests that modern migration occurred with domesticated dogs, likely due to human colonisation of the Americas by Europeans. Overall, our findings shed light on the global population dynamics and evolutionary history of heartworms, which can aid future surveillance and control efforts for this important veterinary parasite.

Introduction

The heartworm *Dirofilaria immitis* is a parasitic nematode responsible for causing life-threatening cardiopulmonary disease in a broad range of *Carnivora* species across tropical, subtropical and temperate regions worldwide¹. Heartworms are transmitted by mosquitoes, with microfilarial stages of the parasite found in the host bloodstream and adult worms residing in the heart and pulmonary arteries². These adult worms can cause lesions, vascular damage, pulmonary hypertension, and potentially right-sided congestive heart failure². Heartworms are predicted to increase in prevalence and expand into new regions due to climate change, pet travel, and habitat alterations³. The emergence of resistance to anthelmintic drugs further exacerbates these issues⁴. Such challenges are not unique to heartworm but are relevant to many vector-borne pathogens. To effectively manage these pathogens in the future, an understanding of their historical origins, dispersal, and current population structure is essential for developing targeted control strategies.

Previous studies suggest heartworms originated in Europe⁵ or Asia² and spread globally via human-dog movements in recent centuries. However, these claims lack historical and genomic evidence. While human-driven animal movements have indeed facilitated the spread of many pathogens^{6,7}, assuming this scenario without sufficient evidence may misguide critical surveillance and control efforts. Such assumptions overlook the deep co-evolutionary history that some pathogens, particularly parasites, have shared with their hosts and environments for millennia. The broad host range of heartworm indicates a long coevolutionary history with *Carnivora* spp., especially canids^{8,9}, whose lineage dates back approximately 40 Mya¹⁰, predating both *Homo* spp. (~2.8 Mya)¹¹ and the domestication of modern dogs (14 – 40 Kya)^{12, 13, 14}. Given that mosquito vectors have existed since the early Triassic (217 Mya)¹⁵, it is possible that heartworms originated and spread during ancient times. We propose that heartworms offer a compelling case study for assessing whether current pathogen distributions were shaped by anthropogenic activities or perhaps possess a much deeper evolutionary history than previously recognised.

Determining the origin of heartworms is challenging due to its complete absence in the fossil record. However, valuable insights can still be obtained by sequencing the genomes of modern specimens collected across a broad geographic range, as demonstrated for other helminths^{7, 16}. This approach can be used to determine whether heartworms were spread (i) relatively recently with dogs, (ii) during ancient times with ancestral *Carnivora*, or (iii) through a combination of both scenarios. To obtain high-quality parasite data, sequencing the genomes of adult stages is recommended¹⁷. However, this approach can be challenging as extracting adults from the heart requires invasive surgery or post-mortem, which are often unfeasible for companion animal and wildlife hosts.

Here, we present the most extensive population genomics study of heartworms to date. Using whole genome sequencing of 127 adult worms obtained from nine countries, we reveal the diversity,

genetic structure, population demographics, and phylogeny of modern heartworms, and infer their historical origins and dispersal. Our analyses support an ancient origin and dispersal scenario, likely shaped by the distribution of ancestral canid hosts throughout time, along with evidence of recent human-driven migration.

Results and Discussion

Intercontinental genome dataset of heartworm

This study presents a global whole genome dataset of 127 adult heartworms (*Dirofilaria immitis*) spanning five broad geographical regions: Australia, the USA, Central America, Europe, and Asia (Fig. 1a). Samples were collected from dogs, cats, foxes, a ferret, a golden jackal, a leopard, and a wildcat. Overall, we obtained an average coverage per sample of 75.47× for the nuclear genome, 13119.80× for the mitochondrial genome, and 1047.09× for the *Wolbachia* endosymbiont genome (Supplementary Table 1, Supplementary Fig. 1–4). Variant calling, joint genotyping, and filtering of heartworm samples revealed 264,558 nuclear single nucleotide polymorphisms (SNPs) (3.3 SNPs/kb), 32 mitochondrial SNPs (2.3 SNPs/kb), and 280 *Wolbachia* SNPs (0.3 SNPs/kb) (Extended Data Fig. 1). The SNP frequencies in the nuclear and mitochondrial genomes are comparable, whereas the frequency in the *Wolbachia* genome is considerably lower. Similar SNP frequencies have been found in other *Wolbachia*-infected filarial worms, such as *Onchocerca volvulus*¹⁸. Despite the global distribution of our heartworm samples, their overall SNP counts are very low compared to other species, such as the livestock nematode *Haemonchus contortus*, for which a global study identified over 23 million SNPs⁷.

Distinct continental partitioning based on variants in the nuclear genome

Nuclear diversity analyses of the heartworm cohort revealed clear groupings of samples based on broad geographical regions. To explore the genetic diversity of the dataset, we first performed principal component analysis (PCA) on 196,550 high-quality SNPs in the autosomal genome. The top two principal components (PCs) (PC1 variance: 12.6%, PC2 variance: 10.69%) revealed four distinct clusters which corresponded to the continents from which the samples were collected (Fig. 1b). A similar pattern of geographical clustering was shown by the third and fourth PCs (PC3 variance: 5.76%, PC4 variance: 4.39%), but the signal was comparatively weaker, and the European and Central American samples formed separate clusters (Extended Data Fig. 2a). To determine whether a specific chromosome was driving the continental grouping, we performed independent PCAs for each chromosome. We found the same continental clustering pattern across all chromosomes, including the sex-linked X chromosome (Extended Data Fig. 2b–f). Similar findings were observed in a previous study of 31 heartworm samples from Australia, Italy, and the USA, where samples from each country formed distinct clusters¹⁹. Most heartworms in the present study were obtained from dogs, but we also included samples from foxes, cats, a golden jackal, a ferret, and a leopard to evaluate genetic variation among different host species. By doing so, we found that geography rather than the host species drove variation in the global heartworm population. This suggests there is no host-specificity, and that

transmission is likely between domestic animals and wildlife (Extended Data Fig. 3a,b). Technical replicates clustered closely with their respective original samples (Extended Data Fig. 3c).

The distinctness of each heartworm population was further highlighted in the linkage disequilibrium (LD) decay plot (Fig. 1c). Each population exhibited different rates of LD decay and baseline LD, with Asia and the USA showing the highest and lowest LD, respectively. These variations in LD between populations may indicate fundamental differences in their population diversity. Estimations of admixture provided further support for continental partitioning in heartworms. To infer the ancestry proportions of samples, we used NGSadmix with a range of K clusters ($K = 2-10$; Extended Data Fig. 4). At $K = 3$ (optimal value), high degrees of shared ancestry were shown between the following pairs: Asia and Australia, Asia and the USA, and Europe and Central America. As the number of K clusters increased, a distinct continental grouping of samples emerged (Fig. 1d), consistent with the findings in our nuclear PCA. The strong population structure in the nuclear genome of heartworms suggests that spatial spread has been restricted during the species' history, resulting in distinct genetic profiles on each continent.

Ancient origin of heartworms with canids

Demographic history analysis of heartworms revealed some population divergences that pre-dated dog domestication. Since continental subpopulations were apparent, we inferred their effective population size histories and split times using SMC++. We found that current effective population sizes are smaller than estimates of past population sizes for all continents except Central America (Fig. 2a). Notably, the first three estimated population divergences occurred with Asia, which split from Central America, the USA, and Europe between ~54 and 86 Kya. Interestingly, these divergences are older than the suggested dates for dog domestication (i.e. ~14 – 40 Kya)^{12, 13, 14}. Together, the population structure and demographic history of our heartworm samples support an 'old' dispersal scenario involving ancient hosts rather than modern dogs.

To hypothesise which ancient host groups may have harboured and dispersed heartworms before the emergence of dogs, we considered the evolutionary history of extant primary hosts. It has been theorised that longer co-evolution between parasites and hosts will likely result in the latter being able to carry higher parasite loads²⁰. Consequently, hosts with greater adult heartworm burdens may indicate a longer period of association. Felids, mustelids, and ursids typically carry fewer adult worms and do not support the complete heartworm life cycle, so they are not considered primary reservoirs^{21, 22, 23, 24}. In contrast, higher worm burdens and microfilaremia are typically found in wild canids such as jackals, foxes, and raccoon dogs²⁵, with wolves and coyotes harbouring the highest numbers of adult worms (Extended Data Fig. 5). These findings imply that canids were most likely the primary hosts for

heartworm during ancient times²⁶. This was further supported by our Bayesian phylogenetic analysis of the mtDNA genome using BEAST2 (Fig. 2b), which revealed that heartworms diverged from other *Dirofilaria* species ~40 Mya during the Eocene, which coincides with the origin of canids in North America^{10, 27}. Given that mosquito vectors were already well-established at this time¹⁵, this correspondence suggests that heartworms and canids may have diverged together millions of years ago and shared a long co-evolutionary history.

Heartworm could be a relic of the past, analogous to ‘living fossils’ like the Wollemi Pine and the coelacanth. The Wollemi Pine, an ancient tree from the age of the dinosaurs, was recently discovered in an undisturbed canyon in Australia²⁸. Similarly, the coelacanth fish has persisted in deep-sea environments for millennia, largely due to a static habitat that reduced the need for adaptation²⁹. Comparably, heartworm has evolved to exploit a unique niche in the heart and bloodstream of canids. These stable environments have likely remained unchanged for millennia, allowing heartworm to persist to the present day without the need for significant adaptation. This study provides compelling evidence that parasites can be ‘frozen in time’, particularly those which lack a free-living stage and have relied entirely on these stable host environments for survival.

Ancient connectivity and fragmentation of heartworm populations

After canids emerged in North America, they migrated across the Bering Land Bridge and the Isthmus of Panama into Eurasia and South America, respectively, during the Miocene (~11 Mya)³⁰. Fossil records and genomic data provide robust evidence that the repeated exposure of the Bering Land Bridge throughout history enabled some ancient canids to form a panmictic population across the northern hemisphere. This was demonstrated in Pleistocene wolves, which were highly connected across North America and Eurasia^{12, 31, 32}. Additional evidence exists for the transcontinental distribution of other ancient canids. For example, *Xenocyon lycaonoides* was a large canid distributed across North America and Eurasia in the medial Pleistocene³³, and *Eucyon* was a jackal-sized canid distributed across North America, Eurasia, and Africa in the late Miocene³⁴. The existence of these interconnected ancient canid populations suggests that heartworms may have also formed a highly connected population with their ancient canid hosts. We estimated the timing of this panmictic *D. immitis* population to be >86 Kya, which is before the species diverged on each continent (Fig. 2a). This roughly aligns with the last interglacial period (116 – 130 Kya), where Earth experienced a climate as warm or even warmer than today³⁵. Such an environment could have been suitable for the development and transmission of ancestral populations of heartworm via mosquito vectors.

We hypothesise that the initial panmictic heartworm population was later fragmented by geographic barriers or bottlenecks driven by the ancient canid hosts. Following the warm climates of

the last interglacial period, the Earth began to cool. By Marine Isotope Stage 4 (58-72 Kya), large continental ice sheets had formed across North America, Beringia, and northern Eurasia³⁶. These ice sheets separated ancient canid populations^{31, 37}, potentially fragmenting the associated ancient heartworm population as well. This fragmentation may have created isolated subpopulations of heartworms with limited gene flow for extended periods (as visualised in Fig. 3), which would explain the continental grouping of samples in our study. Another potential scenario is that the once-panmictic heartworm population diverged in parallel with the speciation of canids. Canid groups had geographical centres of diversification, such as North America for wolves, Africa for foxes, and South America for South American canids³⁰. It is possible that heartworm subpopulations formed within different canid groups as they continued to diverge and radiate globally. However, this scenario may be less plausible as it would likely result in host-specificity in heartworms, which we did not observe in our data. A major limitation in deciphering the true evolutionary history of canine heartworm is its complete absence in the fossil record. Since this parasite is typically found in tropical, subtropical, and temperate climates, it's likely that they were not preserved, or we have not yet excavated their fossils.

Following the potential fragmentation of heartworm populations, we found evidence of ancient migration between Asia and the USA. NGSadmix using few clusters ($K = 2-4$) showed large proportions of admixture between the two continents. We used TreeMix to estimate relationships between heartworm subpopulations and infer admixture events. One migration edge was supported ($\Delta m = 3.45$), which confirmed gene flow between Asia and the USA (Fig. 4a). All tree migration edges and their residual heatmaps are presented in Extended Data Fig. 6. *D*-statistics provided further support for excessive allele sharing between the USA and Asia relative to that between the USA and other regions (Fig. 4b). Given that heartworms from the USA and Asia diverged >60 Kya (Fig. 2a), this admixture may reflect multiple waves of migration with canids across the Bering Land Bridge during the Late Pleistocene, during intervals when ice sheets were absent¹².

Modern migration between Europe and Central America

Our genomic analysis of a global heartworm dataset revealed a close genetic relationship between worms in Europe and Central America. This finding was most evident in our PCA of nuclear SNPs, where samples from Greece, Italy, and Romania clustered with those from Costa Rica and Panama (Fig. 1b). Admixture analysis using NGSadmix further supported this trend as the European and Central American samples shared high levels of ancestry at lower *K* values ($K = 2, 3$, and 5 ; Extended Data Fig. 4). Although increasing the number of *K*s helped differentiate worms from these two continents, low levels of admixture were still present (Fig. 1d). These findings were corroborated using *D*-statistics which provided strong evidence of excessive allele sharing between Central America and Europe relative to that between Central America and other regions (Fig. 4c). We used SMC++ to

explore the joint demographic history of these two populations. By doing so, we found that Central American heartworms experienced a dramatic increase in their effective population size between 150 and 200 years ago and had the most recent divergence estimate with Europe out of all population pairs (Fig. 2a). These findings support a modern migration event between European and Central American heartworms which took place after dog domestication.

One plausible example of a modern migration event between Europe and Central America was the transatlantic transportation of domestic animals, including dogs, during the European colonisation of the Americas beginning in the late 15th century. Animals introduced to the Americas during the Columbus voyages were primarily livestock species, including cattle, sheep, pigs, goats, horses, and mules, but dogs such as greyhounds and mastiffs were also included for hunting, guarding, protection, shepherding, and battle purposes^{38, 39, 40}. Historical records indicate the presence of at least 20 dogs on board during Columbus' second voyage to Hispaniola³⁸. Therefore, infected dogs from Europe may have transported local strains of this parasite to the Americas. Alternatively, the shared ancestry between European and Central American heartworms could result from modern migration and tourism by humans and their infected pets. Considering the dates proposed in our demographic analyses, we posit that the first scenario is more biologically plausible and likely occurred over the past few centuries.

The origin and spread of heartworm in Australia

Heartworms are endemic in Australia, but the mechanisms and timing of their arrival in the country have remained an enigma. Our study includes extensive sampling along the East Coast of Australia (Fig. 5a) and previously unsampled regions of the world, such as southeast Asia. Admixture analysis using NGSadmix revealed a highly diverse Australian cohort at $K = 6$ (Fig. 1d), with substantial levels of admixture with heartworms from Asia and low levels of admixture with Central America (which we have concluded has a European origin, see above). The genetic similarity between the Australian and Asian worms was further emphasised using TreeMix, as samples from these regions formed a monophyletic branch (Fig. 4a). *D*-statistics solidified this relationship by showing excessive allele sharing between the two continents relative to that between Australia and other regions (Fig. 5b). These findings point towards an Asian origin of Australian heartworms rather than an introduction through European colonisation ~235 years ago.

Given the evidence for an Asian origin, the most likely scenario is that heartworms were brought to Australia via dingoes. Dingoes are an ancient dog breed from Asia and were the only canids present on the Australian continent until European settlement. Recent morphometric analysis indicates an East Asian origin of dingoes via Melanesia⁴¹, whereas genetic studies suggest that dingoes likely came from Island Southeast Asia and were brought to Australia by humans between 5 and 12 Kya⁴².

This date range is slightly later than our divergence estimate for Asian and Australian heartworms (~29 Kya). This discrepancy likely arises from the limited geographic scope of our Asian samples, which are only from Thailand and Peninsular Malaysia. Sampling across a broader geographic region, particularly throughout the Indonesian archipelago and Melanesia, could bridge the gap between our Australian and Asian samples. Such data may provide further granularity of the Asia-Australia divergence and potentially coincide more closely with the timeline of dingo arrival. Overall, our results reveal an Asian origin for Australian heartworms and demonstrate the extensive pre-colonial history of heartworms in Australia.

Australian heartworms exhibited the most admixed ancestry profiles out of all sampled populations. NGSadmix demonstrated that heartworms from the state of Queensland were highly admixed, with two sources of ancestry not shared with any other continents (Fig. 1d). Interestingly, 12 out of 16 Sydney heartworms had a single fixed ancestry that was detected in Queensland samples. The remaining four Sydney heartworms had another fixed ancestry, which was also present in Queensland and Asia. Previously, it was believed that heartworm cases in temperate Sydney and broader New South Wales were primarily caused by infected pets or mosquitoes travelling from tropical and subtropical Queensland⁴³, where climates are more favourable to heartworm transmission. However, our nuclear PCA of Australian heartworms showed a large cluster of samples primarily from Queensland, alongside two distinct clusters of Sydney heartworms (Fig. 5c). Collectively, these results suggest that Sydney heartworms may not necessarily originate from Queensland. Further investigations are required to determine whether there are local endemic populations of heartworms in Sydney.

Lack of population structure in mitochondrial and *Wolbachia* genomes

The distinct geographical partitioning in our nuclear data was not corroborated in the mtDNA (Fig. 6a) or *Wolbachia* data (Fig. 6b). Assembly and alignment of whole mitochondrial genomes of the heartworms revealed only 195 variable sites (Supplementary Fig 5a–c), with BEAST2 (Fig. 2b) and IQ-TREE (Extended Data Fig. 7) analyses showing low genetic diversity and limited phylogenetic structure. A median-joining network provided further granularity and grouped most samples by geographic region, but some samples clustered with those from other continents (Fig. 6c). Minimum spanning, TCS, and SplitsTree networks produced similar outcomes.

We propose that the unusually low mitochondrial diversity relative to that of the nuclear genome is due to an endosymbiont-driven selective sweep-like phenomenon in heartworms, analogous to those in insect systems. Maternally-inherited *Wolbachia* symbionts infect over 65% of all insect species and often exhibit reproductive manipulations to enhance their vertical transmission in a population^{44, 45, 46}. Linkage disequilibrium between *Wolbachia* and host mtDNA commonly causes the

initially infected mtDNA type to hitchhike through the population, resulting in unexpectedly low mtDNA diversity⁴⁷. These *Wolbachia*-induced selective sweeps have been observed in several insect populations^{48, 49, 50, 51}. Filarial nematodes share an obligatory mutualistic relationship with *Wolbachia* as they often depend on the endosymbiont for survival and reproduction⁵². Although the *Wolbachia*-mtDNA relationship in filarial nematodes remains poorly understood compared to insect systems, *Wolbachia* has been previously suggested to depress mtDNA diversity in heartworms⁵³. The stark contrast between our nuclear and mitochondrial data underscores the potential for endosymbionts to confound mtDNA evolutionary analyses of filarial worms⁴⁷. Alternative explanations include sampling bias and the possibility that mtDNA reflects more recent animal movements, which are not apparent in the nuclear genome.

Concluding remarks

In conclusion, our transcontinental genome-wide analysis of heartworm parasites revealed a much deeper origin and dispersal history than previously documented, with ancestral canid hosts playing a key role. In the context of future climate change, emerging drug resistance, and increased travel, understanding the global genomic landscape of these parasites is vital for developing effective surveillance and control strategies. Our data indicates that such surveillance and control strategies should account for geographical variation between parasite populations, as heartworms appear to be genetically distinct on each continent. Further sampling in unstudied regions like South America and Africa, combined with finer sampling within continents such as Asia, will enhance our understanding of the evolutionary history and population structure of heartworms.

Methods

Sample collection

A total of 120 individual adult *Dirofilaria immitis* were obtained from Australia ($n = 43$), the USA ($n = 37$), Panama ($n = 14$), Greece ($n = 12$), Thailand ($n = 6$), Italy ($n = 3$), Romania ($n = 3$), Costa Rica ($n = 1$), and Malaysia ($n = 1$). The sampling regime aimed to collect adult *D. immitis* from a wide geographical range, encompassing as many regions around the world as possible. Most of these samples were obtained from dogs (*Canis lupus familiaris*; $n = 115$) but also included some from cats (*Felis catus*; $n = 4$), foxes (*Vulpes vulpes*; $n = 4$), a ferret (*Mustela putorius furo*; $n = 1$), a golden jackal (*Canis aureus*; $n = 1$), a leopard (*Panthera pardus*; $n = 1$), and a wildcat (*Felis silvestris*; $n = 1$) (metadata for all samples is presented in Supplementary Table 2). We included five technical replicates (Brisbane: $n = 1$; Sydney: $n = 2$; Malaysia: $n = 1$; Panama: $n = 1$). Adult *D. repens* ($n = 2$) from dogs in Italy, *Dirofilaria* sp. ‘Thailand II’ ($n = 2$) from humans (*Homo sapiens*) in Vietnam and *D. ursi* ($n = 1$) from an American black bear (*Ursus americanus*) in the USA were collected as outgroup samples.

DNA extraction and whole genome sequencing

Dirofilaria specimens were frozen at -20°C or stored in ethanol upon collection and sent to the University of Sydney, Australia. A 1-2 cm segment between the first and second third of the anterior portion of the worms was excised using a sterile scalpel blade and placed in a 1.5 mL Eppendorf tube.

Ethanol-preserved samples. Worm segments were centrifuged at maximum speed for 30 s using a benchtop centrifuge (Eppendorf, Australia) and excess ethanol was removed. The segments were washed in 500 mL of phosphate-buffered saline (PBS) and placed on a heat block (Heat Block Eppendorf Thermomixer Comfort or Heat Block Major Science Dry Bath Incubator) at 60°C for 20-40 min, depending on their thickness, to evaporate any remaining ethanol.

Frozen samples. Worm segments were defrosted and washed three times in PBS.

Genomic DNA was extracted from the dried tissue segments using a Monarch[®] Genomic DNA Purification Kit (New England Biolabs, Australia) as per the manufacturer's protocol. Extracted DNA was shipped to Novogene (HK) Co., Ltd for indexing, library preparation, and whole genome sequencing using the NovaSeq6000 platform. Overall, 128/130 and 2/130 DNA samples were sequenced at expected depths of $\sim 10\text{G}$ and $\sim 1\text{G}$, respectively, depending on sample quality.

Publicly available data

Two publicly available datasets of individual *D. immitis* were included: (i) five adults from Sydney, Australia (SRA NCBI: SRR13154013-7)⁵⁴; (ii) one adult from Georgia, USA (ENA Accession: ERR034940) and one adult from Pavia, Italy (ENA Accession: ERR034941)⁵⁵.

Raw data processing and mapping

Multiple FastQ files were generated for some samples and merged before data analysis. The quality of all raw paired-end sequencing reads was inspected using FastQC (version 0.11.8; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and the output was summarised using MultiQC⁵⁶ (version 1.17). The raw paired-end reads were scanned with a 10-base sliding window and trimmed to have a minimum average quality score of 20 per base and a minimum read length of 50 bp using Trimmomatic⁵⁷ (version 0.39). We used a Nextflow⁵⁸ mapping pipeline (mapping-helminth/v1.0.9)⁵⁹ to map the trimmed paired-end reads to a combined reference genome containing *D. immitis* (dimmitis_WSI_2.2¹⁹) and the domestic dog (GenBank accession: GCA_014441545). The dimmitis_WSI_2.2 genome has been previously supplemented with the *D. immitis* mitochondrial and *D. immitis*-associated *Wolbachia* endosymbiont genomes¹⁹. The mapping pipeline was designed for the standard mapping of helminth genomes using Minimap2⁶⁰ (version 2.17) as a mapper and GATK⁶¹ (version 4.1.4.1), Samtools⁶² (version 1.14), and Sambamba⁶³ (version 1.0) for further data processing. Reads aligning to only the *D. immitis* genome were extracted for downstream analysis using Samtools.

Sequencing Coverage

Sequencing coverage was examined using Bamtools⁶⁴ (version 2.5.1), Bedtools makewindows⁶⁵ (version 2.31.0), and Samtools bedcov. Coverage statistics were obtained for the nuclear, mitochondrial, and *Wolbachia* genomes of *D. immitis*. To visualise nuclear *D. immitis* sequencing coverage for each sample, the scaffold, mitochondrial and *Wolbachia* genomes of *D. immitis* were removed, along with any chromosomes of the domestic dog genome. The ratio of X chromosome to autosome coverage was visualised to determine the sex of the *D. immitis* samples, identified by a drop in coverage along the X chromosome (ratio ~ 0.5) indicative of a male parasite¹⁹.

Variant calling

Variants were identified using GATK⁶¹ (version 4.1.4.1) HaplotypeCaller, CombineGVCFs, and GenotypeGVCFs. GVCF files were first produced for each sample and then combined into a cohort-

level GVCf file, which was then used for joint genotyping. To optimise this process, separate jobs for each sample and scaffold were executed in parallel, with the results subsequently merged to produce the final cohort VCF file. Outgroup samples were removed to obtain a *D. immitis* cohort VCF file. Variants in the nuclear, mitochondrial, and *Wolbachia* datasets were filtered separately using GATK SelectVariants, GATK VariantFiltration, and VCFtools⁶⁶ (version 1.16). The distribution of the following quality metrics was established (QUAL, DP, MQ, SOR, QD, FS, MQRankSum, and ReadPosRankSum), and the relevant upper and/or lower 1% distribution tails were removed. The single nucleotide variants (SNVs) were further filtered based on the following parameters: bi-allelic, depth ≥ 3 , Hardy-Weinberg Equilibrium p-value $\geq 1e-06$ (nuclear), and minor allele frequency ≥ 0.02 . Indels were not included in subsequent analyses. Samples with $<50\%$ of the total variants were removed, and an optimal threshold for missing data of 0.9 was established (nuclear, mitochondrial, and *Wolbachia*). Additional VCF files were generated from the filtered nuclear data for autosomes (chr1-4), the sex-linked X chromosome, and Australian samples. The same filtering parameters were applied to the VCF file containing both *D. immitis* and outgroup samples.

Population structure

To explore the broad-scale genetic relatedness between *D. immitis* individuals and populations, we performed principal component analysis (PCA) using the R package SNPRelate⁶⁷ (version 1.38.0). PCA was conducted for the nuclear (autosomes together, autosomes separate, and chromosome X separate), mitochondrial, and *Wolbachia* data.

Linkage disequilibrium (LD) statistics were obtained from the nuclear data using VCFtools. First, we filtered and thinned the data (--maf 0.02 --thin 1000 --max-missing 1). We then calculated the squared correlation coefficient between genotypes (--ld-window-bp 1000000 --max-alleles 2 --min-alleles 2 --min-r2 0.1 --geno-r2). To visualise patterns of LD decay, the output was plotted per 0.1 kb for each population.

Admixture

The global ancestry and admixture proportions of *D. immitis* were inferred using NGSadmix⁶⁸ from the ANGSD package. First, genotype likelihoods were extracted from the filtered autosomal *D. immitis* VCF file using VCFtools (--max-missing 1 --BEAGLE-PL). Genotype likelihoods were extracted separately for each chromosome and merged into a single dataset. NGSadmix (-minMaf 0.02 -misTol 0.9) was performed using multiple combinations of different clusters ($K = 2$ to 10) and seeds (1 to 5).

We used TreeMix⁶⁹ (version 1.12) to produce a maximum likelihood tree and infer population splits and admixture events in the *D. immitis* dataset. To generate the input data, VCFtools was used to filter the cohort SNV VCF file (--max-missing 1), retaining only autosomal data and the same samples as in the previous variant calling filtration step. The variants were pruned for linkage disequilibrium (10 kb windows, shifting by 10 kb, LD threshold 0.1), and the VCF file was converted to the appropriate format using custom scripts ('ldPruning.sh' and 'vcf2treemix.sh') obtained from <https://github.com/speciationgenomics/scripts>. TreeMix was run across a range of migration edges (0 to 5) and seeds (0 to 10), with *D. ursi* included as an outgroup. The optimal number of migration edges was estimated using the R package OptM (version 1.8; <https://github.com/cran/OptM>).

To further investigate admixture in the cohort, *D*-statistics were inferred using AdmixTools⁷⁰ (version 5.1). First, we converted our filtered nuclear VCF file into the required eigenstrat format using a custom script ('convertVCFtoEigenstrat.sh') obtained from <https://github.com/speciationgenomics/scripts/>. Additional tools required for file conversion were VCFtools and PLINK⁷¹ (version 1.90b6.18). qpDstat was used to perform *D*-statistic testing for all population pairs relative to the other populations (PopX), with *D. ursi* as the outgroup taxa. *Z*-scores with an absolute value >3 were considered significant.

Population demography

We estimated the population size history of *D. immitis* and divergence times between subpopulations using SMC++⁷² (version 1.15.3). Samples in the filtered autosomal VCF were grouped by continent (*i.e.*, Asia, Australia, Central America, Europe, and USA) and analysed separately before being plotted together. First, the VCF file was converted to the SMC++ input format using *vcf2smc*. The model was fitted using *estimate* with a timepoint range of 1 to 5,000,000 and the nematode *Caenorhabditis elegans* mutation rate of 2.7e-9 per site per generation. The *plot* command (-g -c) was used to visualise the output, with the -g parameter specifying the generation time of the target population. *D. immitis* takes ~1 year to produce offspring and can live for up to 7 years in the host's cardiopulmonary system. The development and transmission of this parasite are also highly dependent on local climate and vector availability. To account for potential variability in the generation time of *D. immitis*, we tested and compared multiple generation times (-g 1, 2, 4, or 6) (Extended Data Fig. 8) and determined that a 4-year interval was the most appropriate estimate. In addition, we used *split* to estimate divergence times between all pairs of subpopulations.

Mitochondrial DNA phylogeny

The whole mitochondrial genomes of all samples were assembled from the raw FastQ data using GetOrganelle⁷³ (version 1.7.7.1). Specifically, the ‘get_organelle_from_reads’ pipeline was implemented to assemble the mitochondrial genomes from genomic skimming data (-F animal_mt, -R 20, --reduce-reads-for-coverage 80, 120, or 500). The complete mitogenomes were aligned in CLC Main Workbench 23 (CLC bio, Qiagen, Clayton, Australia) with publicly available complete mtDNA sequences for *D. immitis* (GenBank accession NC_005305⁷⁴, mDi_Athens_2.1 and mDi_Pavia_2.1⁵⁵), *D. repens* (GenBank accessions KR071802, KX265047–KX265049⁷⁵), *Candidatus* *Dirofilaria hongkongensis* (GenBank accession KX265050⁷⁵), *Dirofilaria* sp. “Thailand II” (GenBank accessions MH823370–MH823372⁷⁶), *O. volvulus* (GenBank accessions AF015193⁷⁷, AP017695, KT599912⁷⁸), *O. ochengi* (GenBank accessions AP017693–AP017694, KX181289–KX181290), and *O. flexuosa* (GenBank accessions AP017692, HQ214004⁷⁹). All mtDNA sequences were circularised and manually edited to begin at the cytochrome c oxidase subunit 1 (*COXI*) start codon before alignment. Separate alignments were created for each of the 12 coding regions, and samples with substantial gaps were excluded. The number of variable sites and the average pairwise genetic distances were calculated per gene for the *D. immitis* sequences using MEGA11⁸⁰.

We performed phylogenomic inference by maximum likelihood (ML) for the complete mtDNA and *COXI* alignments using IQ-TREE⁸¹ (version 1.6.12). A total of 155 sequences were included for analysis, with the complete mtDNA and *COXI* datasets consisting of 14,066 and 1,647 positions, respectively. The ModelFinder⁸² option of IQ-TREE was used to select the best-fitting substitution models according to the Bayesian information criterion (BIC). The best model for the complete mtDNA alignment was GTR+F+R4, with a BIC score of 96915.657 (-LnL = 47077.635). For the *COXI* alignment, the best model was TIM3+F+I, with a BIC score of 10043.191 (-LnL = 4754.954). The ultrafast bootstrap approximation (UFBoot)⁸³ option in IQ-TREE was used to estimate branch supports (-bb 1000). The ML trees were visualised using FigTree (version 1.4.4; available at: <http://tree.bio.ed.ac.uk/software/figtree/>).

To improve the resolution of our mtDNA phylogeny, we first used PopART⁸⁴ (version 1.7) to compute median-joining, minimum spanning⁸⁵, and TCS⁸⁶ networks (default parameters) from the complete mtDNA alignment of our *D. immitis* sequences (n = 122) with *D. ursi* as an outgroup. We then analysed only the SNPs (2,234 sites) using SplitsTree⁸⁷ (version 6.3.32). The P Distance⁸⁸ method (default options) was applied to generate a 123 x 123 editDistance matrix, and the Neighbor Net⁸⁹ method (default options) was used to obtain 210 splits (cyclic). The Show Splits method (default options) was used to generate a Split Network visualisation.

We generated a dated phylogenetic tree using Bayesian Evolutionary Analysis by Sampling Trees (BEAST)⁹⁰ (version 2.7.7). This was based on our complete mtDNA alignment of *D. immitis* and

all *Dirofilaria* and *Onchocerca* outgroups. First, we created an ‘.xml’ file using the corresponding Bayesian Evolutionary Analysis Utility (BEAUti) (version 2.7.7) software. To infer the tree likelihood site model, we used the bModelTest⁹¹ (version 1.3.3) package. We implemented a strict clock model and Yule model, running the Markov chain Monte Carlo (MCMC) for 50,000,000 generations with a trace log every 50,000 generations. The mitochondrial mutation rate of *C. elegans*⁹² was adopted and adjusted to reflect a *D. immitis* generation time of one year by slowing the rate down by 52×, resulting in a mutation rate of 3.07E-9 ($\pm$ 5.96E-10). Tree priors were verified using Tracer⁹³ (version 1.7.2), with effective sample size (ESS) values >200 considered acceptable. A maximum clade credibility tree was generated using TreeAnnotator (version 2.7.7) with 10% burn-in. The dated phylogenetic tree was visualised in FigTree.

The general workflow for this study is summarised in Extended Data Fig. 9.

Data availability

Raw FastQ sequences generated in this study will be made available at SRA NCBI BioProject PRJNA1104412 once this manuscript is submitted for publication.

Code availability

The code used to analyse the data in this study is available from GitHub: https://github.com/rosemondepower/heartworm_genome.git.

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Figures

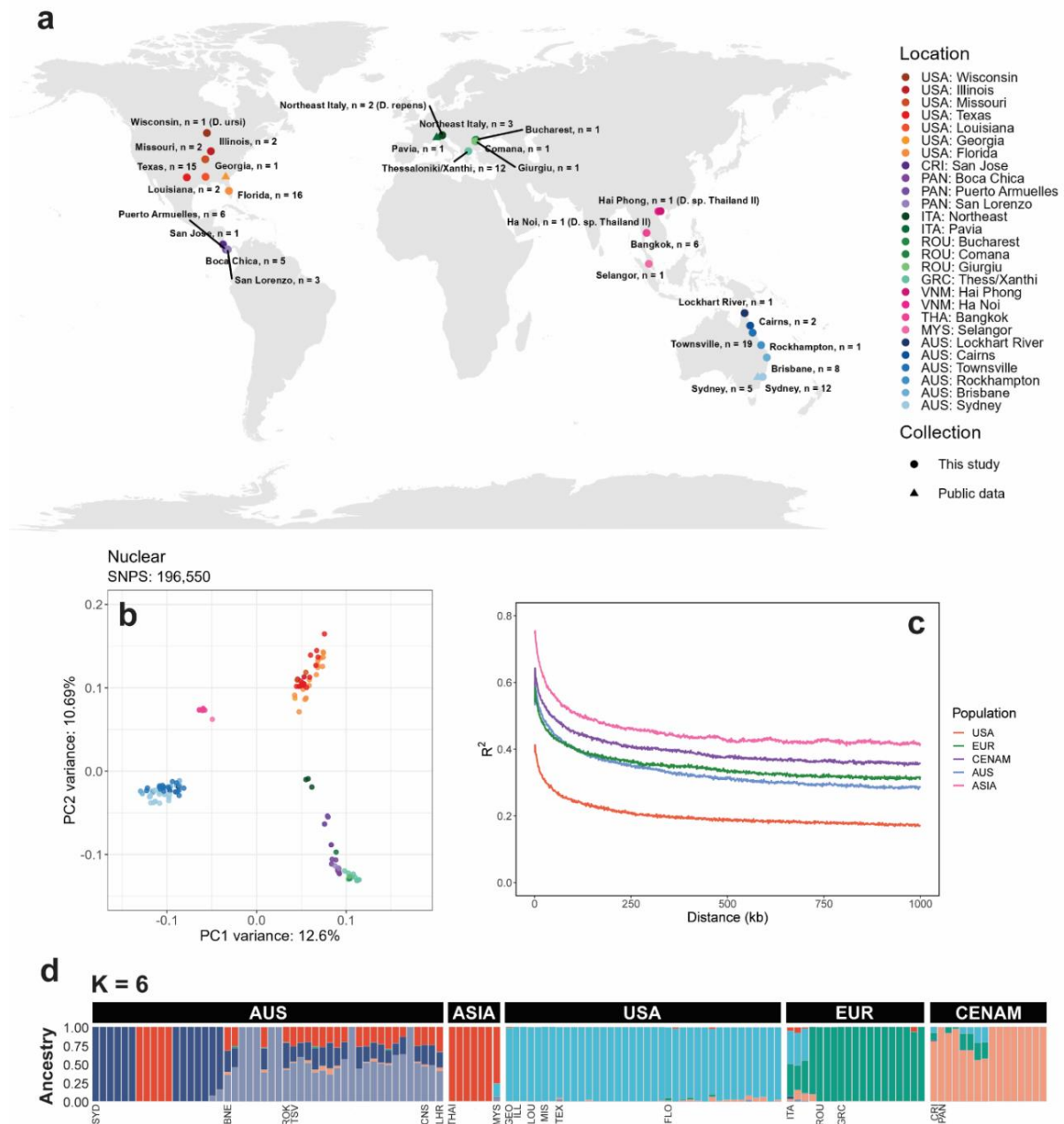


Fig. 1: Global sampling map and population structure of heartworms. **a**, World map showing the location of adult heartworm specimens used in the study. Adult *Dirofilaria ursi*, *D. repens*, and *D. sp.* ‘Thailand II’ were included as outgroup samples. Country abbreviations: USA = United States of America; CRI = Costa Rica; PAN = Panama; ITA = Italy; ROU = Romania; GRC = Greece; VNM = Vietnam; THA = Thailand; MYS = Malaysia; AUS = Australia. **b**, Principal component analysis (PCA) of 196,550 nuclear single nucleotide polymorphisms (SNPs) from 124 heartworms. Replicate samples were included in the analysis. Principal component 1 (PC1) and principal component 2 (PC2) explained 12.6% and 10.69% of the variation, respectively. **c**, Linkage disequilibrium (LD) decay plot for the heartworm cohort, grouped by broad geographic region. **d**, Admixture inferred using NGSadmix with 6 clusters (K). Each vertical bar represents an individual sample, with colours representing the clusters. Labels at the top of the bar charts indicate the broad geographic origins of samples, while those at the bottom show their more specific locations.

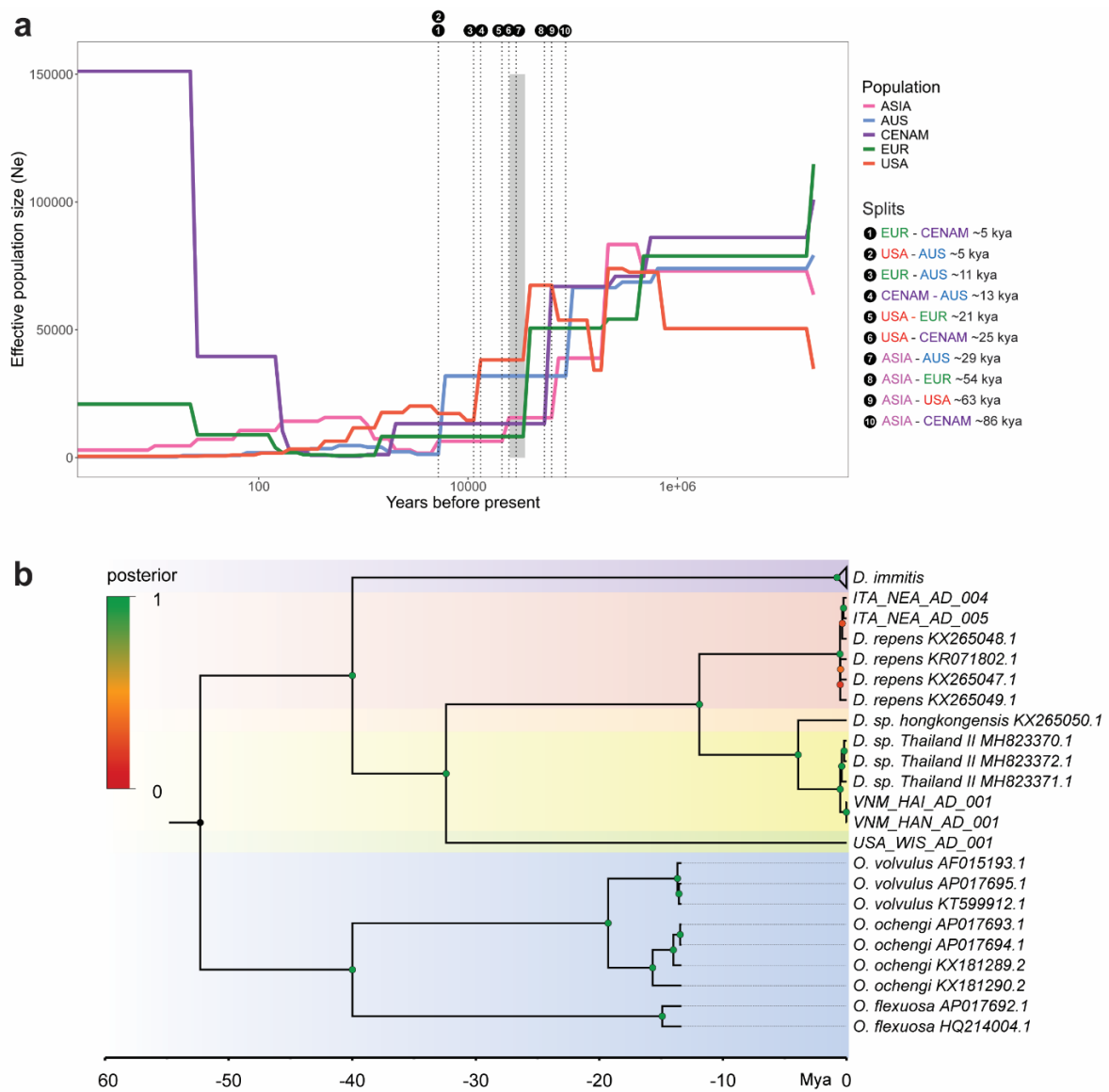


Fig. 2: Demographic history of heartworms and phylogenetic relationships among Onchocercidae. a, Size history of heartworm populations inferred using SMC++ based on a four-year generation time. Each line represents the effective population size of heartworms on each continent over time. The grey box indicates previously suggested date ranges for dog domestication (~14 – 40 Kya). Vertical dotted lines and associated numbers represent divergence times between populations. Details of these population splits and their timing are provided on the right. Abbreviations: AUS = Australia; CENAM = Central America; EUR = Europe; USA = United States of America. **b,** Bayesian inference phylogenetic tree of 155 sequences (14,066 positions) constructed using BEAST2. The tree includes the heartworms and outgroups (ITA_NEA_AD_004, ITA_NEA_AD_005, VNM_HAI_AD_001, VNM_HAN_AD_001, USA_WIS_AD_001) from this study and other published mitochondrial genomes of *Dirofilaria* and *Onchocerca* species. Heartworm samples formed a clade with short branch lengths, which were collapsed for better visualisation. The BEAST2 run used strict clock and Yule models, with a *Caenorhabditis elegans* mutation rate⁹² adjusted to reflect a one-year heartworm generation time ($3.07\text{E-}9 \pm 5.96\text{E-}10$). Posterior probability values for each node are indicated by the colour of the circles, with a legend in the top left corner. Background colours are used to group samples by species within *Dirofilaria* and by genus for *Onchocerca*.

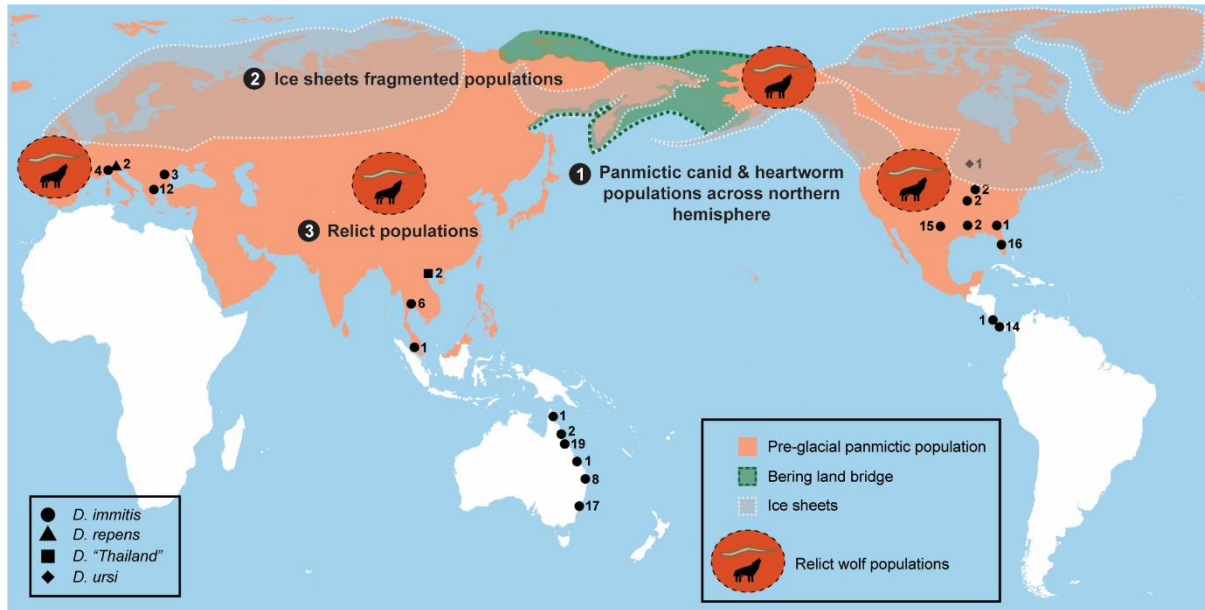


Fig. 3: Ancient dispersal scenario for heartworms. Hypothetical scenario describing an ancient global dispersal of heartworms occurring before dog domestication. Pink regions represent a panmictic population of ancient canids harbouring heartworms across North America and Eurasia. These continents were connected by the periodic exposure of the Bering Land Bridge (green) during the Pleistocene. The formation of large continental ice sheets (grey polygons) in the northern hemisphere subsequently fragmented this panmictic population, resulting in relict populations of canids and their heartworms in various regions of the world. Small black shapes with adjacent numbers indicate the geographic origin of samples collected in this study and their sample size. The legend in the bottom left corner indicates the parasite species, including the heartworm *Dirofilaria immitis* ($n = 127$) and outgroups *D. repens* ($n = 2$), *D. sp. "Thailand II"* ($n = 2$), and *D. ursi* ($n = 1$).

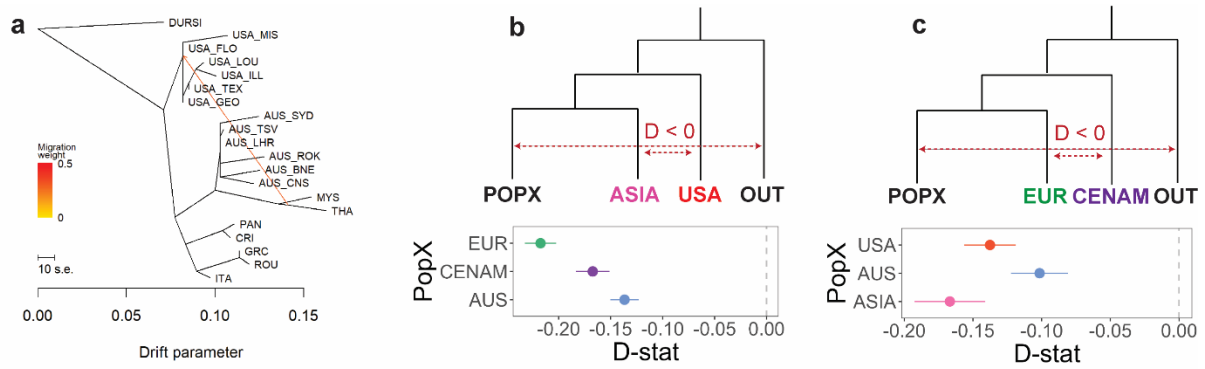


Fig. 4: Admixture events in a global heartworm cohort. **a**, Maximum likelihood tree of heartworm generated using TreeMix, with *Dirofilaria ursi* as an outgroup and one migration edge. **b–c**, D -statistics for hypothetical allele sharing scenarios between heartworm populations. Calculations were performed using Admixtools qpDstat with 21,088 single nucleotide polymorphisms (SNPs) and *D. ursi* as the outgroup (OUT). All z -scores in the figure were significant (>3 or <-3). Red dotted lines along hypothetical trees show the direction of gene flow, while vertical grey dotted lines in the plots indicate where $D = 0$. **b** shows excessive allele sharing between USA and ASIA relative to that between USA and other regions (PopX), and **c** shows excessive allele sharing between Central America (CENAM) and Europe (EUR) relative to that between CENAM and other regions (PopX).

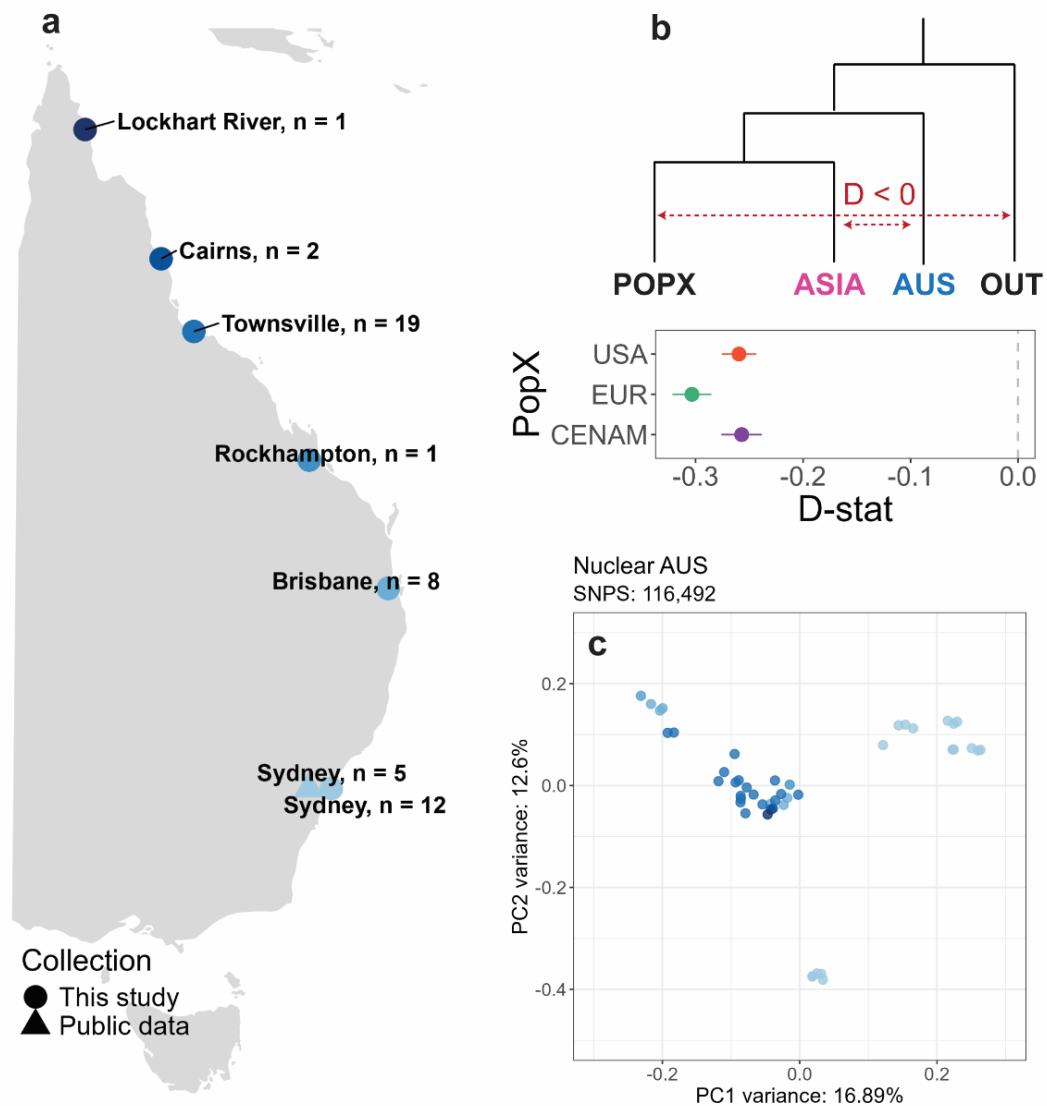


Fig. 5: Gene flow and population structure of Australian heartworms. **a**, Distribution of adult heartworm samples ($n = 48$) collected along the east coast of Australia. **b**, D -statistics showing excessive allele sharing between Australia (AUS) and ASIA relative to that between AUS and other regions (PopX). Admixtools qpDstat was used to analyse 21,088 single nucleotide polymorphisms (SNPs), with *Dirofilaria ursi* as the outgroup (OUT). All z -scores depicted were significant (>3 or <-3). Red dotted lines along hypothetical trees show the direction of gene flow and vertical grey dotted lines in the plots indicate where $D = 0$. **c**, Principal Component Analysis (PCA) of 116,492 nuclear SNPs in the Australian cohort. Principal component 1 (PC1) and principal component 2 (PC2) explained 16.89% and 12.6% of the variation, respectively.

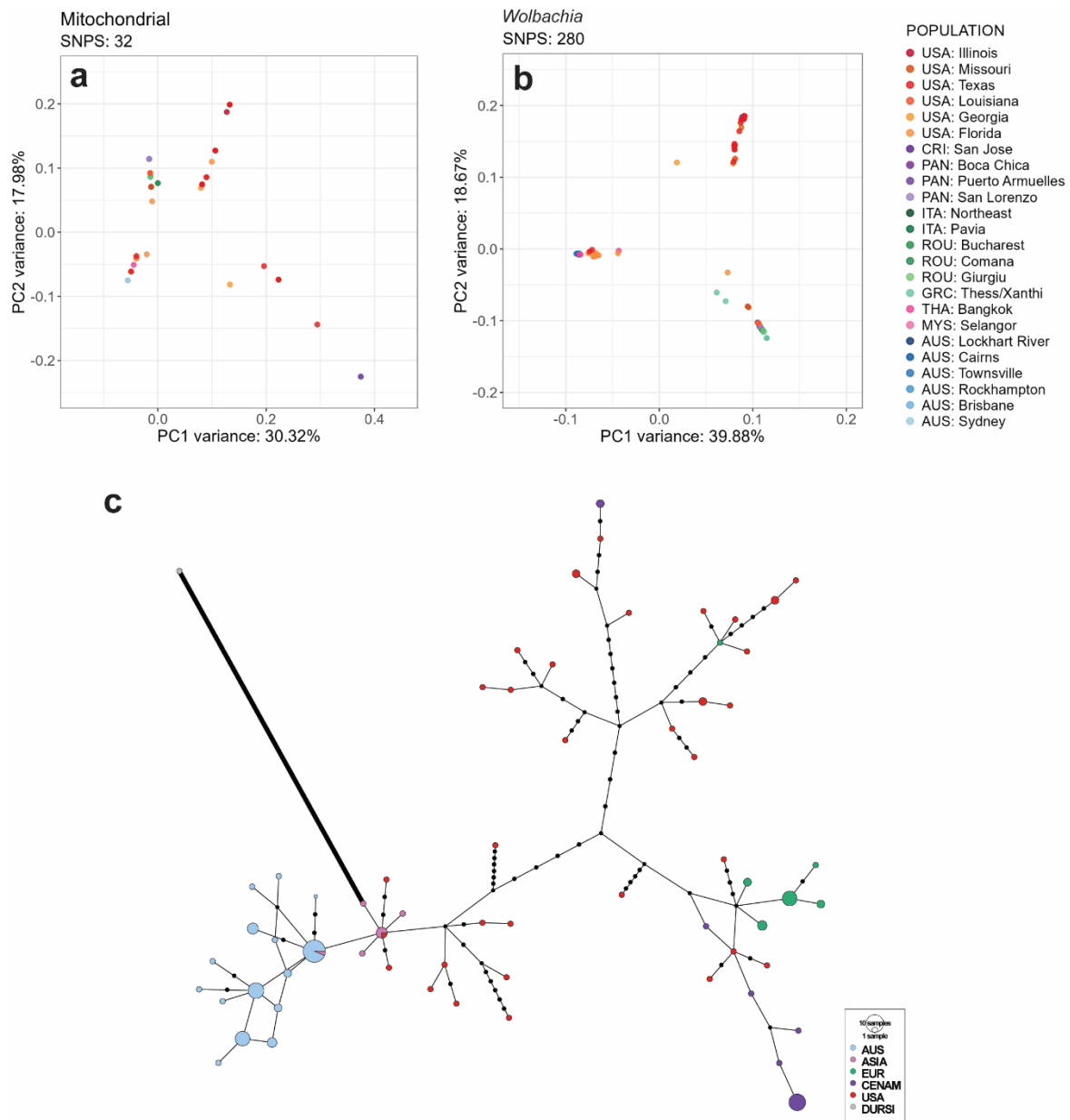
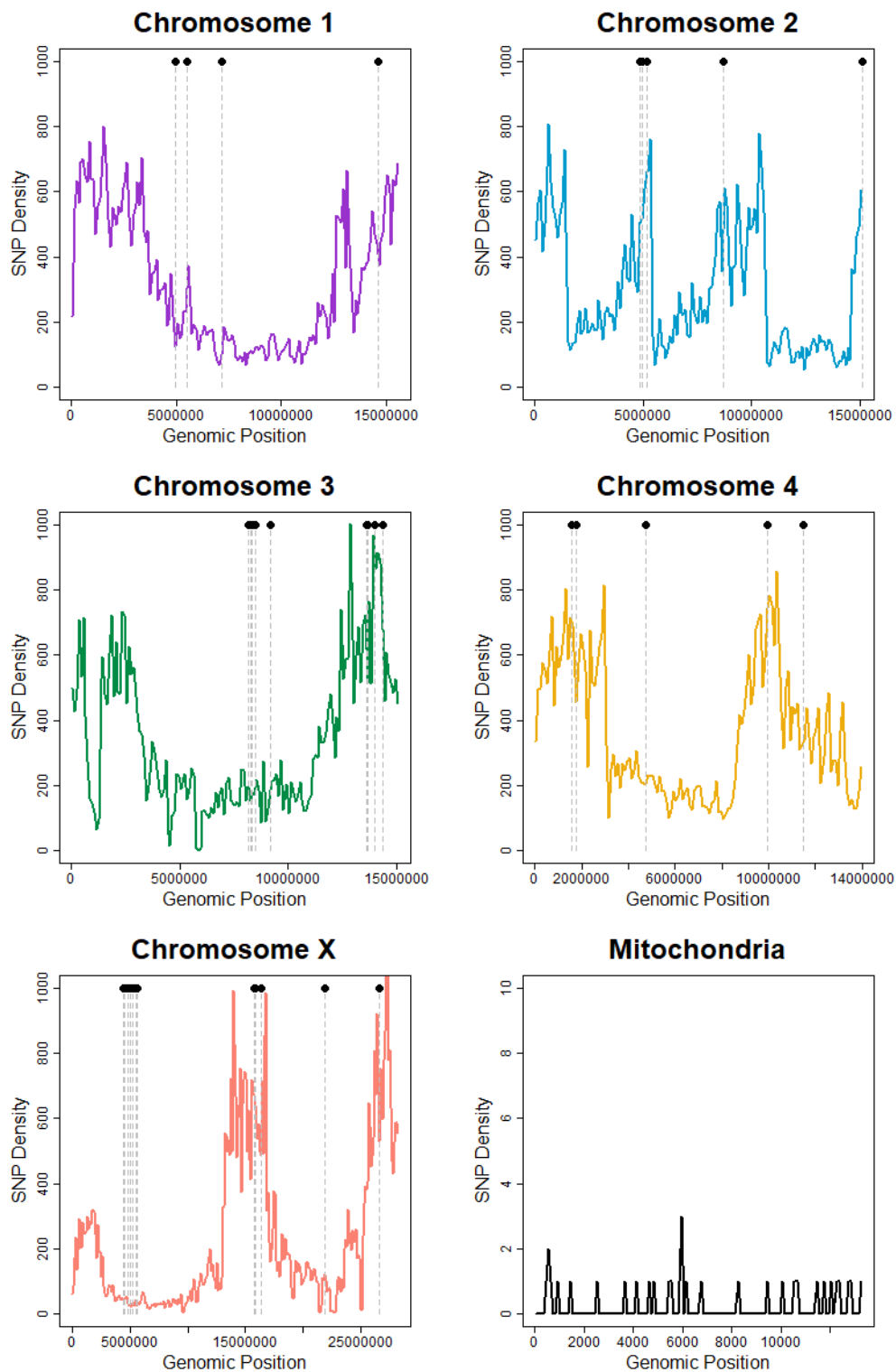
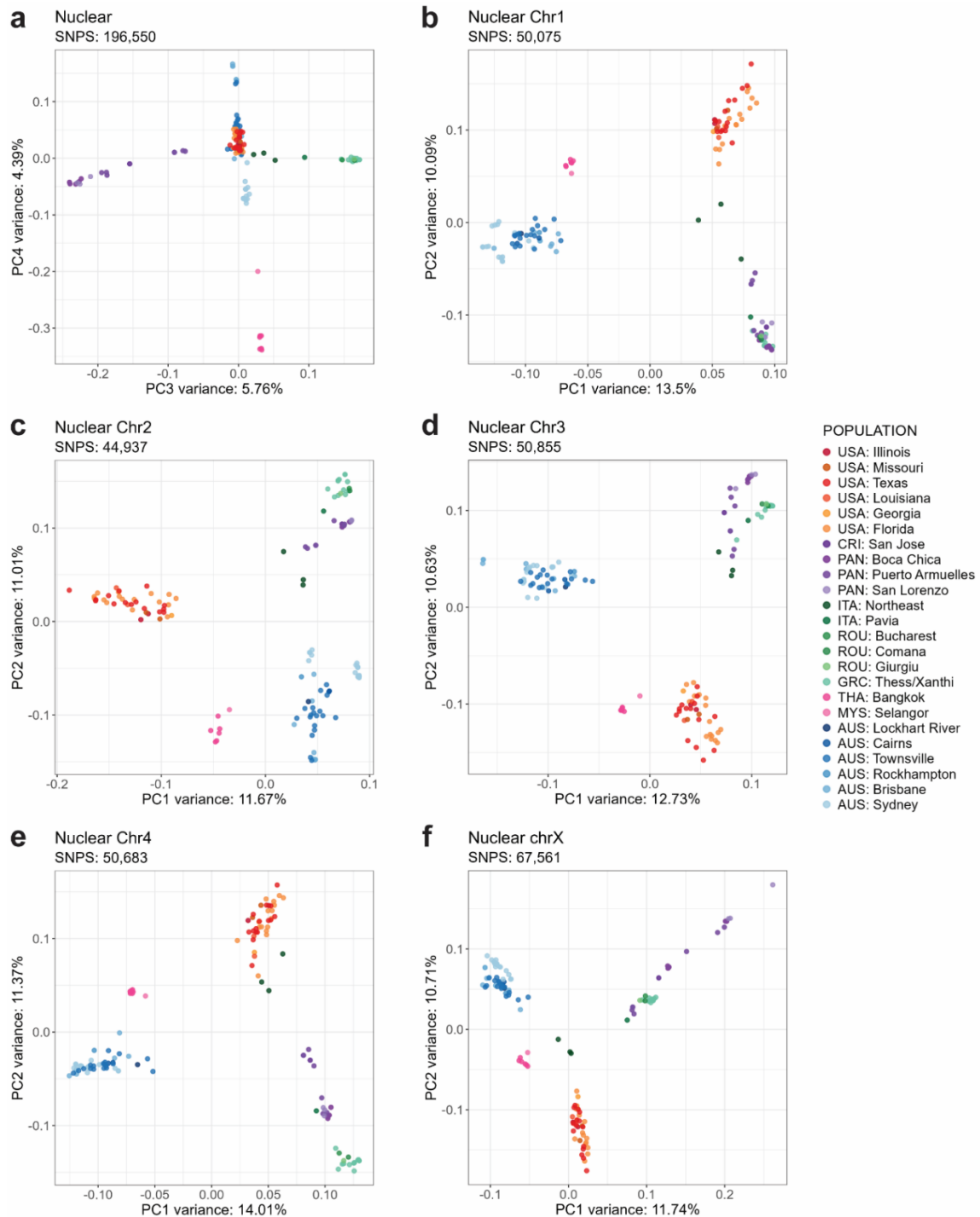


Fig. 6: Genetic relatedness of the mitochondria and *Wolbachia* endosymbionts of heartworms. Abbreviations: USA = United States of America; CRI = Costa Rica; PAN = Panama; ITA = Italy; ROU = Romania; GRC = Greece; VNM = Vietnam; THA = Thailand; MYS = Malaysia; AUS = Australia. **a**, Principal component analysis (PCA) of 32 mitochondrial single nucleotide polymorphisms (SNPs) from 127 heartworms. Principal component 1 (PC1) and principal component 2 (PC2) explained 30.32% and 17.98% of the variation, respectively. **b**, PCA of 280 *Wolbachia* endosymbiont SNPs from 121 samples. PC1 and PC2 explained 39.88% and 18.67% of the variation, respectively. Replicate samples were included in all PCAs. The legend on the right indicates the geographic origin of samples (i.e. COUNTRY: city or region). **c**, Median-joining network of 122 complete heartworm mtDNA genomes (13,938 positions) with *Dirofilaria ursi* (DURSI) as an outgroup, constructed using PopART with an epsilon value of 0. The legend in the bottom right corner indicates the geographic origin of samples and sample sizes.

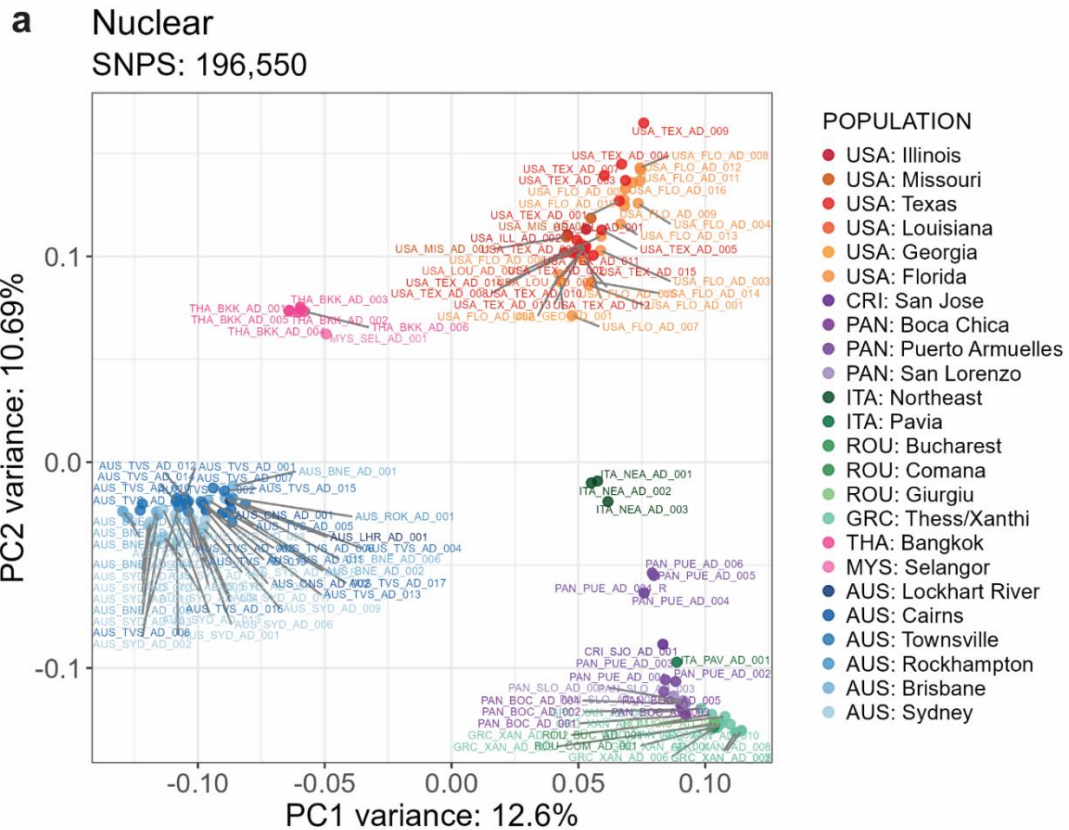
Extended Data Figures



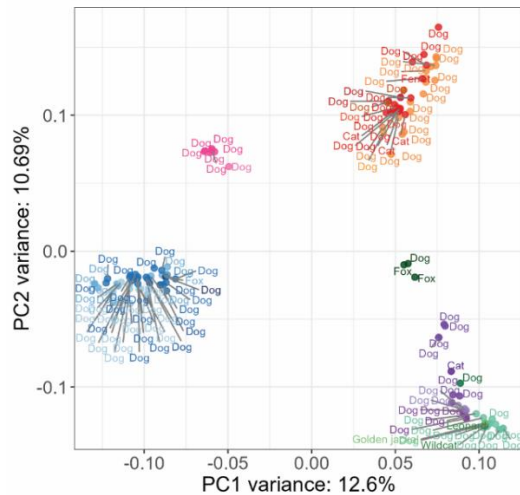
Extended Data Fig. 1: Distribution of single nucleotide polymorphisms (SNPs) in a global cohort of heartworms. The coloured lines indicate the density of SNPs across chromosome 1 (50,075 SNPs), chromosome 2 (44,937 SNPs), chromosome 3 (50,855 SNPs), chromosome 4 (50,683), the sex-linked X chromosome (67,561 SNPs), and the mitochondrial genome (32 SNPs). Vertical dotted lines with small black circles represent the location of SNPs previously associated with drug resistance in heartworms from the USA.



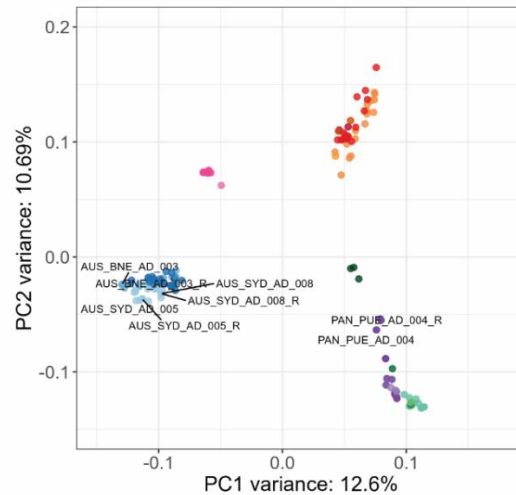
Extended Data Fig. 2: Principal component analysis (PCA) of 124 heartworms from around the world based on the nuclear genome. Samples were collected from Australia (AUS), the USA, Panama (PAN), Greece (GRC), Thailand (THA), Italy (ITA), Romania (ROU), Costa Rica (CRI), and Malaysia (MYS). Replicate samples were included in the analyses. The legend on the right indicates the geographic origin of samples (i.e. COUNTRY: city or region). The principal components (PCs) are shown on the x and y axes. **a**, PCA of 196,550 nuclear single nucleotide polymorphisms (SNPs) showing PC3 and PC4, which explained 5.76% and 4.39% of the variation, respectively. **b–f**, PCAs of 44,937 – 67,561 SNPs in chromosome 1, chromosome 2, chromosome 3, chromosome 4, and the sex-linked X-chromosome of canine heartworm. PC1 and PC2 explained 11.67 – 14.01% and 10.09 – 11.37% of the variation, respectively.



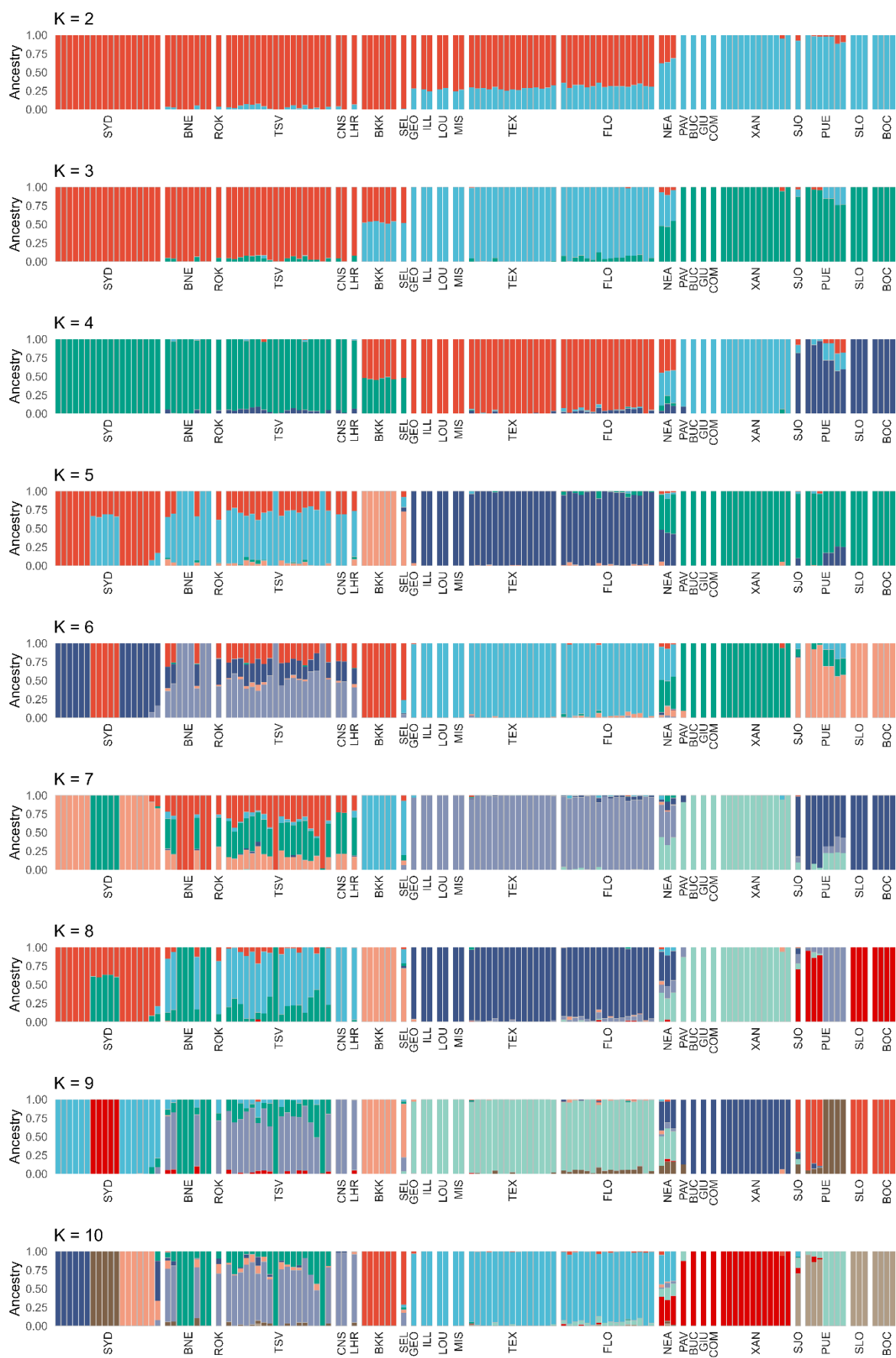
b Nuclear
SNPS: 196,550



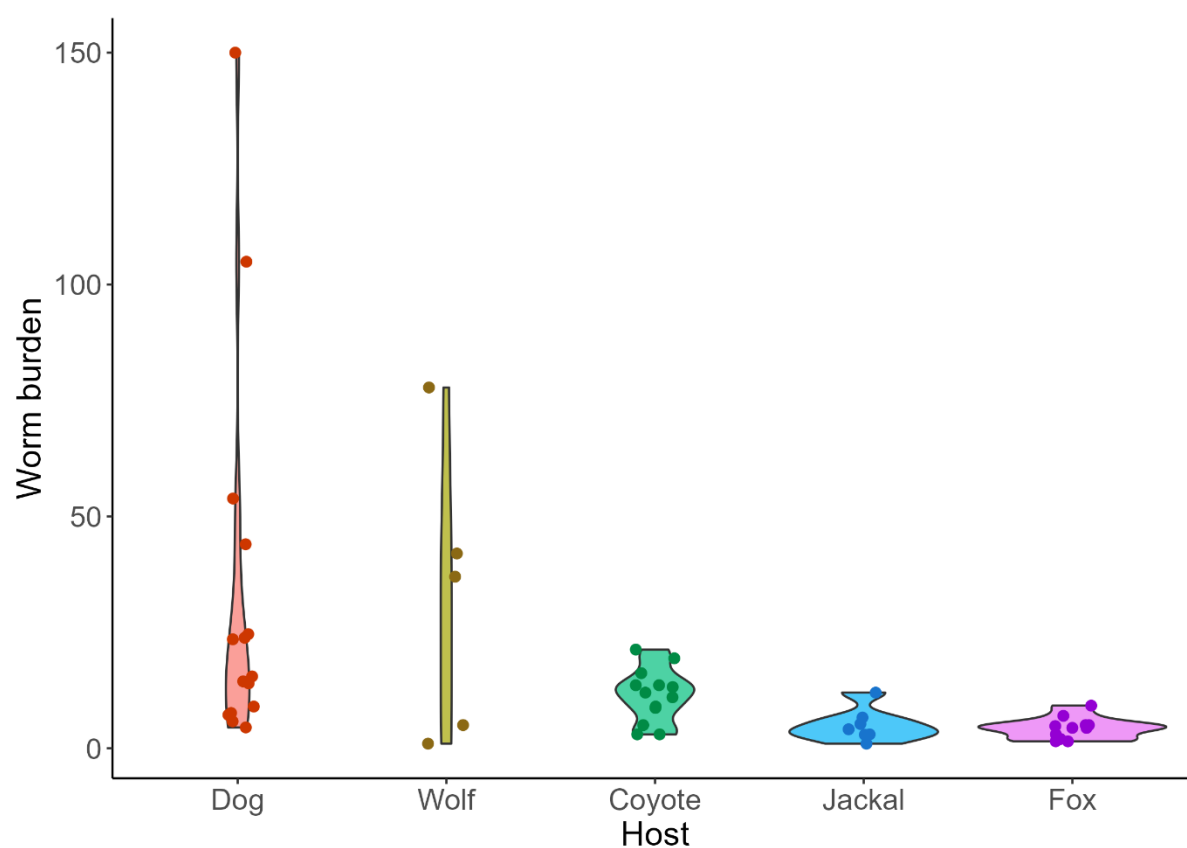
c Nuclear
SNPS: 196,550



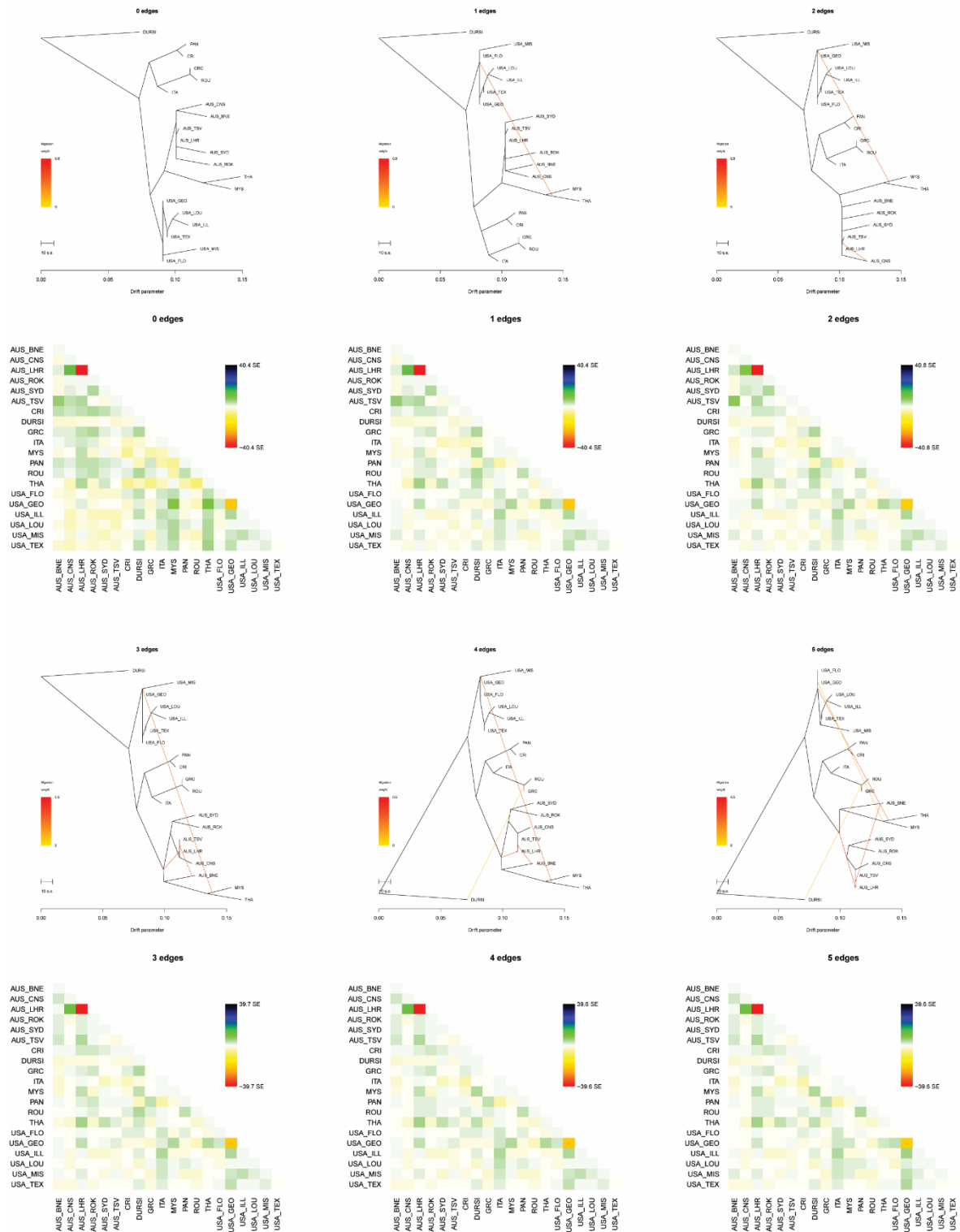
Extended Data Fig. 3: Principal component analysis (PCA) of 196,550 nuclear single nucleotide polymorphisms (SNPs) from 124 heartworms with labelled metadata. Samples were collected from Australia (AUS), the USA, Panama (PAN), Greece (GRC), Thailand (THA), Italy (ITA), Romania (ROU), Costa Rica (CRI), and Malaysia (MYS). Replicate samples were included in the analyses. The legend on the right indicates the geographic origin of samples (i.e. COUNTRY: city or region). Principal component 1 (PC1) and principal component 2 (PC2) are shown on the x and y axes, and explained 12.6% and 10.69% of the variation, respectively. These figures are identical to Fig. 1b but include **a**, sample labels, **b**, host labels and **c**, indication of technical replicate samples.



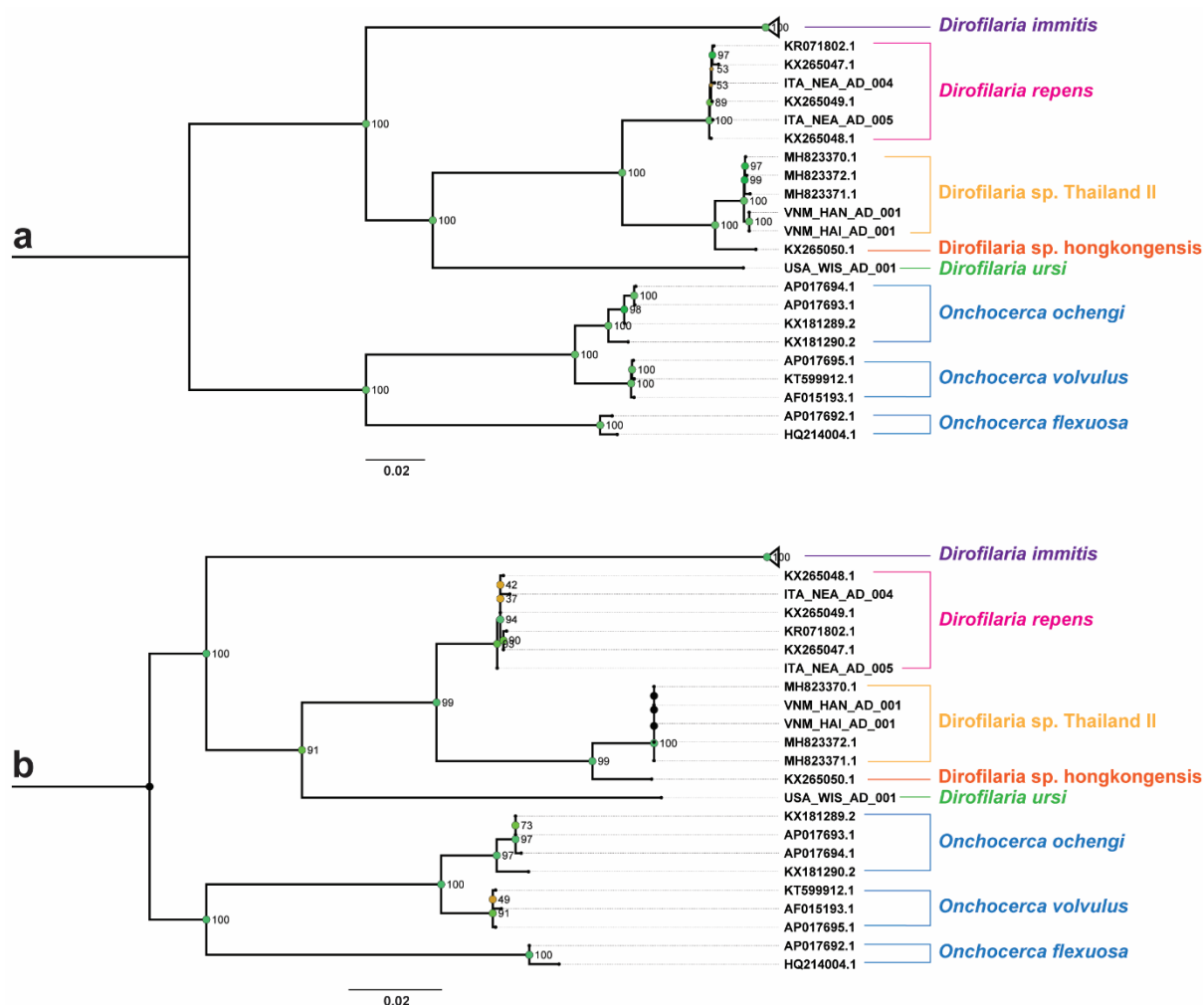
Extended Data Fig. 4: Admixture analysis of adult heartworms. Analysis was performed in NGSadmix across a range of K values (2 – 10) (colours) for 124 heartworms (vertical bars). Geographic origins are indicated along the bottom of each chart. Abbreviations: Australia (Sydney: SYD, Brisbane: BNE, Rockhampton: ROK, Townsville: TSV, Cairns: CNS, Lockhart River: LHR); Thailand (Bangkok: BKK); Malaysia (Selangor: SEL); USA (Georgia: GEO, Illinois: ILL, Louisiana: LOU, Missouri: MIS, Texas: TEX, Florida: FLO); Italy (Northeast: NEA, Pavia: PAV); Romania (Bucharest: BUC, Giurgiu: GIU, Comana: COM); Greece (Thessaloniki/Xanthi: XAN); Costa Rica (San José: SJO); Panama (Puerto Armuelles: PUE, San Lorenzo: SLO, Boca Chica: BOC).



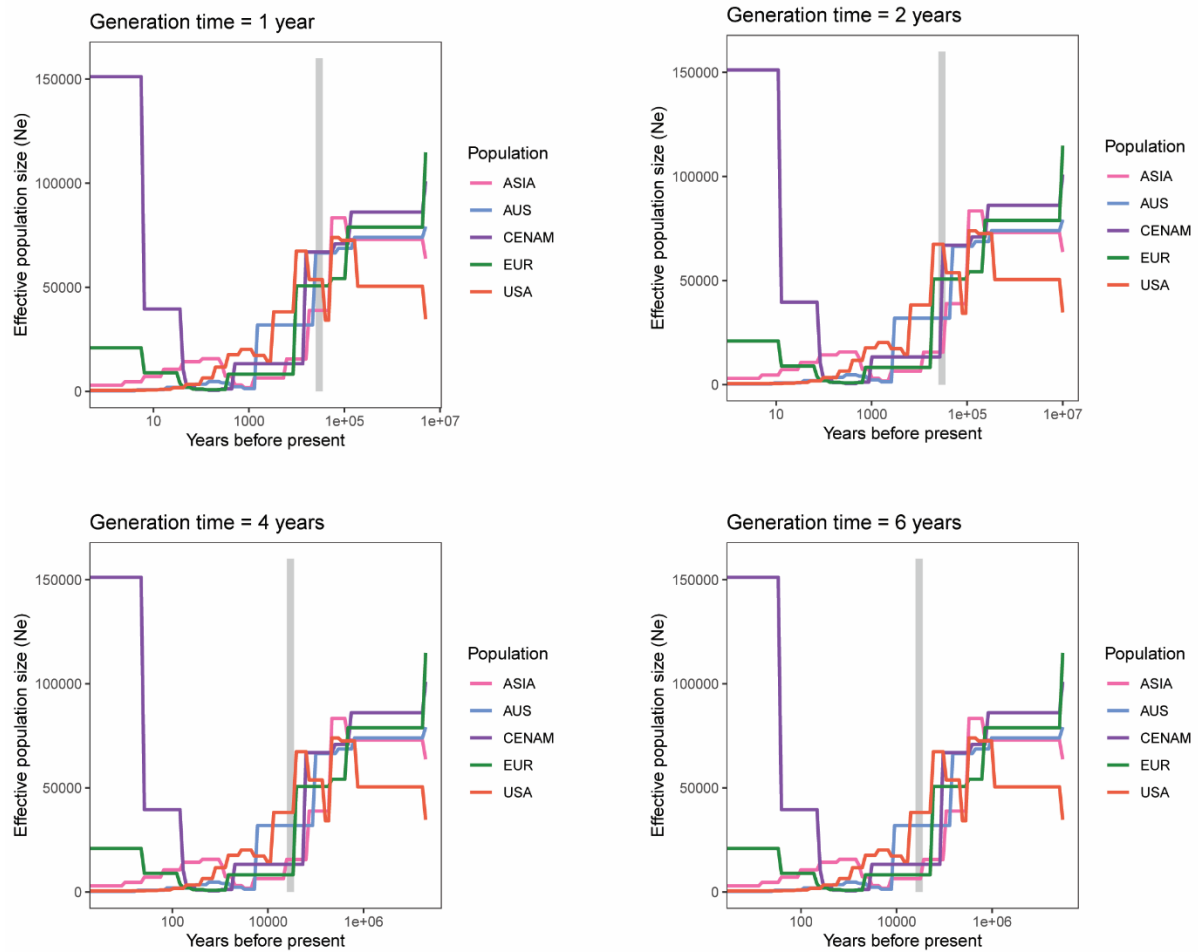
Extended Data Fig. 5: Violin plot comparing adult heartworm burdens in canid primary hosts. Data was obtained from previous literature, with each point representing an individual report.



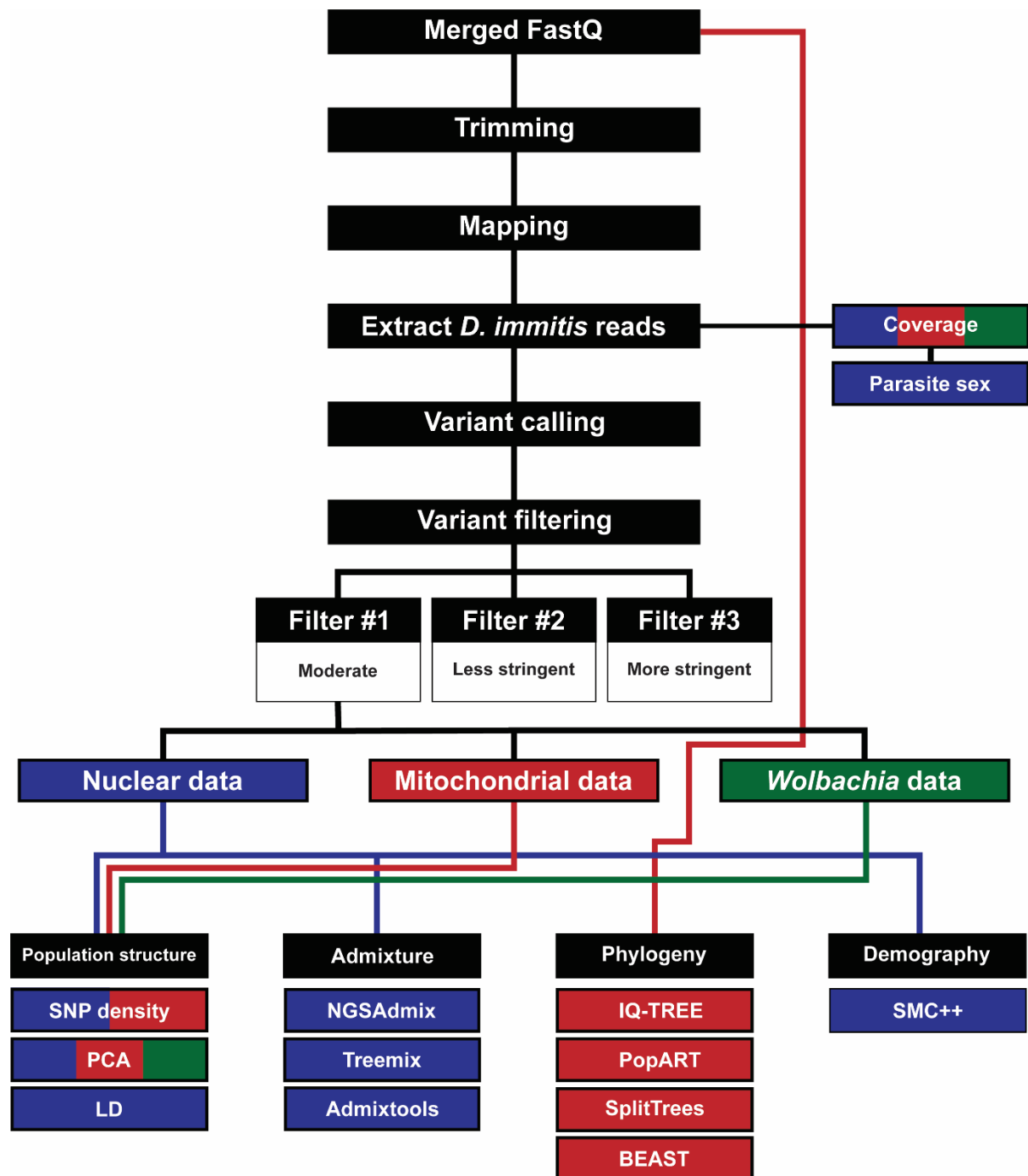
Extended Data Fig. 6: TreeMix analysis of nuclear heartworm data. Maximum likelihood trees are shown with migration edges ranging from 0 to 5, using *Dirofilaria ursi* as an outgroup. Coloured lines represent inferred migration events, with the colour indicating support levels. Below each tree is a heatmap of pairwise residuals. Smaller residuals indicate a better model fit, while the sign of the residuals (positive or negative) suggests whether the model's estimate of gene flow is underestimated (positive residual) or overestimated (negative residual).



Extended Data Fig. 7: Maximum likelihood (ML) phylogeny of Onchocercids based on mitochondrial DNA sequences. Phylogenetic trees were generated using IQ-TREE from a total of 155 sequences. These sequences included *Dirofilaria immitis* and other *Dirofilaria* species collected in this study, as well as publicly available mitochondrial sequences of *Dirofilaria* and *Onchocerca*. Branch support values are indicated next to the nodes and are colour-coded in the circles (red = lower support, green = higher support). The *D. immitis* samples, forming a clade with short branch lengths, were collapsed for visualisation. **a**, ML tree of complete mtDNA sequences (14,066 positions) constructed using the GTR+F+R4 model (BIC = 96915.657; -LnL = 47077.635). **b**, ML tree of *COXI* sequences (1,647 positions) constructed using the TIM3+F+I model (BIC = 10043.191; -LnL = 4754.954).



Extended Data Fig. 8: Demographic history analysis of heartworms using a range of generation times. Size histories were inferred using SMC++, with each line representing a heartworm subpopulation. A range of generation times were tested for *Dirofilaria immitis* (i.e. 1, 2, 4, and 6 years). The grey box indicates previously suggested date ranges for dog domestication (~14–40 Kya). Abbreviations: AUS = Australia; CENAM = Central America; EUR = Europe; USA = United States of America.



Extended Data Fig. 9: Summary diagram of the population genomics workflow in this study. Major data processing steps and analyses are shown for nuclear (blue), mitochondrial (red) and *Wolbachia* (green) data. Multi-coloured boxes indicate analyses conducted on multiple datasets.

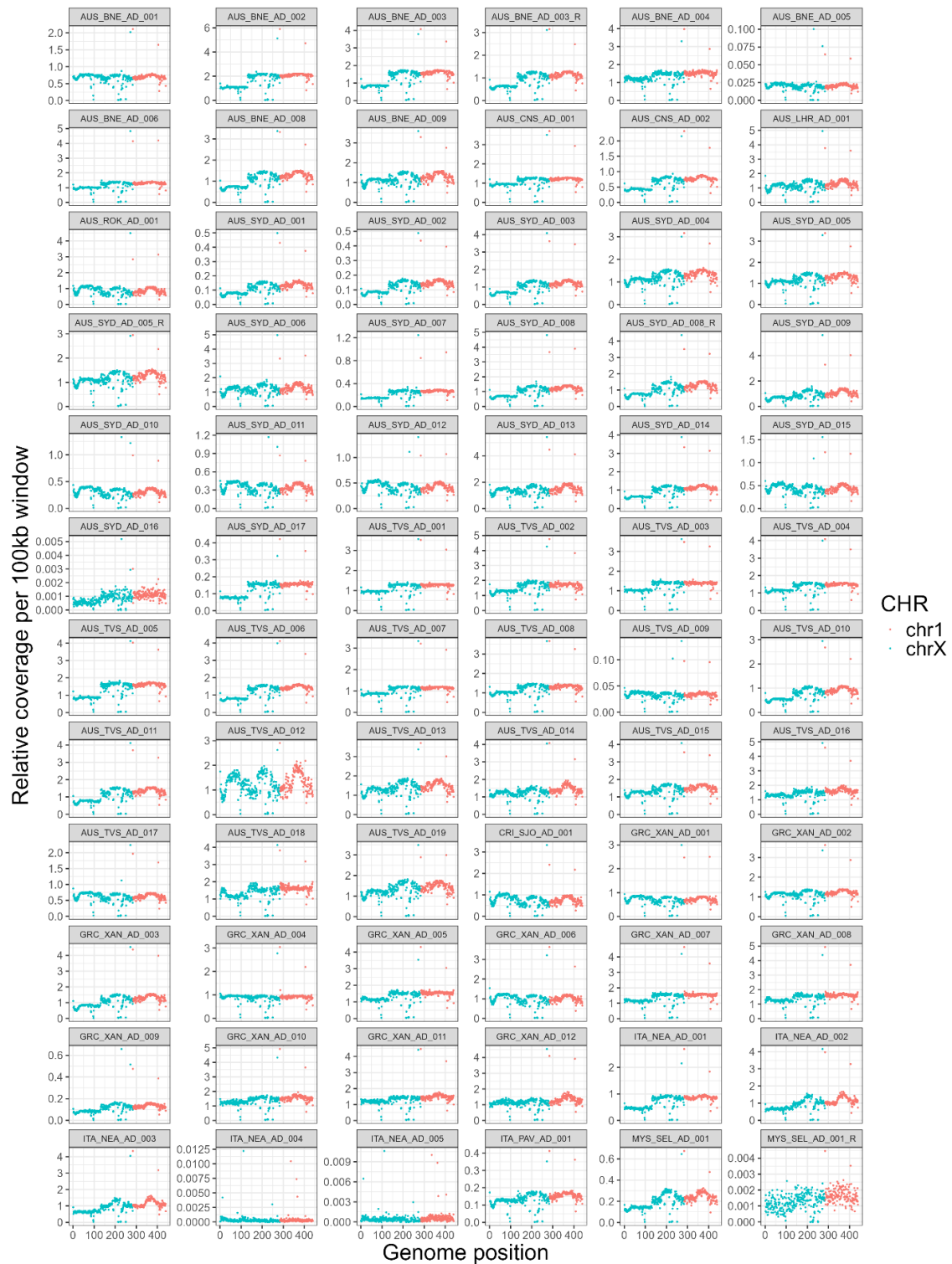
Supplementary Figures



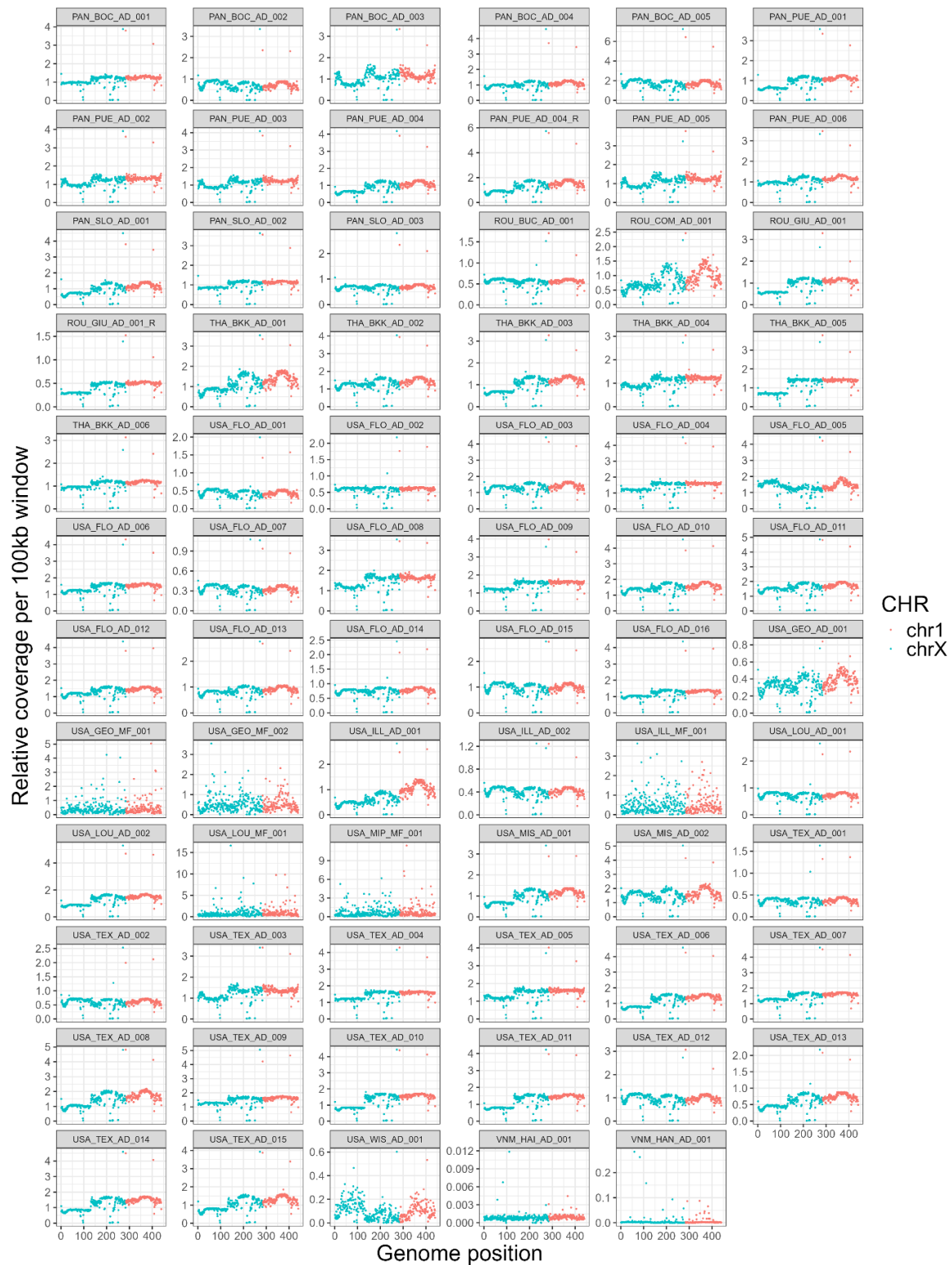
Supplementary Fig. 1: Raw nuclear sequencing coverage per sample (Part 1/2). Coverage was calculated using 100 kb sliding windows for all mapped samples. Each chromosome is shown in a different colour, with a legend on the right. The remaining samples are shown in Supplementary Fig. 2.



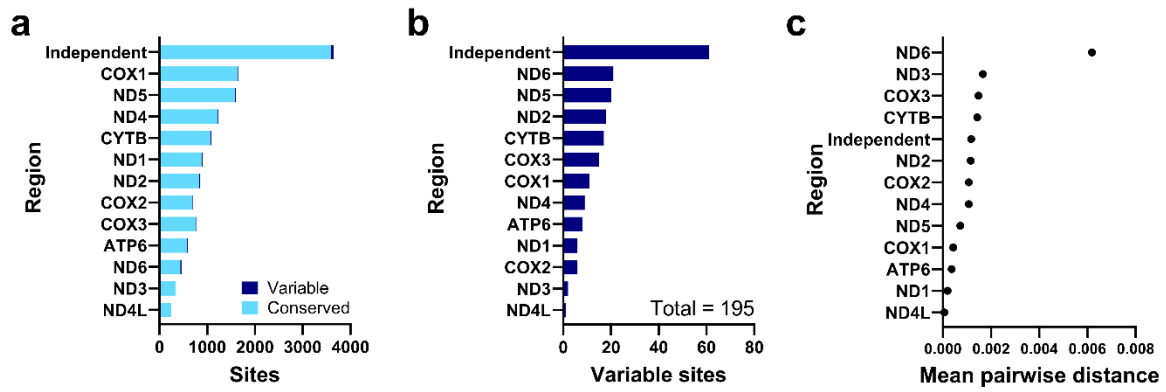
Supplementary Fig. 2: Raw nuclear sequencing coverage per sample (Part 2/2). Coverage was calculated using 100 kb sliding windows for all mapped samples. Each chromosome is shown in a different colour, with a legend on the right.



Supplementary Fig. 3: Sex determination of samples based on relative coverage of sex-linked vs autosomal chromosomes (Part 1/2). Sequencing coverage was assessed using a 100kb sliding window and compared to the overall median coverage. Coverage of the sex-linked X chromosome (blue) was compared to an autosome (chromosome 1, red) to determine the parasite's sex. Male canine heartworms show reduced coverage on chromosome X (ratio ~0.5), while females show similar coverage across sex-linked and autosomal chromosomes (ratio ~1). Ambiguous results were observed in samples with low coverage. The remaining samples are shown in Supplementary Fig. 4.



Supplementary Fig. 4: Sex determination of samples based on relative coverage of sex-linked vs autosomal chromosomes (Part 2/2). Sequencing coverage was assessed using a 100kb sliding window and compared to the overall median coverage. Coverage of the sex-linked X chromosome (blue) was compared to an autosome (chromosome 1, red) to determine the parasite's sex. Male canine heartworms show reduced coverage on chromosome X (ratio ~ 0.5), while females show similar coverage across sex-linked and autosomal chromosomes (ratio ~ 1). Ambiguous results were observed in samples with low coverage.



Supplementary Fig. 5: Summary of complete mitochondrial DNA variants in a global cohort of heartworms. Samples included 133 complete mitochondrial genomes of heartworms, three of which were reference sequences obtained from published literature. **a**, Comparison of the number of variable and conserved sites per region. **b**, Number of variable sites per region. **c**, Average pairwise genetic distances per region.

Connecting statement 3

The population genomics study presented in Chapter 3.1 used DNA extracted from adult heartworms, which resulted in sufficient coverage of the *Dirofilaria immitis* genome (average coverage per sample: nuclear = 75.47×, mitochondrial = 13119.80×). Although they yielded high-quality sequencing data, adult heartworms are difficult to obtain because they reside in the heart and pulmonary arteries of their hosts. Extraction of these adult worms therefore requires either surgery or necropsy, which are invasive and may not occur frequently. In addition, cultural and religious factors may limit the performance of necropsies and prevent human contact with animal hosts. These limitations were evident in Chapter 3.1, where notable sampling gaps were present in South America, Africa, and much of Asia.

To circumvent the need for adult heartworms, it may be easier to obtain more accessible life stages, i.e. microfilariae, which circulate freely in the host's bloodstream and require only standard blood collection. This approach is used in Chapter 3.2, where individual microfilariae are extracted from dog blood and purified from other host blood components. Whole genome amplification and sequencing are then performed to evaluate the potential of microfilariae samples for population genetic analyses. The same workflow from Chapter 3.1 is applied here to compare the overall success of this method in obtaining high-quality sequencing data with sufficient coverage of the heartworm genome (ideally ~30×).

———— Chapter 3.2 ————

Whole genome amplification and sequencing of individual
Dirofilaria immitis microfilariae

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Rosemonde Isabella Power in the preparation and submission of the following manuscript:

Power, R. I., Doyle, S. R., & Šlapeta, J. (2024). Whole genome amplification and sequencing of individual *Dirofilaria immitis* microfilariae. *Experimental Parasitology*, 263-264, 108806.
<https://doi.org/10.1016/j.exppara.2024.108806>

Contribution to the manuscript of all authors is as follows:

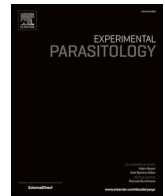
Task	Contributing co-authors
Conceptualisation	RIP, JŠ
Data collection and curation	RIP
Formal analysis	RIP
Funding acquisition	JŠ
Investigation	RIP
Methodology	RIP, SRD
Supervision, discussion and consultation	JŠ, SRD
Preparation of figures used in the manuscript	RIP
Writing of the original draft	RIP
Critical review and editing of the manuscript	RIP, SRD, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct.

Kind regards,

Professor Jan Šlapeta

26th September 2024



Whole genome amplification and sequencing of individual *Dirofilaria immitis* microfilariae

Rosemonde I. Power^a, Stephen R. Doyle^b, Jan Šlapeta^{a,c,*}

^a Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales, 2006, Australia

^b Wellcome Sanger Institute, Cambridgeshire, CB10 1SA, United Kingdom

^c Sydney Infectious Diseases Institute, The University of Sydney, New South Wales, 2006, Australia

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ABSTRACT

Dirofilaria immitis is a filarial parasitic nematode of veterinary significance. With the emergence of drug-resistant isolates in the USA, it is imperative to determine the likelihood of resistance occurring in other regions of the world. One approach is to conduct population genetic studies across an extensive geographical range, and to sequence the genomes of individual worms to understand genome-wide genetic variation associated with resistance. The immature life stages of *D. immitis* found in the host blood are more accessible and less invasive to sample compared to extracting adult stages from the host heart. To assess the use of immature life stages for population genetic analyses, we have performed whole genome amplification and whole-genome sequencing on nine ($n = 9$) individual *D. immitis* microfilaria samples isolated from dog blood. On average, less than 1% of mapped reads aligned to each *D. immitis* genome (nuclear, mitochondrial, and *Wolbachia* endosymbiont). For the dog genome, an average of over 99% of mapped reads aligned to the nuclear genome and less than 1% aligned to the mitochondrial genome. The average coverage for all *D. immitis* genomes and the dog nuclear genome was less than 1, while the dog mitochondrial genome had an average coverage of 2.87. The overwhelming proportion of sequencing reads mapping to the dog host genome can be attributed to residual dog blood cells in the microfilariae samples. These results demonstrate the challenges of conducting genome-wide studies on individual immature parasite life stages, particularly in the presence of extraneous host DNA.

1. Introduction

Canine heartworm disease, caused by *Dirofilaria immitis*, poses a significant threat to the health of dogs and cats. *Dirofilaria immitis* is distributed globally across tropical, subtropical, and temperate regions, with infections documented in the Americas, Europe, Australia, Asia, and some areas of Africa (Dantas-Torres et al., 2023; Genchi and Kramer, 2020; Noack et al., 2021; Simón et al., 2012). Macrocyclic lactone (ML) anthelmintic drugs are essentially the only class of drugs available for canine heartworm prevention. Although these drugs have worked effectively for 30 years, resistance has been confirmed in the USA (Blagburn et al., 2016; Blagburn et al., 2011; Bourguinat et al., 2015; McTier et al., 2017; Pulaski et al., 2014). In response to this alarming discovery, researchers have identified a suite of single nucleotide polymorphisms (SNPs) that can be used for screening purposes to diagnose resistance based on a genetic comparison of phenotypically susceptible and drug resistant isolates from the USA (Ballesteros et al., 2018;

Bourguinat et al., 2017a; Bourguinat et al., 2015; Bourguinat et al., 2017b). These diagnostic SNPs were determined to be associated with resistance but were not the casual mutations responsible for resistance (Bourguinat et al., 2015).

The confirmation of phenotypic and genetic resistance to ML treatment in the USA has sparked concerns about the potential emergence of resistance in other parts of the world. In Australia, suspicions of ML resistance arose when dogs reportedly on 'rigorous' prevention tested positive for *D. immitis* infection (Nguyen et al., 2016). Further investigations revealed delayed microfilariae reduction after ML treatment suggestive of resistance, and yet, the parasites were classified as genetically susceptible based on genotyping using the predictive SNPs developed in the USA (Power and Šlapeta, 2022). In the first whole-genome study of *D. immitis* in Australia, no adult worms showed a similar genetic profile to any of the previous phenotypically well-characterised susceptible or resistant isolate from the USA (Lau et al., 2021). Furthermore, the diagnostic resistance-associated SNPs

* Corresponding author. Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, New South Wales, 2006, Australia.

E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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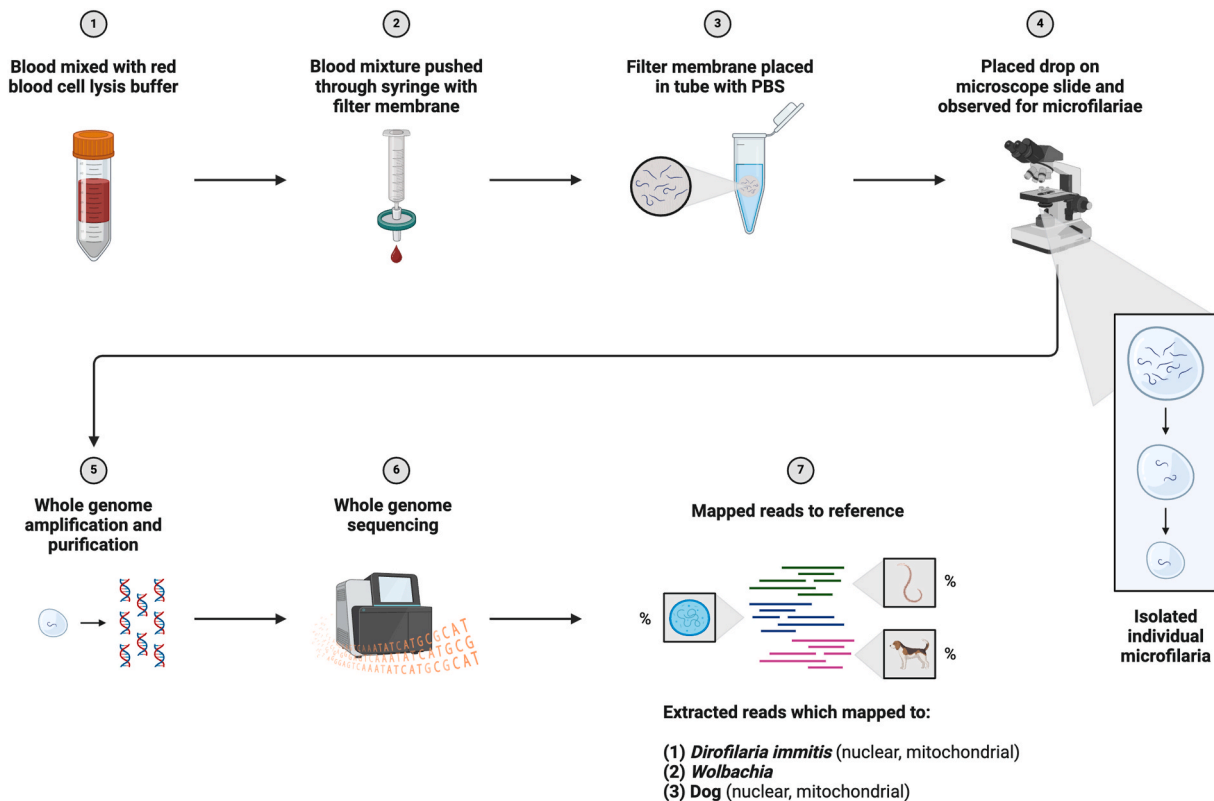


Fig. 1. Workflow for whole genome amplification and whole-genome sequencing of single *Dirofilaria immitis* microfilariae. Created with BioRender.com.

found in USA samples were deemed unlikely to be present in Australian and Italian samples (Gandasegui et al., 2023). These findings indicate that the suite of diagnostic SNPs from the USA may have limited diagnostic relevance outside of the USA and are unsuitable for monitoring ML resistance within Australia. To understand the genetics of resistance, including the evolution and transmission of resistant variants, we must gain a deeper understanding of the genetic structure of this parasite and assess the genetic similarity between endemic populations.

The most comprehensive approach for conducting population genomic studies involves sequencing the whole genome of single individuals (Small et al., 2019). This allows for the accurate genotyping of individuals, which is beneficial for understanding population structure, genomic diversity, and demographic history (Choi et al., 2016; Sallé et al., 2019; Small et al., 2019). One option to isolate DNA from a single *D. immitis* is to extract adult worms from an infected animal. Although a 1 cm segment of the worm yields sufficient DNA for genome sequencing, this method is highly invasive, requiring either surgery or necropsy to extract the adult stages. Alternatively, microfilariae stages of *D. immitis* are readily accessible in the blood of infected animals, requiring only a simple blood collection. The relative ease of sampling microfilariae would facilitate a more extensive genome-wide study of *D. immitis* within Australia and on a global scale. However, individual microfilariae contain minute amounts of DNA that are generally insufficient for direct whole-genome sequencing (WGS) (Doyle et al., 2019).

This study aimed to (1) generate sufficient DNA from individual *D. immitis* microfilariae for WGS and (2) determine the suitability of this data for population genomic analyses. To do so, we utilised whole genome amplification (WGA) technology to amplify the genomes of single microfilariae before WGS. The results of this study reveal valuable insights into the applicability of WGA for genome-wide studies using early parasite life stages.

2. Materials and methods

2.1. Sample collection

Blood samples ($n = 8$) were collected from *D. immitis*-positive dogs ($n = 7$) at a veterinary clinic in Queensland, Australia between March and August 2021. A registered veterinarian collected these blood samples as part of a larger study (Power and Šlapeta, 2022).

2.2. Isolating single *D. immitis* microfilaria

Single *D. immitis* microfilariae were isolated from whole dog blood. A filtration procedure was implemented to separate the *D. immitis* microfilariae from the other blood components, as previously described (Bourguinat et al., 2015; Power and Šlapeta, 2022). Briefly, filtration involves lysis of red blood cells using Red Blood Cell Lysis Buffer (8 g/L sodium carbonate, 5 mL/L Triton X-100), filtration through a polycarbonate membrane filter (pore size 20 μm , filter diameter 25 mm; Sterlitech® Corporation, WA), followed by membrane flushing with phosphate-buffered saline (PBS). The membrane was placed into a 1.5 mL Eppendorf tube, and 500 μL of PBS was added. The tube was shaken several times to release the *D. immitis* microfilariae from the membrane. A small drop of the PBS mixture was placed onto a microscope slide and examined for microfilariae under a microscope (BX40, Olympus, Australia) using 4 $\times$ and 10 $\times$ objectives. Once microfilariae were observed in the field of view, a small group of microfilariae was aspirated along with the surrounding PBS and placed onto the microscope slide once again as a separate drop. This process was repeated until only a single microfilaria remained in the final drop (Fig. 1). The final drop was aspirated and placed into a clean 1.5 mL Eppendorf tube. Single microfilaria samples were stored at -20°C before WGA.

Table 1Proportions of *Dirofilaria immitis*, *Wolbachia*, and dog sequencing reads.

Sample ID	qPCR (C _t -value)		Total Sequencing reads	<i>D. immitis</i> nuclear		<i>D. immitis</i> mtDNA		<i>D. immitis</i> <i>Wolbachia</i>		Dog nuclear		Dog mtDNA	
	<i>D. immitis</i>	Dog		Reads	%	Reads	%	Reads	%	Reads	%	Reads	%
JS6089	23.24	18.51	9,131,485	10,928	0.120	36	0.0004	205	0.00224	9,115,157	99.82	446	0.0049
JS6090	22.36	18.38	6,290,159	101,169	1.608	126	0.0020	2281	0.03626	6,181,733	98.28	407	0.0065
JS6091	24.99	17.13	14,848,583	4650	0.031	16	0.0001	20	0.00013	14,834,911	99.91	909	0.0061
JS6092	22.71	16.77	10,817,398	1654	0.015	29	0.0003	81	0.00075	10,809,373	99.93	458	0.0042
JS6093	23.54	16.97	9,716,364	1306	0.013	55	0.0006	8	0.00008	9,708,544	99.92	284	0.0029
JS6094	N/A	36.59	6,738,448	1293	0.019	0	0	390	0.00579	6,733,876	99.93	10	0.0001
JS6095	24.01	17.27	12,221,332	12,504	0.102	51	0.0004	4	0.00003	12,200,173	99.83	317	0.0026
JS6096	20.99	17.83	11,055,065	44,213	0.400	343	0.0031	14	0.00013	11,002,280	99.52	641	0.0058
JS6097	24.82	18.24	11,542,790	431	0.004	42	0.0004	6	0.00005	11,534,875	99.93	214	0.0019

2.3. Whole genome amplification of single *D. immitis* microfilariae

To amplify the genomes of single *D. immitis* microfilariae ($n = 9$), WGA was performed using the GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma-Aldrich, Australia). This kit is designed to amplify the genome of a single cell, potentially leading to a million-fold amplification of DNA, resulting in a yield suitable for sequencing. A negative control sample of ddH₂O was included to ensure the absence of environmental contamination. The lysis, fragmentation, OmniPlex library generation, and PCR amplification steps of the kit were performed sequentially without pause according to the manufacturer's instructions. The whole genome amplified DNA was purified using the QIAquick PCR Purification Kit (QIAGEN, Australia) with the recommended protocol and stored at -20°C until further use.

2.4. Real-time quantitative PCR to detect *D. immitis* and dog DNA

To verify the presence of *D. immitis* DNA in the whole genome amplified DNA samples, a diagnostic TaqMan probe real-time quantitative PCR targeting the *cox1* gene was performed. This *D. immitis* assay used primers (S0624_F, S0626_R) and a probe (S0625_P) previously reported by Panetta et al. (2021) and Power and Šlapeta (2022). To determine whether dog DNA was present in the WGA DNA samples, a real-time PCR targeting the partial canine glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) region was performed. This dog assay used previously reported primers (S0631_DOG_F, S0634_DOG_R) and probe (S0632_DOG_P) (Orr et al., 2020; Panetta et al., 2021; Power and Šlapeta, 2022). All primer and probe sequences used in this study are available in Supplementary Table S1. The reactions for both the *D. immitis* and dog assays were run at a final volume of 20 μL consisting of 10 μL of Luna® Universal Probe qPCR Master Mix (New England Biolabs, Australia), primers at a concentration of 400 nM, probes at a concentration of 100 nM, and 2 μL of template DNA. The PCR cycling conditions consisted of 95°C for 1 min followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each qPCR run included a no-template control (NTC) of PCR-grade water. The real-time PCRs were performed on a CFX96 Real-Time PCR system with the corresponding Bio-Rad CFX Maestro Software (BioRad, Australia). The qPCR threshold was auto-calculated, and cycle threshold (C_t)-values were reported.

2.5. Whole-genome sequencing

The whole genome amplified DNA obtained from each single *D. immitis* microfilaria was aliquoted (30 μL) into a 1.5 mL Eppendorf tube and submitted to Novogene (HK) Co., Ltd for indexing, library preparation and WGS to generate approximately 1 Gb of sequencing data per sample. Sequencing was performed using the Illumina NovaSeq 6000 sequencing platform using 150 bp paired-end sequencing chemistry.

2.6. Trimming, mapping, and genome coverage analysis

Raw FastQ files generated from the same sample were concatenated before analysis, keeping the forward and reverse reads separate. Raw paired-end sequencing reads were examined for quality using FastQC (version 0.11.8) (Andrews, 2010) and trimmed using Trimmomatic (version 0.39) (Bolger et al., 2014) (parameters: SLIDINGWINDOW:10:20 MINLEN:50). Bwa (version 0.7.17) (Li, 2013) *mem* was used to map the trimmed reads to a combined reference genome for *D. immitis* (dimmitis_WSI_2.2) and the domestic dog (can-Fam6; GenBank accession: GCA_000002285.4) (Gandasegui et al., 2023). The dimmitis_WSI_2.2 genome has been previously supplemented with the *D. immitis* mitochondrial and *D. immitis*-associated *Wolbachia* endosymbiont genomes (Gandasegui et al., 2023). The resulting Sequence Alignment Map (SAM) files were converted to Binary Alignment Map (BAM) format whilst filtering out alignments with mapping quality scores <15 using Samtools (version 1.17) (Danecek et al., 2021). The BAM files were sorted by their leftmost coordinates and indexed. Sequencing coverage for the genomes of *D. immitis* (nuclear, mitochondrial, and *Wolbachia*) and the domestic dog (nuclear and mitochondrial) was analysed using bamtools (version 2.5.1) (Barnett et al., 2011), bedtools (version 2.31.0) (Quinlan and Hall, 2010) *make-windows*, samtools (version 1.17) (Danecek et al., 2021) *bedcov*, *datamash* (version 1.7) (Free Software Foundation, 2014) and R (version 4.3.2) (R Core Team, 2023) with the *tidyverse* (Wickham et al., 2019), *ggpubr* (Kassambara, 2023), *ggsci* (Xiao, 2024), and *stringr* (Wickham, 2023) packages.

2.7. Calculating the proportion of reads aligning to *D. immitis*, *Wolbachia*, and dog genomes

To assess the relative proportions (%) of *D. immitis* and dog reads in each sequenced sample, the mapped reads which aligned to the reference genomes of (i) *D. immitis*, (ii) the *D. immitis*-associated *Wolbachia* endosymbiont, and (iii) the domestic dog were extracted using Samtools (version 1.17). Alignment and filtering statistics were obtained throughout the analysis using Samtools *Flagstat* (version 1.17) and the results were compiled for all samples using MultiQC (version 1.15. dev0). The workflow for this project is illustrated in Fig. 1.

3. Results

3.1. Presence of *Dirofilaria immitis* and dog DNA using real-time PCR

Whole genome amplified DNA obtained from single *D. immitis* microfilariae samples were subjected to real-time qPCR targeting the *cox1* gene of *D. immitis* and the *GAPDH* gene of the domestic dog. A total of 8/9 DNA samples were positive for *D. immitis*, returning a mean C_t-value of 23.33 (range: 20.99–24.99) (Table 1). Dog DNA was amplified in all samples (9/9) with a mean C_t value of 19.74 (range: 16.77–36.59) (Table 1).

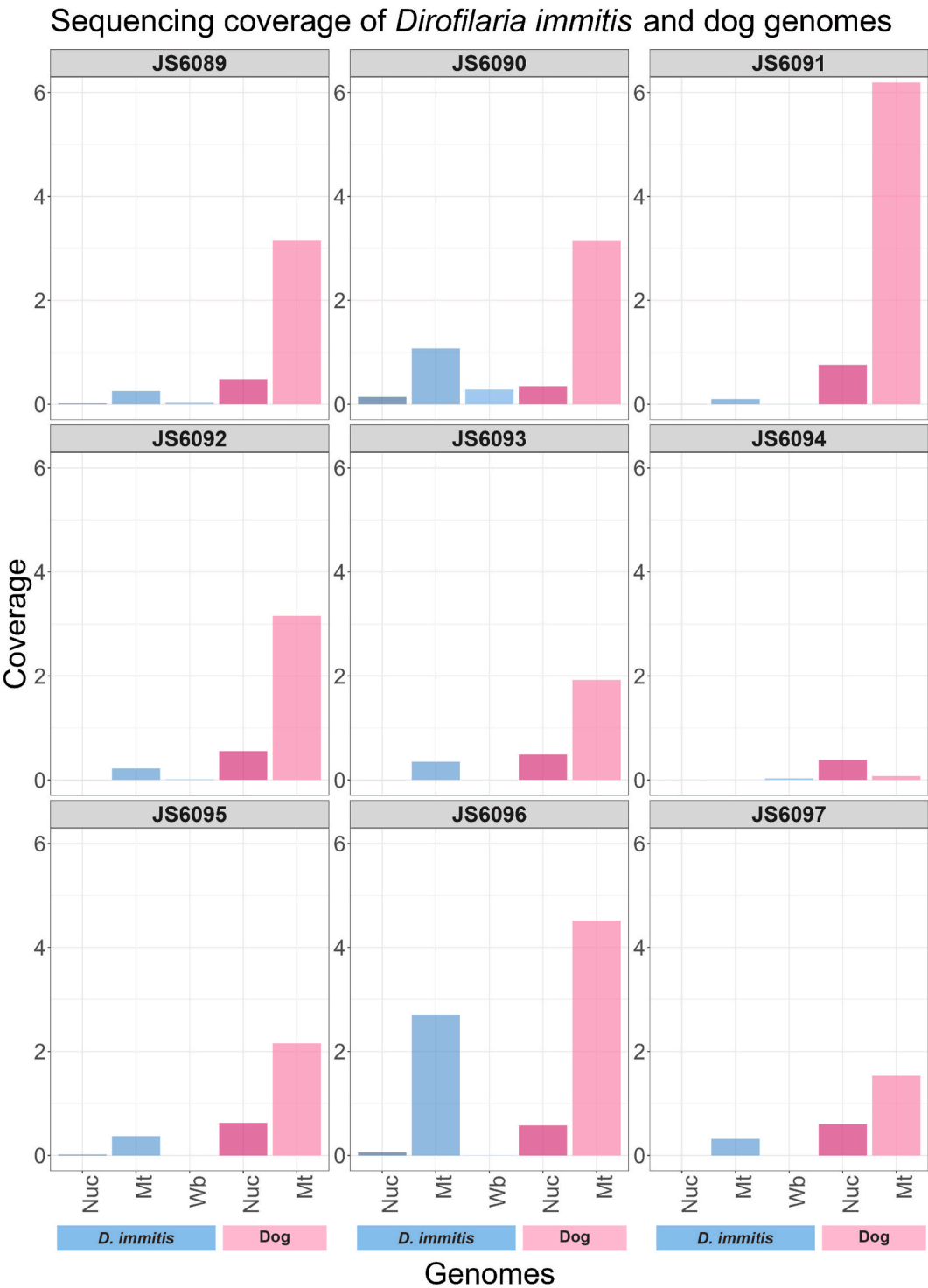


Fig. 2. Sequencing coverage of the *Dirofilaria immitis* (nuclear, mitochondrial, *Wolbachia* endosymbiont) and domestic dog (nuclear, mitochondrial) genomes from individual microfilariae samples. Genomes of *D. immitis* and the domestic dog are indicated in blue and pink, respectively.

3.2. Predominance of dog reads from single *D. immitis* microfilariae samples

WGS of the whole genome amplified DNA was performed to determine the suitability of the microfilariae isolation and WGA technique described in the study. All samples retained >90% of their reads after the initial trimming and filtration step (Supplementary Table S2). An average of 11,237,446 trimmed, QC-passed reads were mapped to the combined reference genome for *D. immitis* and the domestic dog. An

average of 11,051,886 (98%) were successfully mapped to this combined reference genome, with an average of 10,262,403 mapped reads (93%) remaining after filtering. Calculation of mapping statistics revealed averages of 19,794 (0.26%), 78 (0.0008%), and 334 (0.005%) reads aligned to the nuclear, mitochondrial, and *Wolbachia* genomes of *D. immitis*, respectively (Table 1). For the domestic dog, an average of 10,235,658 (99.7%) reads aligned to the nuclear genome and an average of 410 (0.004%) reads aligned to the mitochondrial genome (Table 1). Sequencing coverage analysis from 1 Gb data per sample revealed

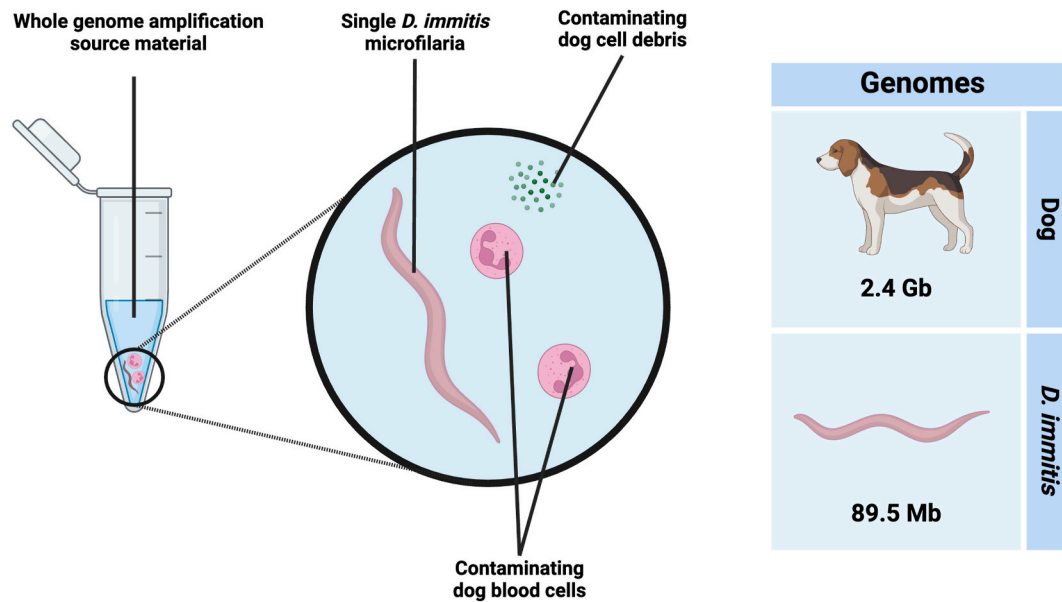


Fig. 3. The presence of contaminating dog blood cells in single *Dirofilaria immitis* microfilaria samples results in a predominance of dog sequencing reads (>98%). Created with [BioRender.com](https://www.biorender.com).

average coverages of <1 for all genomes of *D. immitis* (nuclear, mitochondrial, and *Wolbachia*) and the nuclear genome of the domestic dog (Fig. 2, Supplementary Table S3). An average coverage of 2.87 was obtained for the mitochondrial genome of the domestic dog (Fig. 2, Supplementary Table S3).

4. Discussion

Recent advancements in high-throughput sequencing technologies have made genome-wide approaches more accessible for global parasite population studies (Doyle and Cotton, 2019). To examine the genetic diversity and connectivity of *D. immitis* populations and the probability of resistance arising worldwide, conducting genome-wide studies using samples from a broad geographical range is imperative. Obtaining microfilariae stages from the blood of infected dogs is the most convenient and ethical sampling method for *D. immitis*. Here, we have obtained the DNA of multiple single *D. immitis* microfilariae using a whole-genome amplification (WGA) technique. WGS of the DNA resulted in an overwhelming proportion of sequencing reads aligning to the dog host genome, with poor proportional coverage of the nuclear, mitochondrial, and *Wolbachia* endosymbiont genomes of *D. immitis*. Similar outcomes were observed in Gandasegui et al. (2023) where pooled microfilariae samples collected from dog blood generated a high proportion of dog reads and low numbers of *D. immitis* reads.

The high level of host contamination in our data was not attributed to cross-contamination or contamination of the laboratory environment as qPCR of the negative control revealed no presence of dog or *D. immitis* DNA. Instead, host contamination in the microfilariae samples can be explained by residual dog blood cells and the considerable differences in genome sizes. Despite efforts to obtain clean, blood-free microfilariae, the final samples still contained residual dog blood cells or debris (Fig. 3). Since the dog genome (2.4 Gb) is over 26 times larger than that of *D. immitis* (89.5 Mb), it is plausible that indiscriminate amplification during WGS led to a predominance of dog reads in the data (Gandasegui et al., 2023).

Other techniques have previously been used to separate microfilariae from host blood components or preferentially amplify the microfilariae DNA. In Franks and Stoll (1945), centrifugation and saponin were used to draw off the white blood cell layer and haemolyse red blood cells to obtain pure *D. immitis* microfilariae from dog blood. WGS of *D. immitis*

microfilariae purified using the saponin approach has previously resulted in successful sequencing of the target genome (Doyle et al., 2019; Shin et al., 2020). Nevertheless, it is recommended to test whether the saponin method has indeed removed all host components before proceeding with WGS. This can be done using DNA quantification tools such as qPCR. Other studies have used saponin for the initial lysis of red blood cells, followed by microbial and digestive enzymes to lyse the remaining cellular components (Sawyer and Weinstein, 1963). Alternatively, Taylor (1960) collected *D. immitis* microfilariae in serum separated from clotted blood. Additional studies conducting WGS on microfilariae obtained via these alternative purification techniques are required to evaluate their effectiveness in the removal of host cell components. Unlike the metagenomic approach used in the current study, a selective whole genome amplification (sWGA) technique was employed by Small et al. (2019) to conduct a genome-wide study on *Wuchereria bancrofti*, a filarial nematode causing lymphatic filariasis. The sWGA technique specifically enriched *W. bancrofti* DNA in single microfilariae isolated from human blood whilst limiting extraneous amplification of the host genome. By doing so, 42 genomes of *W. bancrofti* were obtained and 90% of the sequencing reads were mapped to the target parasite genome, highlighting its potential use for *D. immitis*.

Despite the relative ease of collecting blood samples from *D. immitis*-infected dogs, there are clear challenges in obtaining sufficient DNA from single microfilariae. The most reliable method for obtaining high coverage of the *D. immitis* genome while minimising host contamination involves using DNA extracted from adult worms, as demonstrated previously (Gandasegui et al., 2023; Godel et al., 2012; Gomes-de-Sá et al., 2022; Lau et al., 2021). Although promising, the difficulty of obtaining adult *D. immitis* must be considered when conducting population genomic studies on a global scale. Since the adult stages typically reside in the pulmonary arteries, extraction of these specimens requires invasive surgery or necropsy, which is not usually feasible (Wolstenholme et al., 2015). This limitation may restrict the sampling size and narrow the geographical sampling range.

5. Conclusions

This study reveals the challenges encountered when conducting genome-wide studies on single *D. immitis* microfilariae in the presence of extraneous host DNA. Further improvements to remove contaminating

host DNA prior to amplification, or selective amplification of the target DNA, will likely improve the viability of the use of microfilariae for WGS. While this study did not obtain sufficient enrichment of sequencing reads targeting the *D. immitis* genome, a genome-wide population study of *D. immitis* was successfully conducted by Gandasegui et al. (2023) using 31 samples from Australia, Italy, and the USA. These samples were comprised of adult worms, single microfilariae, and pooled microfilariae. A unique opportunity exists for future studies to build on this existing collection by sequencing additional *D. immitis* specimens from around the world, which could utilise optimised WGS of microfilariae. Of particular interest will be *D. immitis* genomes from previously unexplored regions such as Asia, Latin America, and Africa.

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Animal ethics

The research described in this study was approved by the University of Sydney's Animal Research Committee (Protocol Number: 2020/1746).

CRediT authorship contribution statement

Rosemonde I. Power: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Stephen R. Doyle:** Writing – review & editing, Validation, Supervision, Resources, Methodology. **Jan Šlapeta:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Raw FastQ sequences generated in this study are available at SRA NCBI BioProject: PRJNA1097760. Code and analyses are available at https://github.com/rosemondempower/heartworm_mf.git.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exppara.2024.108806>.

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———— Chapter 4 ————

General discussion

4.1 Thesis overview and significance

The macrocyclic lactone (ML) drug class has been the cornerstone of heartworm prevention for the past 30-40 years. Widespread use of these MLs can lead to the selection of parasite resistance—a problem already identified in the USA (Pulaski et al., 2014). In response to emerging reports of apparent ineffectiveness of heartworm prevention, various phenotypic and genotypic resistance screening tools have been developed. This includes an ante-mortem phenotypic tool called the 7-day in-vivo microfilarial suppression test (MFST), which uses microscopy (i.e., the modified Knott's test) to measure reductions in microfilaremia after microfilaricidal treatment (Geary et al., 2011). Genotypic tools include a set of clinically validated single nucleotide polymorphisms (SNPs) strongly correlated with resistance (Ballesteros et al., 2018; Bourguinat et al., 2017). Prior to this thesis, a study in Queensland (QLD), Australia found heartworm-positive dogs despite claims of 'rigorous' heartworm prevention (Nguyen et al., 2016). Such findings have certainly stirred suspicion about the possible emergence of resistance in Australia. Yet, no phenotypic testing and only limited genotypic testing have been conducted to determine whether resistance has arisen in the country (Lau et al., 2021; Nguyen et al., 2016). Early detection of resistance will be essential to managing its spread and prolonging the effectiveness of MLs. We have barely scratched the surface of understanding ML-resistance in Australian heartworms—much remains to be uncovered.

This thesis aimed to discover the origins of Australian heartworms and expose the potential reality of macrocyclic lactone resistance in these populations. Chapter 2.1 worked towards confirming the perceived presence of phenotypic drug resistance in Australian heartworms using traditional microscopy-based tools, with this confirmation being ultimately achieved in Chapter 2.2 using the MFST. Chapters 2.1 and 2.2 revealed that resistance-associated SNPs from the USA were absent in Australian heartworms. Chapter 3.1 discovered that Australian heartworms originated from Asia and possess genomic profiles distinct from those in other regions, including the USA. Finally, Chapter 3.2 highlighted the limitations of whole genome sequencing for microfilarial stages due to contaminating host data. The chapters of this thesis delve into different aspects of heartworm diagnostics and resistance, coming together to form a cohesive narrative that propels our understanding of ML resistance in Australian heartworms.

4.1.1 Confirming the perceived presence of phenotypic drug resistance in Australian heartworms using traditional microscopy-based diagnostic tools

Despite suspicions of ML resistance in Australian heartworms, no phenotypic studies have been conducted to investigate this. In Chapter 2.1, I address these concerns by using the modified Knott's test to enumerate microfilaremia levels in Australian dogs before and after ML treatment (Knott, 1939; Power & Šlapeta, 2022). Unexpectedly, a large portion of dogs demonstrated sub-optimal ($\leq 90\%$)

reductions in microfilaremia ~30 days after treatment. Compared to a previous study where dogs showed microfilariae clearance (>90% reduction) 28 days after receiving the same ML (McTier et al., 2017), the sub-optimal responses observed in Chapter 2.1 were indicative of suspect ML-resistance. While Chapter 2.1 revealed sub-optimal responses of Australian *D. immitis* to MLs, it did not provide definitive evidence of phenotypic ML-resistance due to limitations in sampling and drug administration. Chapter 2.2 directly addressed these shortcomings by conducting the first in-vivo 7-day microfilarial suppression test (MFST) on Australian heartworms (Power & Šlapeta, 2024), adhering to the criteria proposed by Geary et al. (2011). This led to the discovery of two dogs from Townsville, Australia showing <40% reductions in microfilaremia, providing tangible phenotypic evidence of ML-resistance in Australia (Bowman et al., 2015; Geary et al., 2011). Although microfilaremia was eventually cleared in these dogs after 70 days, these delayed clearances, which have also been observed in Greece (Diakou et al., 2018), raise the question of whether true ML-resistance exists in Australia, or if there are fundamental phenotypic differences between Australian and USA heartworms. If fundamental differences do exist, then the MFST developed for USA populations may not yield the same results in Australia.

Recognising the labour-intensive and subjective nature of the traditional modified Knott's test, Chapter 2.1 developed rapid real-time polymerase chain reaction (qPCR) assays targeting *D. immitis*, *Wolbachia*, and dog DNA. By applying these qPCR assays to samples collected before and after ML treatment, I demonstrated that they are comparable to the modified Knott's test for monitoring microfilaremia levels and post-ML responses. These qPCRs were further streamlined into duplex and triplex assays in Appendix 2, which reduced resources and maximised sample throughput (Lau et al., 2024). The duplex qPCR was then implemented in Chapter 2.2, verifying its ability to effectively monitor microfilaremia changes and screen for phenotypic resistance in the field.

4.1.2 Revealing whether Australian heartworms possess genetic signatures associated with drug resistance in the USA

Resistance-associated SNPs have been used for diagnostic screening of resistance in the USA (Fisher et al., 2024) and internationally (Curry et al., 2022). Given the suspicion of resistance in Australia, these SNPs presented an opportunity to screen for genetic signatures of resistance in Australian heartworms. Prior to this thesis, only one study had applied these SNPs to Australian heartworms, which lacked phenotypic data (Lau et al., 2021). In Chapters 2.1 and 2.2, I used an Illumina metabarcoding approach to analyse these SNPs in heartworm-positive dogs from Australia (Power & Šlapeta, 2022, 2024). The results revealed a susceptible genotype in all samples, including the two case study dogs that demonstrated a resistant phenotype in the MFST (see section 4.1). The discrepancy between phenotype and genotype in these two dogs again raises the question of whether the heartworms

were genuinely genetically susceptible, or if fundamental genetic differences between Australian and USA heartworms render these SNPs non-informative in Australia.

Next-generation sequencing has typically been used to target the resistance-associated SNPs, often involving complex workflows and long turnaround times (Ballesteros et al., 2018; Power & Šlapeta, 2022). To overcome this, Chapter 2.2 developed an in-vitro rhAmpTM SNP qPCR assay targeting the two most predictive resistance SNPs (L15709_A and L30575) (Ballesteros et al., 2018; Kumar et al., 2023). Assay performance was validated using synthetic control templates and compared to MiSeq sequencing. The outcome is a simple assay for rapid and accurate genotyping of susceptible, resistant, and heterozygous infections, facilitating early detection and monitoring of resistance. A similar droplet digital PCR (ddPCR) assay has been developed (Kumar et al., 2023) and deployed on USA and European isolates (Kumar et al., 2024), further showcasing the utility and convenience of these SNP PCRs for rapidly genotyping heartworm strains. Given the possible irrelevance of these SNPs outside the USA, I recommend adapting my SNP qPCR from Chapter 2.2 to incorporate new genetic markers that may be identified in the future, particularly those that will be validated in Australian strains.

4.1.3 Uncovering the ancestral origin and genetic background of Australian heartworms using a whole genome sequencing approach

Earlier chapters of this thesis (Chapters 2.1 and 2.2) alluded to potential phenotypic and genotypic differences between Australian and USA heartworms (see Sections 4.1.1 and 4.1.2). Building on this, the first large-scale population genomics study of *D. immitis* ($n = 32$) provided initial evidence of genetic differences between heartworms from Australia, the USA, and Italy (Appendix 3) (Gandasegui et al., 2024). In Chapter 3.1, I conducted whole genome analyses on 127 adult heartworms from Australia, the USA, Europe, Central America, and Asia. The key outcome was the confirmation that heartworms on each continent are genetically distinct from one another, based on their nuclear genomes. Consequently, I posit that the resistance-associated SNPs from the USA are unsuitable for use in Australia and other regions, as previously speculated (Gandasegui et al., 2024). Another key finding was that heartworm was introduced to Australia from Asia, most likely via dingoes, rather than by European colonisation. Finally, it was previously believed that heartworm infections in Sydney originated from QLD (McKeever et al., 2021). Chapter 3.1 challenges this dogma by revealing a highly admixed Australian cohort with a clear separation between Sydney and QLD individuals, indicating the possibility of local relic populations existing in Sydney.

Overall, the population genomics study presented in Chapter 3.1 is one of the most comprehensive for a parasite species, aside from *Haemonchus contortus* (Sallé et al., 2019). All genetic data generated in this study will be publicly available upon publication, enabling researchers to build upon this dataset and further explore the global diversity of heartworm. A notable limitation of my

genome database is the lack of information pertaining to the resistance phenotype for most samples, as many were opportunistically collected from animals with unknown treatment histories.

The challenges of collecting adult heartworm samples (Chapter 3.1) are addressed in Chapter 3.2. In this chapter, I explored whole genome amplification and sequencing of individual microfilariae extracted from the bloodstream (Power et al., 2024). While this approach was more accessible and ethical compared to collecting adult worms, the overwhelming presence of non-target host reads in the data made it unsuitable for genome-wide studies.

4.2 Future directions

This thesis has addressed several knowledge gaps regarding heartworm resistance in Australia and raised many new questions. In this discussion, I propose three key avenues for future research that I believe are crucial for advancing our understanding of heartworm biology and resistance both in Australia and globally.

The need for an experimental infection study in Australia

The MFST and SNPs used in this thesis serve as proxy tools for detecting drug resistance in heartworms. Definitive confirmation of resistance in Australia would require an experimental infection study, identical to those conducted in the USA (see Appendix 1). Once resistance is confirmed in Australia, new local genetic markers correlated with resistance can be identified and used for surveillance, as the USA-based markers may not be applicable abroad. A major limitation that has prevented experimental infection studies from being conducted in Australia is the cost. There are currently no established facilities in Australia for conducting these experiments, and even if such infrastructure is developed, the study would cost at least US \$15,000 (Wolstenholme et al., 2015). An alternative approach would be to ship blood samples from Australia to the USA for experimental infection studies. Although feasible, this comes with additional challenges such as ensuring that microfilariae remain viable throughout transport, and that USA mosquitoes are suitable vectors for Australian isolates. Recent research has demonstrated that immunodeficient mice can sustain heartworm infections, allowing the parasite to enter the heart and develop into adult stages (Hess et al., 2023; Risch et al., 2024). These mice can offer a more ethical in-vivo model for heartworm resistance testing in the future, potentially reducing the use of more sentient species, such as dogs, by $\geq 50\%$ (Dagley et al., 2024).

Global heartworm genome database—The missing pieces of the puzzle

Despite curating the world's largest database of heartworm genomes to date, there are noticeable sampling gaps in heartworm-infected regions such as South America, Africa, and Asia (Fig. 1). In Asia and Africa, cultural factors may limit veterinary necropsies where adult heartworms can be collected. In some regions, dogs are considered a food source (Ekanem et al., 2013; Vu et al., 2021), while in others, such as Muslim countries, they are typically avoided due to perceptions of their uncleanness (Berglund, 2014). In addition, rabies is a major public health risk in both continents which further discourages human contact with dogs (World Health Organization (WHO), 2024). The absence of samples from Africa could simply reflect the limited presence of the parasite, as suggested in Fig. 1. I propose prioritising future sampling in two regions: (1) South America to explore how the unique evolutionary history of South American canids has shaped that of heartworm, and (2) Island Southeast Asia and Melanesia, from which Australian dingoes are believed to have migrated, to further clarify the specific origins of Australian heartworms (Fillios & Taçon, 2016; Koungoulos et al., 2024).

Dirofilaria immitis in dogs in the 21st century

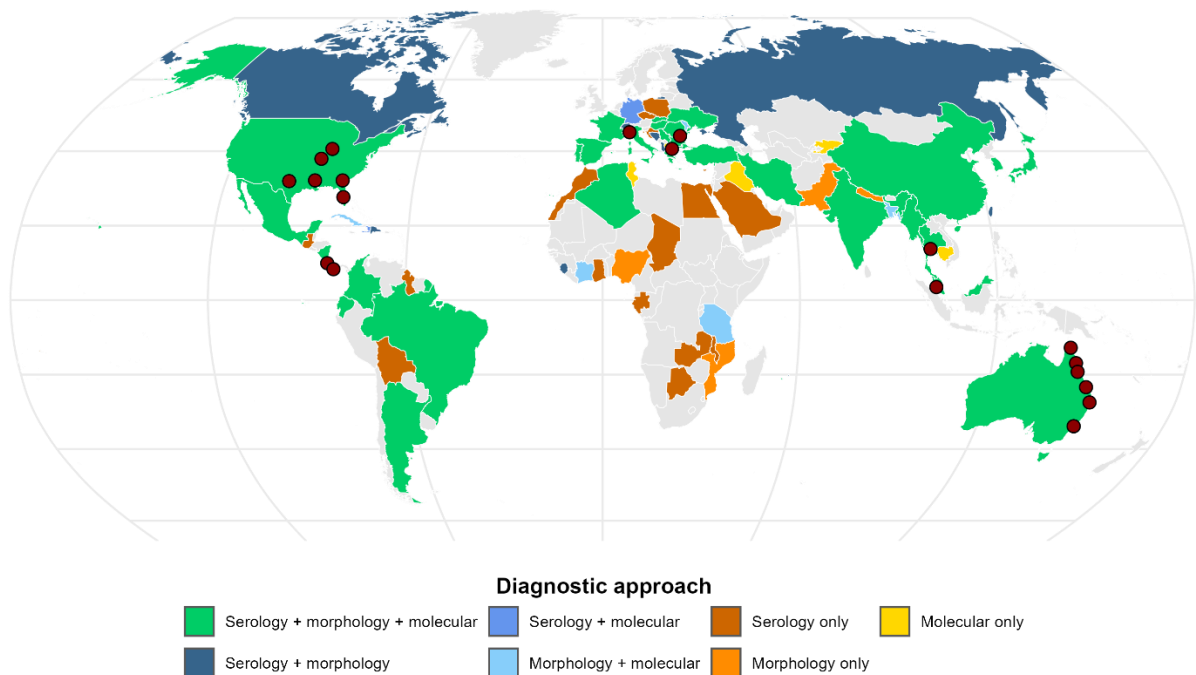


Fig. 1 Geographical distribution of *Dirofilaria immitis* in dogs during the 21st century. Data was systematically collected for each country using the following PubMed search terms: ((*dirofilaria immitis*) OR (canine heartworm) OR (dirofilariasis)) AND (country of interest). Data was categorised by diagnostic approach, i.e., serology, morphology (microfilariae or adult worms), or molecular. Dark red points on the map indicate the adult heartworm specimens collected for genomic analysis in Chapter 3.1.

Elucidating the low mitochondrial diversity of heartworms

In this thesis, heartworm mitochondrial genomes exhibited low genetic diversity and did not align with the continental clustering pattern observed in the nuclear genomes, as initially noted in Appendix 3 (Gandasegui et al., 2024). The cause of this discrepancy remains unclear. Although I theorised in Chapter 3.1 that the low mtDNA diversity might result from a *Wolbachia*-driven mitochondrial selective sweep, this hypothesis has not been formally tested. Validation of a *Wolbachia*-driven mitochondrial sweep requires comparisons of mtDNA diversity between infected and uninfected specimens from a population (Duploux, 2023). These comparisons are not possible for *D. immitis* as it relies on the endosymbiont for survival, and thus all individuals are presumed to be infected. It should be noted that if such a selective sweep exists, it would have occurred very recently across multiple populations, which appears inconsistent with the nuclear genome data. Further investigations are necessary to identify the factors driving reduced mtDNA diversity in heartworms, and it will be interesting to explore whether similar scenarios are observed in other filarial worms.

4.3 Conclusion

ML resistance is a looming threat in Australia and may already be quietly circulating in heartworm populations, yet to be detected. A recent report of resistance in Europe (Traversa et al., 2024), likely originating from the USA, underscores the growing global risk of resistance, with Australia being no exception. Since dogs imported to Australia are not currently required to undergo heartworm testing or treatment prior to entry, the importation of resistant isolates is possible (Department of Agriculture, Fisheries and Forestry [DAFF], 2024). However, it remains uncertain whether Australian mosquitoes would be capable vectors of these foreign *D. immitis* isolates. This thesis successfully discovered an Asian origin for Australian heartworms and uncovered phenotypic evidence of resistance in the country using the USA-based MFST. Yet, this phenotypic evidence of resistance was not supported by USA-based genetic markers. Genome-wide analyses of a global heartworm cohort in this thesis revealed notable genetic differences between heartworm populations across continents, highlighting the limitations of USA-based tools in detecting resistance in other countries. To unequivocally confirm resistance in Australia, we urgently need to conduct experimental infection studies. This will support the development of locally tailored screening tools, allowing for the rapid detection and management of resistance in the future.

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— **Appendix** —

———— **Appendix 1** ————

Dirofilaria immitis isolates from the USA
demonstrating <100% macrocyclic lactone
efficacy in clinical experimental infection studies

Isolate	Origin	ML	Product	Dosage	Efficacy (%)	Reference
AMAL-2014*	AL	MOX	ProHeart® tablets	1 dose (3 µg/kg)	61.6	(McTier, Six, et al., 2017)
Jd2009-2*	AR	IVM	N/A	5 monthly doses (0.007-0.009 mg/kg)	71.3	(Bourguinat et al., 2015)
		IVM	N/A	9 monthly doses (0.006-0.013 mg/kg)	<100	
		MOX	N/A	1 dose (0.17 mg/kg)	21.6	
		MO	N/A	Unpublished	<100	
		SEL	N/A	Unpublished	<100	
JYD-34*	IL	IVM	Heartgard® Plus Chewables	3 monthly doses (6.2-11.9 µg/kg)	29.0	(Blagburn et al., 2016)
		MO	Trifexis® Chewables for Dogs	3 monthly doses (0.5-1.0 mg/kg)	52.2	
		SEL	Revolution®	3 monthly doses (6.6-13.1 mg/kg)	28.8	
		MOX	Advantage Multi® for Dogs	1 dose (2.8-6.7 mg/kg)	100	(McTier, Six, et al., 2017)
		MOX	ProHeart® tablets	1 dose (3 µg/kg)	19	
		MOX	ProHeart® tablets	3 monthly doses (3 µg/kg)	44.4	
		MOX	ProHeart® 6	1 dose (0.17 mg/ kg)	99.5	(Bowman et al., 2017)
		MOX	ProHeart® tablets	1 dose (3 µg/kg)	19	(McTier et al., 2019)
		MOX	ProHeart® tablets	1 dose (6 µg/kg)	25.5	
		MOX	ProHeart® tablets	1 dose (12 µg/kg)	33.3	
		MOX	ProHeart® tablets	1 dose (24 µg/kg)	53.2	(Kryda et al., 2020)
		MOX	ProHeart® tablets	3 monthly doses (3 µg/kg)	44.4	
		MOX	ProHeart® tablets	3 doses (24 µg/kg)	98.8	
		MOX	ProHeart® tablets	3 doses (40 µg/kg)	100	(McTier et al., 2021)
		MOX	ProHeart® tablets	3 doses (60 µg/kg)	100	
		MOX	N/A	4 monthly doses (24 µg/kg)	95.9	
		MOX	N/A	6 monthly doses (24 µg/kg)	99.3	(McTier et al., 2021)
		IVM	Heartgard® Plus	6 monthly doses (6.0-11.8 µg/kg)	63.9	
		MO	Interceptor® Plus	6 monthly doses (0.5-1.0 mg/kg)	54.6	
		MOX	ProHeart® 12	1 dose (0.5 mg/kg)	100	

		MOX	ProHeart® 12	1 dose (0.5 mg/kg)	98.3	
		IVM	Heartgard® Plus	6 monthly doses (6.5-11.5 µg/kg)	10.5	
		IVM	Heartgard® Plus	12 monthly doses (6.5-11.5 µg/kg)	37.7	
		MO	Interceptor® Plus	6 monthly doses (0.51-1.1 mg/kg)	14.6	
		MO	Interceptor® Plus	12 monthly doses (0.51-1.1 mg/kg)	34.9	
		MOX	N/A	3 monthly doses (30 µg/kg)	97.9	(Martin et al., 2024)
		MOX	N/A	3 monthly doses (50 µg/kg)	99	
		MOX	N/A	1 dose (100 µg/kg)	91.1	
		MOX	N/A	5 monthly doses (9 µg/kg)	98.8, 94.1	
		MOX	N/A	5 monthly doses (6 µg/kg)	83.3, 90.2	
LSU 10*	LA	IVM	Ivomec® 1% Injection	6 monthly doses (12 µg/kg)	<100	(Pulaski et al., 2014)
LSU 10-II*	LA	IVM	Ivomec® 1% Injection	3 monthly doses (24 µg/kg)	<100	
LSU 13*	LA	IVM	Ivomec® 1% Injection	6 monthly doses (12 µg/kg)	<100	
Metairie-2014**	LA	IVM	Heartgard® Plus	6 doses, monthly (weight-appropriate dose)	N/A	(Maclean et al., 2017)
MP3	GA	IVM	Heartgard® Plus Chewables	1 dose (6.09-11.90 µg/kg)	95.6	(Blagburn et al., 2011)
		MO	Interceptor® Flavor Tabs	1 dose (0.53-1.00 mg/kg)	95.4	
		SEL	Revolution®	1 dose (6.61-12.97 mg/kg)	95.5	
		MOX	Advantage Multi® for Dogs	1 dose (3.03-6.86 mg/kg)	100	
		MO	Interceptor® Flavor Tabs	1 dose (0.5-0.75 mg/kg)	<100	(Snyder, Wiseman, Cruthers, et al., 2011)
		IVM	Heartgard® Plus Chewables	1 dose (6-9 µg/kg)	<100	
		MO	N/A	1 dose (0.5-0.75 mg/kg)	98.9-99%	(Snyder, Wiseman, Bowman, et al., 2011)
		MO	N/A	2 monthly doses (0.5-0.75 mg/kg)	99.6-100	
		MO	N/A	3 monthly doses (0.5-0.75 mg/kg)	100	
		MOX	ProHeart® tablets	1 dose (3 µg/kg)	100	(McTier, Six, et al., 2017)
Td2008-2*	LA	IVM	N/A	9 monthly doses (0.006-0.013 mg/kg)	23.8	(Bourguinat et al., 2015)
		MO	N/A	Unpublished	<100	
		SEL	N/A	Unpublished	<100	
Yazoo-2013**	MS	IVM	Heartgard® Plus	6 doses, monthly (weight-appropriate dose)	N/A	(Maclean et al., 2017)
ZoeLA*	LA	MOX	ProHeart® tablets	1 dose (3 µg/kg)	54	(McTier, Six, et al., 2017)

		MOX	ProHeart® tablets	1 dose (3 µg/kg)	44.4	(McTier et al., 2019)
		MOX	ProHeart® tablets	1 dose (60 µg/kg)	88.2	
		MOX	ProHeart® tablets	3 doses (24 µg/kg).	99.5	
		MOX	ProHeart® tablets	3 doses (40 µg/kg).	99.5	
		MOX	ProHeart® tablets	3 doses (60 µg/kg).	100	
		MOX	N/A	4 doses, monthly (24 µg/kg)	96.8	(Kryda et al., 2020)
		MOX	N/A	6 doses, monthly (24 µg/kg)	96.1	
		IVM	Heartgard® Plus	6 doses, monthly (6.2-15.5 µg/kg)	18.7	
		MO	Interceptor® Plus	6 doses, monthly (0.5-1.2 mg/kg)	21.2	(Myers et al., 2022)
		MOX	Simparica Trio®	6 doses, monthly (23.1-44.9 µg/kg)	97.2	
		IVM	Heartgard® Plus	6 doses, monthly (5.6-11.8 µg/kg)	8.5	
		MO	Interceptor® Plus	6 doses, monthly (0.5-1.0 mg/kg)	35.9	
		MOX	ProHeart® tablets	1 dose (3 µg/kg)	82.7	(McTier, Six, et al., 2017)
		MOX	ProHeart® 6	1 dose (0.17 mg/kg)	0	

ZoeMO*	IL	MOX	ProHeart® SR-12	1 dose (0.5 mg/kg)	19	(McTier, Pullins, et al., 2017)
		MOX	N/A	1 dose (0.25 mg/kg)	14	
		MOX	ProHeart® tablets	1 dose (3 µg/kg)	82.1	(McTier et al., 2019)
		MOX	ProHeart® tablets	3 doses (24 µg/kg)	99.5	
		MOX	ProHeart® tablets	3 doses (40 µg/kg)	100	
		MOX	ProHeart® tablets	3 doses (60 µg/kg)	100	
		MOX	ProHeart® tablets	3 doses (60 µg/kg)	100	
		MOX	ProHeart® tablets	3 doses (60 µg/kg)	100	

* Considered phenotypically resistant

** Considered phenotypically resistant but was not confirmed via necropsy

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———— Appendix 2 ————

Exploring multiplex qPCR as a diagnostic tool for detecting microfilarial DNA in dogs infected with *Dirofilaria immitis*: A comparative analysis with the modified Knott's test

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Rosemonde Isabella Power in the preparation and submission of the following manuscript:

Lau, D. C.-W., Power, R. I., & Šlapeta, J. (2024). Exploring multiplex qPCR as a diagnostic tool for detecting microfilarial DNA in dogs infected with *Dirofilaria immitis*: A comparative analysis with the modified Knott's test. *Veterinary Parasitology*, 325, 110097.
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Contribution to the manuscript of all authors is as follows:

Task	Contributing co-authors
Conceptualisation	DC-WL, RIP, JŠ
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Formal analysis	DC-WL, RIP
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Methodology	DC-WL, RIP
Supervision, discussion and consultation	JŠ
Preparation of figures used in the manuscript	DC-WL
Writing of the original draft	DC-WL
Critical review and editing of the manuscript	DC-WL, RIP, JŠ

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct.

Kind regards,

Professor Jan Šlapeta

26th September 2024



Exploring multiplex qPCR as a diagnostic tool for detecting microfilarial DNA in dogs infected with *Dirofilaria immitis*: A comparative analysis with the modified Knott's test

Daisy Ching-Wai Lau^a, Rosemonde Isabella Power^a, Jan Šlapeta^{a,b,*}

^a Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales 2006, Australia

^b The University of Sydney Institute for Infectious Diseases, New South Wales 2006, Australia

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ABSTRACT

Current recommendations to diagnose cardiopulmonary dirofilariosis in dogs caused by *Dirofilaria immitis* involves tandem antigen and circulating microfilariae tests. The modified Knott's test is an important tool in heartworm diagnosis, allowing identification of circulating microfilariae. However, the subjective nature of the modified Knott's test affects its accuracy and diagnostic laboratories usually do not provide a quantitative outcome. Quantitative enumeration of microfilariae enables clinicians to track treatment progress and acts as a proxy for detecting emerging macrocyclic lactone resistance. There is a need for better diagnostic tools suitable for routine use to efficiently and accurately quantify the presence of *D. immitis* microfilaremia. The aim of this study was to determine whether the quantitative modified Knott's test can be substituted by multiplex quantitative polymerase chain reaction (qPCR) targeting *D. immitis* and associated *Wolbachia* endosymbiont DNA in canine blood samples. To do this, genomic DNA samples (n = 161) from Australian dogs, collected as part of a previous 2021 study, were assessed in a TaqMan qPCR targeting DNA of *D. immitis*, *Wolbachia* sp. and *Canis lupus familiaris*. Of the 161 genomic DNA samples, eight were considered positive for *D. immitis* microfilariae. The qPCR assay demonstrated good efficiency (E = 90 to 110%, R² > 0.94). Considering the performance and efficient use of bench time, this TaqMan qPCR assay is a suitable alternative to the modified Knott's test for quantitative enumeration of microfilariae (Cohen's kappa coefficient [κ]: κ = 1 using *D. immitis* qPCR marker, κ = 0.93 using *Wolbachia* qPCR marker). The qPCR data demonstrated a comparable result to that of the quantitative modified Knott's test in a 2022 survey of *D. immitis* in Australian dogs (n = 23) before and after macrocyclic lactone (ML) administration. Improving the detection and diagnosis of canine heartworm infections will assist veterinarians in better managing and controlling disease outcomes and will be valuable for tracking the spread of ML resistance in Australia.

1. Introduction

Dirofilaria immitis (canine heartworm) is a widespread mosquito-borne filarial nematode of veterinary importance (Noack et al., 2021; Simón et al., 2012). Adult heartworms colonise the pulmonary arteries, and in more severe cases, the right ventricle, which can cause fatal cardiopulmonary disease if left untreated (Bowman and Atkins, 2009). Canine heartworm infection is commonly diagnosed using tests detecting *D. immitis* antigen in the blood of dogs or by identifying circulating *D. immitis* microfilariae (Little et al., 2018; Power and Šlapeta, 2022). The modified Knott's test is routinely used by diagnostic laboratories to detect microfilaremia of *D. immitis*, and species-level identification is

possible by identifying specific morphometric features (Geary et al., 2011; Rojas et al., 2015). The modified Knott's test is a concentration technique that has high sensitivity compared to a direct smear or buffy coat examination (Martini et al., 1991; Ranjbar-Bahadori et al., 2007). The modified Knott's test, however, is labour-intensive, potentially subjective and the accuracy of results varies depending on the skill of the technician (Magnis et al., 2013). Due to increasing canine dirofilariosis reports in both endemic and non-endemic areas and emerging resistance to common preventatives, alternative molecular diagnostic methods such as the use of polymerase chain reaction (PCR) have gained more attention (Bourguinat et al., 2015; Diakou and Prichard, 2021; Pulaski et al., 2014; Simón et al., 2012). There is a need for a more rapid and

* Corresponding author at: Sydney School of Veterinary Science, Faculty of Science, University of Sydney, New South Wales 2006, Australia.

E-mail address: jan.slapeta@sydney.edu.au (J. Šlapeta).

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cost-effective diagnostic tool suitable for routine use in diagnostic laboratories to efficiently and accurately quantify the presence of *D. immitis* microfilaremia.

Using qPCR, we can directly target *D. immitis* DNA, but *D. immitis* is also one of several parasitic filarial nematodes from the family Onchocercidae that harbour endosymbiotic intracellular bacteria from the genus *Wolbachia* (Sironi et al., 1995). *Wolbachia* is involved in the immunopathogenesis of *D. immitis* infections and is essential for its maturation, long-term survival, and fertility (McHaffie, 2012). Large colonies of *Wolbachia pipientis* are located in the hypodermal lateral chords of male and female worms (Kozek, 2005; Kramer et al., 2003). The bacteria also take residence in the reproductive tract of females, oocytes, and circulating microfilariae (McHaffie, 2012; Taylor and Hoerauf, 1999). Targeting *Wolbachia* sp. and *D. immitis* DNA using multiplex qPCR has been shown to be an efficient tool for detecting canine heartworm infection (Crowder et al., 2012; Laidoudi et al., 2020; Negron et al., 2022; Simsek and Ciftci, 2016). However, there is no information about direct comparison between quantification using qPCR and quantification via the modified Knott's test.

Quantitative enumeration of microfilariae has recently gained prominence even in clinical practice (Geary et al., 2011). The macrocyclic lactone (ML) endectocides for the prevention of canine heartworm infection must be 100% efficacious to be registered (Hampshire, 2005). However, presence of ML-resistant *D. immitis* clinical isolates in the USA have been confirmed (Bourguinat et al., 2015; Diakou and Prichard, 2021; Pulaski et al., 2014). Emergence of ML-resistance is a notable concern for the veterinary industry as a loss of ML efficacy compromises canine health. A definitive test for ML resistance does not yet exist, but the microfilarial suppression test (MFST), initially proposed by Geary et al. (2011), can be used to presumably diagnose phenotypic ML resistance. The MFST requires quantification of circulating *D. immitis* microfilariae before and after ML administration (at microfilaricidal dose) two to four weeks apart (Prichard, 2021). An observation of < 90% reduction of circulating *D. immitis* microfilariae has been clinically validated as suspect ML-resistance in association with single nucleotide polymorphisms (SNP) that were highly predictive for ML resistance in US isolates (Ballesteros et al., 2018; Bourguinat et al., 2011; Bourguinat et al., 2015).

The objective of this study was to determine whether the quantitative modified Knott's test can be substituted by multiplex qPCR targeting *D. immitis* and associated *Wolbachia* endosymbiont DNA in canine blood samples for a more rapid and accurate measurement of microfilariae levels. To do so, a TaqMan quantitative PCR (qPCR) assay targeting *D. immitis* DNA, *Wolbachia* sp. of *D. immitis* DNA and dog DNA was used to quantify microfilariae DNA in samples from dogs in Australia.

2. Material and methods

2.1. Sample collection

In Panetta et al. (2021), blood samples were collected from shelter dogs in Queensland, Australia during 2020. Genomic DNA (gDNA) was isolated from 200 µL of frozen whole blood using the mammalian blood protocol of the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia). After elution into 100 µL, 161 of these gDNA samples were stored at -20 °C before being reused in the current study. gDNA of adult *D. immitis* (138 ng/L) collected from adult dogs from Sydney, Australia were used as positive controls for calibration (Lau et al., 2021).

2.2. TaqMan quantitative PCR targeting *D. immitis*, *Wolbachia* sp. of *D. immitis* and *Canis lupus familiaris*

TaqMan qPCR targeting the cytochrome c oxidase subunit 1 gene (*cox1*) of *D. immitis* was carried out using primers and probe as previously described by Panetta et al. (2021). The qPCR targeting the *ftsZ* gene of *Wolbachia* spp. associated with *D. immitis* was performed

following the methodology described by Laidoudi et al. (2020). The presence of canine DNA was verified by amplifying the partial canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as previously described (Orr et al., 2020; Panetta et al., 2021). The primers for *cox1*, *ftsZ* and GAPDH were each labelled with a different coloured fluorophore of FAM, HEX and Cy5, respectively.

2.3. Relative quantification of DNA using quantitative simplex, duplex and triplex TaqMan qPCR reactions

All qPCR reactions were prepared using the Myra Liquid Handling System (Bio Molecular Systems, Australia). Each simplex, duplex and triplex qPCR reaction was assessed with 10-fold serial dilutions of a known concentration (138 ng/L) of adult *D. immitis* DNA and with nuclease-free water as a negative (no template) control. The final qPCR reaction mixture for the 161 samples was carried out in 10 µL final volumes of Luna® Universal Probe qPCR Master Mix (New England Biolabs, Australia) containing 1 µL of sample DNA, forward and reverse primers at a final concentration of 400 nM and probe at 100 nM. The PCR cycling conditions included an initial denaturation step at 95 °C, 1 min; followed by 40 two-step cycles of 95 °C for 15 sec and 60 °C for 30 sec. qPCR testing was performed using a CFX96 Touch™ Real-Time PCR Detection System with the corresponding CFX Manager v.3.1 software (BioRad Laboratories Pty Ltd.) to construct standard curves from serial dilutions from the adult *D. immitis* DNA (138 ng/L) and to obtain efficiency (*E*) and *R*² values. qPCR assays were deemed optimised if *E* ranged from 90 to 110% and *R*² > 0.94. Default settings were used to automatically determine the qPCR threshold and the threshold cycle (*C*_q)-values were recorded for each sample. Samples that amplified with a *C*_q-value < 40 were considered positive. Samples were run simultaneously to eliminate the issue with qPCR run-to-run variability.

2.4. TaqMan duplex qPCR targeting *D. immitis* and *Wolbachia* spp. of *D. immitis* with maximal amount of source DNA

To evaluate the impact of DNA template concentration (volume) in the qPCR reaction, the qPCR run protocol in 2.3 was repeated for the eight samples that were positive using the modified Knott's test from the study by Panetta et al. (2021) with the maximum DNA template volume (3.2 µL) in the final qPCR reaction (10 µL). An additional 15 samples that were negative using the modified Knott's test in Panetta et al. (2021) were run using SensiFAST Probe No-ROX Mix (BioLine, Australia).

2.5. TaqMan duplex qPCR targeting *D. immitis* and *Wolbachia* spp. on canine blood samples before and after macrocyclic lactone administration

Samples collected from 23 dogs before and after the administration of doxycycline and macrocyclic lactone (ML) in Power and Šlapeta (2022) were reused in this study. Sixteen of these dogs previously failed to reduce their microfilaremia by >90% after ML treatment using the modified Knott's test but were shown to harbour 7 ML-susceptible SNPs of interest on loci L9400, L15709_A, L20587, L21554, L30575, L42411 and L45689. Six samples were shown to reduce microfilariae by >90%. The remaining sample was amicrofilaremic both before and after ML treatment.

These privately-owned dogs with confirmed or suspect *D. immitis* infections were sampled in Queensland and New South Wales, Australia between 2019 and 2022. Doxycycline antibiotic and ML (HeartGard Plus or ProHeart SR-12) were administered at diagnosis, followed by adulticide injections as per the routine treatment protocol for canine heartworm disease. Blood samples were collected before and after the doxycycline and ML administration. DNA was isolated from 100 µL of each whole blood sample using the mammalian blood protocol of the Monarch® Genomic DNA Purification Kit (New England Biolabs, Australia) and eluted into 100 µL. Eluted DNA was stored at -20 °C until reuse in the current study. Duplex TaqMan qPCR reactions were

performed in triplicate on 46 of these DNA samples from 23 dogs using the run protocol in 2.3 with SensiFAST Probe No-ROX Mix (BioLine, Australia). The arbitrary qPCR baseline thresholds were set to 500 and 100 RFU for the quantification of *D. immitis* and *Wolbachia* DNA, respectively. To evaluate the sensitivity and specificity, we considered only those samples with an initial count of > 1000 microfilariae / mL. This was to address possible inconsistencies with the morphological count in the modified Knott's test and homogenise the data for comparison.

2.6. PCR targeting 7 SNPs associated with ML-resistance in USA

PCR assays using the SensiFAST™ SYBR® No-ROX Kit (BioLine, Australia) were performed on five samples from the study by Panetta et al. (2021) (samples 1847-13, 1847-15, 2216-4, 3072-2, 3072-15) including a positive adult *D. immitis* DNA control to amplify the 7 SNP regions associated with ML-resistance (L9400, L15709_A, L20587, L21554, L30575, L41411 and L45689) according to Bourguinat et al. (2015) and as implemented by Power and Šlapeta (2022). The final qPCR reaction mixture was carried out in 25 µL final volumes containing 2.5 µL of sample DNA, and forward and reverse primers at a final concentration of 100 nM. The number of cycles was optimised empirically to ensure that each sample avoided the plateau phase of amplification. Reaching the exponential phase of amplification limits potential bias resulting from template saturation (Power et al., 2021). The cycling conditions included an initial denaturation at 95 °C for 3 min followed by 28–40 cycles of 95 °C for 5 s, 55 °C for 10 s and then 72 °C for 20 s with a final melt curve. The melt curve was used to verify the presence of a single desired PCR product. Next generation sequencing (NGS) using Illumina MiSeq v2 250 PE was performed at the Ramaciotti Centre for Genomics, University of New South Wales, Australia. FastQ sequences were deposited at SRA NCBI BioProject: PRJNA1015377.

2.7. SNP analysis of NGS samples

FastQ files containing Illumina amplicon data amplified by PCR for each of the seven SNP loci were analysed with RStudio Version 2023.03.1 + 446 and Cutadapt 4.0 (Martin, 2011) to remove primer sequences. The dada2 pipeline (package version 1.26.0) (Callahan et al., 2016) was used to infer the *D. immitis* SNP amplicon sequence variants (ASVs) present in the DNA samples. The dada2 pipeline (1.16) (<https://benjjneb.github.io/dada2/tutorial.html>) as well as workflow implemented by Power and Šlapeta (2022) was followed. Quality of reads were checked with the “plotQualityProfile” tool. Forward and reverse FastQ reads were filtered and trimmed to 180 and 170 nucleotides, respectively, with the “filterAndTrim” tool. From the input reads, > 65%, > 90%, 96%, > 63%, > 64%, > 95% and > 98% were retained and

0.00%, 0.00%, 0.00%, 0.06%, 0.00%, 0.00% and 0.27% of merged reads were removed as chimeras for L9400, L15709_A, L20587, L21554, L30575, L41411 and L45689, respectively. To address potential sample bleeding issues, the proportion of ASVs representing < 2.5% in a sample were removed (Mitra et al., 2015). A reference table containing options for each possible SNP polymorphism at the 7 SNP loci being assessed was created. This reference table was imported into the dada2 package and using the “AssignSpecies” tool, assignments were made between ASVs and the reference sequence if there was exact matching. The CLC Main Workbench (version 22.0) (CLC bio, Qiagen, Australia) was used to visualise the ASVs against their respective SNP reference sequences.

3. Results

3.1. Triplex qPCR assay showed good efficiency and a high true negative rate when compared with the modified Knott's test

The design of each qPCR assay including the simplex qPCR targeting *D. immitis* or *Wolbachia* sp., duplex qPCR targeting *D. immitis* and *Wolbachia* sp. and triplex qPCR targeting *D. immitis*, *Wolbachia* sp. and dog DNA was assessed with 10-fold serial dilutions of a known DNA concentration of adult *D. immitis* DNA collected from a dog in the study by Lau et al. (2021). All qPCR assays, including the triplex assay, showed good efficiency with $E = 90$ to 110% and $R^2 > 0.94$.

Canine blood DNA samples (n = 161) from Panetta et al. (2021) were assessed with the triplex qPCR including eight samples that were considered positive and 15 samples that were considered negative for *D. immitis* microfilariae using the modified Knott's test (Table 1). Initially when we used 1/10 (1 µL) of the qPCR volume (10 µL) for the DNA template, only 4/8 and 2/8 *D. immitis* microfilariae-positive samples returned C_q -values for *D. immitis* and *Wolbachia* DNA, respectively. Increasing the content of the template to 3.2 µL in the qPCR resulted in C_q -values for *D. immitis* DNA in all eight samples and C_q -values for *Wolbachia* DNA in seven samples (Table 1). Selection of 15 modified Knott's test-negative samples were negative for *D. immitis* and *Wolbachia* DNA in the qPCR using 3.2 µL of template. Dog DNA was detected in 158/161 (98%) of the samples.

To evaluate potential ML-resistance associated *D. immitis* SNPs, five of the eight modified Knott's test-positive samples (microfilariae count 28–3208 microfilariae/ mL) from the study by Panetta et al. (2021) underwent Illumina NGS amplicon metabarcoding at 7 SNPs of interest on L9400, L15709_A, L20587, L21554, L30575, L41411 and L45689 loci. All five samples and their 7 SNPs matched the ML ‘susceptible’ reference sequence (Supplementary Information S1).

Analyses of the performance of the TaqMan triplex qPCR assay was compared with the modified Knott's test by calculating the Cohen's kappa coefficient (κ) to assess test agreement. Calculations revealed

Table 1
Quantification of DNA amplified from triplex qPCR using eight samples positive on the modified Knott's test from dogs in Australia.

Sample	Mff/ mL	<i>D. immitis</i> DNA quantity (<i>cox1</i>) 1 µL*	<i>D. immitis</i> DNA quantity (<i>cox1</i>) 3.2 µL*	<i>Wol</i> sp. DNA quantity (<i>ftsZ</i>) 1 µL*	<i>Wol</i> sp. DNA quantity (<i>ftsZ</i>) 3.2 µL*
2212-4	2	-	0.06342	-	0.00693
1847-13	948	0.00000	0.00855	-	0.00020
1847-15	28	-	0.00706	-	0.00046
1847-17	2	-	0.00075	-	-
2216-4	3208	0.36539	0.29495	0.00002	0.01558
3072-2	2600	0.37011	0.30605	0.00012	0.03110
3072-15	3040	0.00466	0.13284	-	0.00540
3072-31	26	-	0.00140	-	0.00026

Abbreviations: Microfilariae (Mff); *Wolbachia* (Wol)

* Total volume of the qPCR reaction was 10 µL

Table 2
Quantification of *D. immitis* DNA and microfilariae numbers before and after ML treatment.

Dog	Mff count before	Mff count after	% change	Mean mff (rq) before	Mean mff (rq) after	% change	Mean Wol (rq) before	Mean Wol (rq) after	% change
1	1	0	-100.00%	0.00	0.00	N/A	0.00	0.00	N/A
15	380	0	-100.00%	139.07	0.00	-100.00%	0.00	0.00	-100.00%
21	175	0	-100.00%	1.39	2.53	+82.14%	37.41	0.00	+54.11%
38	*1500	0	-100.00%	530.23	0.09	-99.98%	539.87	0.00	-100.00%
16	*7625	600	-92.13%	1140.67	191.90	-83.18%	1124.00	84.50	-92.48%
5	*38250	3512	-90.82%	6670.00	1321.33	-80.19%	7434.00	101.27	-98.64%
33	*1570	470	-70.06%	355.47	234.73	-33.96%	400.13	104.97	-73.77%
13	*7450	2475	-66.78%	5362.33	2579.33	-51.90%	4994.67	77.97	-98.44%
6	*7560	2518	-66.69%	3496.67	1356.00	-61.22%	5334.67	402.80	-92.45%
34	*9400	3180	-66.17%	5278.33	505.70	-90.42%	5158.33	29.80	-99.42%
28	*3180	1100	-65.41%	233.33	487.47	+108.91%	430.90	84.15	-80.47%
27	*3420	1350	-60.53%	210.57	330.07	+56.75%	403.27	59.96	-85.13%
37	*2300	960	-58.26%	654.77	111.73	-82.94%	452.07	42.82	-90.53%
35	*11990	7760	-35.28%	8741.67	480.73	-94.50%	5905.00	51.76	-99.12%
40	*3410	2380	-30.21%	846.63	39.03	-95.39%	634.47	8.12	-98.72%
24	420	300	-28.57%	38.00	52.91	+39.23%	46.89	17.95	-61.72%
4	*2856	2217	-22.37%	1235.67	749.77	-39.32%	2022.67	183.84	-90.91%
3	*9540	8208	-13.96%	718.83	140.93	-80.39%	1281.33	36.52	-97.15%
43	*2590	2590	0.00%	181.60	481.97	+165.40%	149.27	50.49	-66.17%
19	0	0	N/A	0.51	0.00	-100.00%	0.26	0.00	-100.00%
7	38	565	+1386.84%	26.32	417.37	+1485.54%	49.47	56.36	+13.92%
2	510	1436	+181.57%	1143.03	601.67	-47.36%	1076.10	130.55	-87.87%
25	*24800	62160	+150.65%	1176.00	309.10	-73.72%	1180.50	374.60	-68.27%

Abbreviations: Microfilariae (Mff); Wol (Wolbachia); relative quantification unit (rq)
* samples with initial microfilaraemia >1000 mff/ml; all samples are reported as means from the triplicate qPCR

excellent test agreement, $\kappa = 1$ and 0.93 between the qPCR assay (Protocol 2.4) using *D. immitis* and *Wolbachia* as the diagnostic marker, respectively, and the modified Knott's test. Resulting frequencies are available from LabArchives (<https://dx.doi.org/10.25833/225t-dp56>).

3.2. Relative concentration of *D. immitis* DNA is a suitable proxy to the microfilariae suppression test

Quantitative qPCR for *D. immitis* and *Wolbachia* sp. DNA was run in triplicate for all 23 paired samples obtained before and after the initiation of heartworm treatment (Table 2). We focused on the samples with initial microfilaraemia levels >1000 microfilaria/ml ($n = 16$) of canine blood. Of these samples, three were previously shown to reduce microfilariae by >90 % and 13 failed to reduce microfilariae by >90 % (Power and Šlapeta, 2022).

For the three samples that reduced microfilariae by > 90%, qPCR targeting *D. immitis* DNA was in 33% (1/3) agreement; the DNA quantity was reduced by 80%, 83% and 100% (Table 2). For the 13 samples that did not reduce microfilariae by > 90% using the modified Knott's test, there was 77% (10/13) agreement, as these samples reduced the DNA by up to 82% or increased the quantity by 165% (Table 2). Considering the modified Knott's test as the reference test, the qPCR for *D. immitis* had a sensitivity of 33% (95% CI 17% to 88%) and specificity of 77% (95% CI 50% to 92%).

For the three samples that reduced microfilariae by > 90%, qPCR targeting *Wolbachia* sp. DNA was in 100% agreement; the DNA quantity was reduced by 92% to 100% (Table 2). For 13 samples that did not reduce microfilariae by >90% using the modified Knott's test, there was only 39% (5/13) agreement, as these samples reduced the DNA by 66% to 85% (Table 2). Considering the modified Knott's test as the reference test, the qPCR for *Wolbachia* sp. had a sensitivity of 100% (95% CI 44% to 100%) and specificity of 39% (95% CI 18% to 65%). A positive correlation was found between *D. immitis* and *Wolbachia* sp. DNA concentrations (Pearson's correlation coefficient: $r[30] = .92$, $p < 0.0001$). No statistically significant correlations were found between the modified Knott's test and *D. immitis* or *Wolbachia* sp. DNA concentrations.

4. Discussion

The modified Knott's test is a powerful tool that enables detection of circulating microfilariae of *D. immitis* and via extension is used as a proxy to detect emerging ML resistance (Knott, 1939; Power and Šlapeta, 2022). The drawback with the modified Knott's test is that this test is nowadays rarely performed by clinicians and not considered an in-clinic test anymore (Goodwin, 1998; Henry et al., 2018). Even if the modified Knott's test is utilised, diagnostic laboratories do not typically provide a quantitative outcome in some regions, such as Australia. The quantitative modified Knott's test is used as a proxy for ML resistance (Power and Šlapeta, 2022). Despite the test having been suggested by Geary et al. (2011), it has been utilised very rarely and only in research pursuits (Power and Šlapeta, 2022). Data from our study reveals that the qPCR can be used as a suitable proxy to the quantitative modified Knott's test in Power and Šlapeta (2022). While there was discordance between the individual percentage change for *D. immitis* DNA in the qPCR and modified Knott's test results, the qPCR detected 77% of the cases that did not reduce microfilariae by > 90% using the modified Knott's test. The contradicting results between the reduction rates obtained from the modified Knott's test and qPCR are likely attributable to the nature of the two different diagnostic methods. The modified Knott's tests utilised in the study by Power and Šlapeta (2022) to calculate reduction in microfilariae count is based on a microscopy technique which may suffer from some degree of subjectivity and human error, including difficulties with enumeration when microfilariae clump in high-count samples. The kinetics of *D. immitis* DNA in canine blood such as the metabolic duration of DNA from dead microfilariae or the turnover of DNA released from circulating microfilariae may have also

affected microfilarial DNA detected before and after ML treatment (Oi et al., 2015). The sensitivity of the multiplex qPCR assay is influenced by its ability to detect the amount of DNA found in a single *D. immitis* microfilaria. The qPCR technique allows up to a billion-fold amplification of small DNA quantities which can greatly enhance the signal in detecting low microfilariae titres (Sinclair, 2002). Samples 1847–17 and 2212–4 contained 2 microfilariae/ mL as identified by the modified Knott's test (Panetta et al., 2021) and was the lowest microfilariae concentration available in this study for assessing the performance of the multiplex qPCR.

Bacteria of the genus *Wolbachia* have an obligatory endosymbiotic relationship with *D. immitis*, are significantly present in all heartworm stages and are a treatment target in canine heartworm disease (Bandi et al., 2001; Carretón et al., 2020). The detection of *Wolbachia* DNA in 100% (16/16) of dogs with initial microfilariae counts >1000 mff/mL from the study by Panetta et al. (2021), indicates that the molecular detection of this endosymbiont is associated with circulating microfilariae and can serve as a positive control in the multiplex qPCR. Inconsistencies in detecting *Wolbachia* DNA may occur, which can be explained by its weaker infection density during the larval stage, thus limiting DNA detection (Rao et al., 2006). The results from this study suggest that the multiplex qPCR diagnostic test could be used to track the clearance of *Wolbachia*. In dogs naturally infected with *D. immitis*, the successful removal of microfilariae from circulating blood has been linked with the complete clearance of *Wolbachia* DNA (Louzada-Flores et al., 2022). Following doxycycline antibiotic and ML treatment, a >90% reduction in the *Wolbachia* sp. DNA load was observed in 69% (11/16) of dogs with initial microfilariae counts >1000 mff/mL (Power and Šlapeta, 2022). A significant positive correlation was detected between the DNA load of microfilariae and *Wolbachia*. The efficacy of doxycycline against *D. immitis* is likely due to its action against *W. pipiens* in all heartworm stages and gradual elimination of the circulating microfilariae biomass (Carretón et al., 2020; McCall et al., 2014).

Molecular SNP markers highly correlated with ML resistance have been used to differentiate between cases of ML resistance from ML susceptible populations in the USA (Ballesteros et al., 2018). Using the same genetic markers, ML resistance has yet to be detected in Australia (Lau et al., 2021; Power and Šlapeta, 2022). All five samples from the study by Panetta et al. (2021) which underwent SNP analysis for 7 SNP loci associated with ML resistance matched with the ML susceptible reference sequence, but were not subjected to ML phenotypic testing. In addition, marked genetic differences were evident when aligned against the *D. immitis* reference genome characterised from the USA. The population of *D. immitis* isolates in Australia and the USA are subjected to different selection pressures due to geographical and ecological variation, genetic bottleneck, selective sweep, or selection by ML anthelmintics. Population structure and heritability differences could hinder the use of the same molecular markers characterised from the USA to detect ML resistance in Australia. Rather to enable accurate detection of ML resistance in Australia, phenotypic and genotypic data from Australian isolates will be necessary for characterisation of ML susceptibility and resistance in the Australian context. The use of PCR assays is one methodology that can be used to conduct genotypic analysis of individual heartworm cases and will help detect and delineate ML-resistance in *D. immitis* populations.

5. Conclusion

The molecular approach developed herein utilising TaqMan multiplex qPCR technology can deliver analogous results to that of the modified Knott's test. However, to determine the reasons for the discordant results, a large sample size and further replication, including replication of blood sample DNA isolations, is required. The multiplex qPCR represents an alternative to the diagnosis and monitoring of canine dirofilariasis, including quantification in dogs infected with

canine heartworm (<30-day old *D. immitis*) from only 100 µL of whole blood. In addition, some advantages compared to conventional parasitology techniques include high throughput testing and less active bench time. The integration of molecular assays in the diagnosis of *D. immitis* infections provides clinicians with an efficient alternative for primary diagnostic heartworm screening.

CRedit authorship contribution statement

Power Rosemonde Isabella: Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **Lau Daisy Ching-Wai:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Šlapeta Jan:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Editorial Board of Veterinary Parasitology (JS).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.110097.

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———— **Appendix 3** ————

Genome structure and population genomics of the canine
heartworm *Dirofilaria immitis*

Sydney School of Veterinary Science
Faculty of Science
The University of Sydney, NSW
Australia 2006

To whom it may concern:

Subject: Author Attribution Statement for Thesis with Publications

I am writing this letter to stipulate the role of Rosemonde Isabella Power in the preparation and submission of the following manuscript:

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Contribution to the manuscript of all authors is as follows:

Task	Contributing co-authors
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Data collection and curation	RIP, EC, DC-WL, AW, JŠ
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Supervision, discussion and consultation	SRD
Preparation of figures used in the manuscript	JG, SRD
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Critical review and editing of the manuscript	JG, RIP, EC, DC-WL, CMO, AW, RP, JŠ, SRD

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the above authorship attribution statements are correct.

Kind regards,

Professor Jan Šlapeta

26th September 2024



Genome structure and population genomics of the canine heartworm *Dirofilaria immitis*



Javier Gandasegui^a, Rosemonde I. Power^b, Emily Curry^c, Daisy Ching-Wai Lau^b, Connor M. O'Neill^d, Adrian Wolstenholme^d, Roger Prichard^c, Jan Šlapeta^b, Stephen R. Doyle^{e,*}

^a Barcelona Institute for Global Health (ISGlobal), Hospital Clínic – University of Barcelona, Barcelona, Spain

^b Sydney School of Veterinary Science, Faculty of Science, The University of Sydney, NSW, Australia

^c Institute of Parasitology, McGill University, Sainte Anne-de-Bellevue, QC, Canada

^d Department of Infectious Diseases, University of Georgia, Athens, GA 30602, USA

^e Wellcome Sanger Institute, Cambridgeshire CB10 1SA, United Kingdom

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ABSTRACT

The heartworm, *Dirofilaria immitis*, is a filarial parasitic nematode responsible for significant morbidity and mortality in wild and domesticated canids. Resistance to macrocyclic lactone drug prevention represents a significant threat to parasite control and has prompted investigations to understand the genetic determinants of resistance. This study aimed to improve the genomic resources of *D. immitis* to enable a more precise understanding of how genetic variation is distributed within and between parasite populations worldwide, which will inform the likelihood and rate by which parasites, and in turn, resistant alleles, might spread. We have guided the scaffolding of a recently published genome assembly for *D. immitis* (ICBAS_JMDir_1.0) using the chromosomal-scale reference genomes of *Brugia malayi* and *Onchocerca volvulus*, resulting in an 89.5 Mb assembly composed of four autosomal- and one sex-linked chromosomal-scale scaffolds representing 99.7% of the genome. Publicly available and new whole-genome sequencing data from 32 *D. immitis* samples from Australia, Italy and the USA were assessed using principal component analysis, nucleotide diversity (Pi) and absolute genetic divergence (Dxy) to characterise the global genetic structure and measure within- and between-population diversity. These population genetic analyses revealed broad-scale genetic structure among globally diverse samples and differences in genetic diversity between populations; however, fine-scale subpopulation analysis was limited and biased by differences between sample types. Finally, we mapped single nucleotide polymorphisms previously associated with macrocyclic lactone resistance in the new genome assembly, revealing the physical linkage of high-priority variants on chromosome 3, and determined their frequency in the studied populations. This new chromosomal assembly for *D. immitis* now allows for a more precise investigation of selection on genome-wide genetic variation and will enhance our understanding of parasite transmission and the spread of genetic variants responsible for resistance to treatment.

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1. Introduction

The heartworm *Dirofilaria immitis* is a filarial nematode that primarily parasitises domestic and wild canids. Heartworm disease, or dirofilariasis, is caused by the presence of adult worms in

pulmonary arteries that can cause inflammation, vascular dysfunction, and pulmonary hypertension, and circulating microfilariae in peripheral blood that cause acute and chronic inflammatory lesions in the lungs and other organs that, if not treated, can progress to congestive heart failure (Ames and Atkins, 2020). The mosquito-borne *D. immitis* can infect a wide range of other mammals and can be zoonotic (McCall et al., 2008), although human infections are relatively rare and are typically asymptomatic (Lee et al., 2010; Dantas-Torres and Otranto, 2013).

Prevention and control of dirofilariasis rely on preventive chemotherapy (PC) using anthelmintics, primarily macrocyclic lactones (MLs) (McCall, 2005). Treatment of the definitive host aims to target the larval stages of the parasite after infection and

* Corresponding author at: Wellcome Sanger Institute, Cambridgeshire CB10 1SA, United Kingdom.

E-mail addresses: javier.gandasegui@isglobal.org (J. Gandasegui), rosemonde.power@sydney.edu.au (R.I. Power), emily.curry@mail.mcgill.ca (E. Curry), dlau9325@uni.sydney.edu.au (D.C.-W. Lau), connoroneill1225@gmail.com (C.M. O'Neill), adrianw1906@gmail.com (A. Wolstenholme), roger.prichard@mcgill.ca (R. Prichard), jan.slapeta@sydney.edu.au (J. Šlapeta), stephen.doyle@sanger.ac.uk (S.R. Doyle).

prevents further development of the parasite to the pathogenic adult stages (Prichard, 2021). However, widespread and regular use of all anthelmintics places significant selection pressure on parasite populations and can promote the emergence of drug resistance (Kaplan and Vidyashankar, 2012; Rose Vineer et al., 2020). In the USA, reports describing the loss of efficacy of MLs for the treatment of *D. immitis* infections have been increasing in frequency since 1998 (Hampshire, 2005) and confirmed by the isolation of an ML-resistant *D. immitis* isolate in 2014 (Pulaski et al., 2014). Several studies have attempted to define genetic variants associated with resistance to be used to monitor its emergence and spread (Bourguinat et al., 2011a, 2011b, 2016, 2017; Mani et al., 2017; Willi et al., 2018; Sanchez et al., 2020; Curry et al., 2022a). The most promising of these studies used pooled worm whole-genome sequencing of multiple phenotypically-defined sensitive and resistant isolates, from which, after validation using independent genotyping, a set of 42 single nucleotide polymorphisms (SNPs) were proposed to be associated with poor treatment response and ML resistance (Bourguinat et al., 2015). Subsequently, this initial set of genetic variants was re-assessed and reduced to 10- and two-SNP panels, validated using clinical samples, that were proposed to discriminate between susceptible and ML-resistant parasites (Ballesteros et al., 2018). This small panel of SNPs has been examined in European and Australian samples; however, additional support for these markers was not provided due to the lack of phenotypically-defined resistant isolates (Curry et al., 2022b; Power and Šlapeta, 2022). One caveat to the genome-to-SNPs approach was that the initial analyses were performed on a fragmented genome assembly and without a firm understanding of the genetic diversity within and between isolates, which can lead to both biological and technical biases that can confound association studies for genetic traits such as drug resistance (Doyle and Cotton, 2019). Improved genome resources and control of genetic diversity have been critical for increasing the genetic resolution to detect drug resistance loci in several helminths (Doyle et al., 2019, 2022; Le Clec'h et al., 2021; Laing et al., 2022; Beesley et al., 2023) and may aid not only in prioritising genetic markers but in enabling the identification of causal variants for ML resistance in *D. immitis*.

There are few studies focused on understanding the population genetics of *D. immitis*. The publication describing the draft genome assembly nDi.2.2 compared two isolates, one from Italy and one from the USA, revealing low levels of genetic divergence (Godel et al., 2012). Within the US, two studies based on microsatellite genotyping report variable levels of population structure and connectivity, from clustering individuals based on geographical origin (Belanger et al., 2011) to a more recent study that found a complete lack of genetic structure based on geographic origin or anthelmintic resistance phenotype (Sanchez et al., 2020). Although limited in number, these studies suggest that microsatellites are insufficient and that higher resolution data such as whole genome sequencing may be necessary to identify fine-scale genetic structure between *D. immitis* populations. A better understanding of population structure and connectivity of *D. immitis* in endemic regions will subsequently inform the likelihood and rate by which parasites, and in turn resistant alleles, might spread.

In this study, we have curated a recently published assembly to generate the first known chromosomal assembly of *D. immitis*, providing new insight into autosome and sex-chromosome structure relative to closely related filarial nematodes. Using this new assembly, we have evaluated publicly available and new whole-genome sequencing data to understand the global population structure of *D. immitis*, from which we have defined levels of genetic diversity within and between populations. Finally, we have reassessed previously described genetic markers for ML resistance, now in a chromosomal context.

2. Materials and methods

2.1. Genome assembly

2.1.1. Curating a chromosome-scale genome assembly

Our motivation to improve the genome assembly of *D. immitis* was driven by the need for high quality contiguous genome resources to understand genome-wide genetic diversity, particularly in mapping genetic variation associated with drug resistance (Doyle and Cotton, 2019; Doyle et al., 2022). Until recently, the available genome of *D. immitis* was highly fragmented (nDi.2.2; $n = 16,061$ scaffolds) (Godel et al., 2012), limiting the interpretation of genome-wide variation to very discrete regions of the genome. The ICBAS_JMDir_1.0 assembly, generated from Nanopore long-read sequencing of DNA from a single worm (Gomes-de-Sá et al., 2022), significantly improved assembly contiguity ($n = 110$ contigs); however, the assembly was still fragmented, lacked chromosomal context and did not differentiate autosomal and sex-linked sequences. To improve the ICBAS_JMDir_1.0 assembly further, the genome was curated as follows.

First, we recognised that the ICBAS_JMDir_1.0 was missing a subset of universally conserved BUSCO genes (BUSCO v.5.4.3, nematoda ob10 lineage, $n = 3,131$ (Manni et al., 2021)) present in the draft nDi.2.2 assembly. In total, 26 contigs/scaffolds containing 30 complete BUSCO genes were recovered from nDi.2.2 and merged with the ICBAS_JMDir_1.0. Gaps in the newly added scaffold sequences were closed using PacBio reads (accession: SRR10533235) with TGS-GapCloser v.1.2.1 (Xu et al., 2020), reducing gaps from 468 to 275. The merged assembly was subsequently scaffolded using chromosomal assemblies of *Brugia malayi* (Foster et al., 2020) and *Onchocerca volvulus* (Cotton et al., 2016) (note: the post-publication “V4 assembly” of *O. volvulus* was used here, in which complete chromosomal scaffolds are resolved) as a reference using RagTag v2.1.0 (Alonge et al., 2022). This revealed greater sequence conservation between *D. immitis* and *O. volvulus* relative to *B. malayi*, consistent with the phylogenetic placement of these species (McCann et al., 2021). Two clear differences were observed between these intermediate assemblies: (i) that the chromosome number differed, with five chromosomal scaffolds after *B. malayi*-led scaffolding and four chromosomal scaffolds after *O. volvulus*-led scaffolding, and (ii) the structure of the sex chromosome differed between *O. volvulus* and *B. malayi*, based on ancestral chromosome fusion and fission, with a conserved sex-specific region and divergent species-specific regions. Given that the *D. immitis* karyotype is $2n = 10$, consistent with the chromosome number of *B. malayi*, we took the following approach: (i) the *O. volvulus*-led X chromosome (OVOC.OM2) was used to guide the delineation of the *D. immitis* X chromosome, and (ii) split the *O. volvulus* OM1a-led scaffold into two *D. immitis* chromosomes, Chr2 and Chr4, based on synteny with distinct *B. malayi* chromosomes. These decisions were supported by detecting intra-chromosomal telomere sequences within the intermediate but misassembled *B. malayi*-led X chromosome and *O. volvulus* OM1a-led scaffolds, which, when corrected, resulted in terminal telomere sequences in the curated *D. immitis* chromosomal sequences. The telomere location and frequency were determined using fastaq search_seq (<https://github.com/sanger-pathogens/Fastaq>) using a dimer of the telomeric monomer sequence “TTAGGC”. An additional round of gap-filling with TGS-GapCloser did not further reduce the number of gaps. Finally, redundant sequences were identified and removed with purge_dups v.1.2.5 (Guan et al., 2020).

2.1.2. Wolbachia and mitochondrial genomes

Wolbachia contigs were removed from the *D. immitis* ICBAS_JMDir_1.0 assembly by the original authors (Gomes-de-Sá et al.,

2022). To retrieve it for analysis here, raw Nanopore reads from the sample accession SRR14299255 were reanalysed. Porechop v.0.2.4 was first used to trim sequence adapters from the nanopore data before assembling the data with flye v.2.9.1-b1780 (parameters: --nano-raw --genome-size 100 M (where “M” is Mb or million base pairs)). A single contig of 915,677 bp was assembled. We confirmed the *Wolbachia* genome by comparing it with the assembled genome from the Di2.2 assembly (wDi22; *Dirofilaria immitis*_wolbachia_2.2.fna) using minimap2 v.2.16-r922. The mitochondrial genome was also missing from the ICBAS_JMDir_1.0. In this instance, the mitochondrial genome scaffold mDi_Athens_2.1 from the nDi.2.2 assembly was used.

2.1.3. Genome comparison between *D. immitis* assemblies and related nematodes

The assembly contiguity and completeness during assembly curation and relative to other *D. immitis* and related filarial nematodes was assessed in several ways. Genome assembly statistics were calculated using assembly-stats (<https://github.com/sanger-pathogens/assembly-stats>). Conserved BUSCOs were calculated using BUSCO v.5.4.3, which uses MetaEuk as the default gene predictor for eukaryotes (parameters: --mode genome --lineage_dataset nematoda_odb10; $n = 3,131$) (Manni et al., 2021). The *D. immitis* assemblies were compared by analysing the cumulative assembly size and sequence lengths generated using samtools faidx. Large-scale comparisons between genome assemblies were performed using minimap2 (parameter: -x asm20), from which paf outputs were visualised in R v.4.0.3 as a pairwise comparison of sequence hit positions and length (minimum (min): 300 bp) as dot plots or in a circular format showing links between chromosomes (min: 5,000 bp) using the R package circlize (<https://github.com/jokergoo/circlize>).

2.2. Samples

In total, data from 31 samples were used. Metadata regarding the samples are provided in [Supplementary Table S1](#).

2.2.1. Publicly available datasets

A total of 12 publicly available datasets were used from the following ENA BioProject IDs, including (i) PRJNA681066: data from five adult samples from New South Wales (NSW), Australia (Lau et al., 2021); (ii) PRJNA588995: data from five microfilaria samples from the USA, including two from Georgia (GEO), one each from Illinois (ILL), Louisiana (LOU), and Mississippi (MIP) (Shin et al., 2020); and (iii) PRJEB2554: two adult samples, one from Georgia (GEO), USA, and one from Pavia (PAV), Italy (Godel et al., 2012).

2.2.2. Additional, unpublished datasets

An additional 19 datasets were curated from collaborators, including:

- (i) Twelve pooled microfilaria samples from Queensland (QLD), Australia. These Australian blood samples (32–38,250 microfilaria.ml⁻¹) and their DNA were included in a previous study of ML resistance in Australia (Power and Šlapeta, 2022). Extracted DNA from filtered microfilariae of *D. immitis* from 2019 (HW3/19, HW4/19) and 2020 (HW5/20 to HW9/20, HW12/20 to HW16/20) were sent to Novogene (HK) Co., Ltd for indexing, library construction and whole-genome sequencing; Illumina NovaSeq sequencing was performed at an expected depth of 6G with 150 bp paired-end reads.
- (ii) Seven pooled microfilaria samples from the USA, including two from Georgia, and one each from Arkansas (ARK), Louisiana (LOU), Michigan (MCH), Tennessee (TEN), and Texas (TEX). DNA libraries were sequenced on a HiSeq X10 using 150 bp paired-end reads.

2.3. Genomic analyses: data preparation, mapping and variant calling

2.3.1. Evaluation of sample contamination

Given the variable sample provenance and heterogeneity, the degree of contamination in the raw sequencing data was evaluated using Kraken2 v.2.1.2 (Wood and Salzberg, 2014). The raw reads were analysed using two approaches; (i) using the minikraken2 8 Gb database to assess sample contamination with bacteria and/or viruses, and (ii) using a custom Kraken database consisting of the *D. immitis* (nDi.2.2) and the domestic dog *Canis lupus familiaris* (GenBank accession: GCA_014441545) genomes. The output of Kraken2 was summarised using MultiQC v.1.14 (Ewels et al., 2016).

2.3.2. Trimming, mapping and processing of sequencing data

Quality-based read trimming was performed to remove poor quality reads and contaminating adapter sequences using trimmomatic v.0.39 (Bolger et al., 2014) (parameters: ILLUMINACLIP:Illumina-adapters.fa:2:30:10 SLIDINGWINDOW:10:20 MINLEN:50). Trimmed reads were mapped to the improved *D. immitis* reference genome (supplemented with mitochondrial and *Wolbachia* genomes) using minimap2 v.2.16-r922 (Bolger et al., 2014; Li, 2018). Sambamba v.0.6.6 was used to mark duplicate reads. For samples with multiple read sets, mapped reads were combined using samtools merge (v.1.14).

2.3.3. Genome coverage analysis

Sequencing coverage throughout the genome was determined using a combination of bamtools (Barnett et al., 2011), bedtools makewindows (v2.29.0) (Quinlan, 2014), and samtools bedcov (v.1.14) (Li et al., 2009), in sliding windows and per chromosome. The ratio of X chromosome to autosome coverage was used to infer sample sex. Nuclear, mitochondrial and *Wolbachia* genome coverage are shown in [Supplementary Table S2](#). Samples from Queensland showed very low nuclear and *Wolbachia* coverage, whereas mitochondrial coverage was higher in comparison. This can be expected since the number of mitochondria per cell increases the number of DNA copies, which facilitates mapping its genome. However, coverage was highly variable in all cases given that the sample collection, sample type, and sequencing approaches differed between samples.

2.3.4. Variant calling and filtering

Variant calling was performed using GATK v.4.1.4.1 (Van der Auwera and O'Connor, 2020). Initially, variants were identified, and GVCF files were generated for each BAM file using the tool HaplotypeCaller. Then variants were consolidated, and GVCF files merged using CombineGVCFs before joint-call cohort genotyping using GATK GenotypeGVCFs. Finally, a single multisample variant call format (VCF) file containing all initial variants and samples was generated.

Variant filtering was performed using GATK SelectVariants and VariantFiltration. Nuclear, mitochondrial and *Wolbachia* variants were filtered separately and following the same workflow. A data-led approach was employed to determine the distribution of quality metrics, including QUAL, DP, MQ, SOR, FS, QD, MQRankSum, and ReadPosRankSum, by removing relevant tails of variant distributions (typically the upper and/or lower 1–5%).

Variants were further filtered using vcftools v.0.1.16 (Danecek et al., 2011) to ensure they met the following criteria: minimum and maximum alleles = 2; minor allele frequency (MAF) > 0.02; Hardy Weinberg Equilibrium = >1e-6 (nuclear variants) and per genotype depth > 3. Considering the MAF threshold and our sample size, only variants with at least two alleles in the population were considered. Missingness, defined by vcftools as a way to describe the proportion of variants with missing data, was calculated in

two ways; (i) per-sample missingness (--missing-indv) was evaluated to identify samples with high levels of missing data, and (ii) per-site missingness (--missing-site) was evaluated to establish a threshold for all subsets of variants. Only SNPs located in chromosome 1 to chromosome 4 were retained for the population genetic analyses, excluding variants in the shorter scaffolds (which constitute ~0.3% of the genome) and the sex-linked X chromosome.

2.4. Genomic analyses: population genomics

2.4.1. Principal component analysis (PCA)

Given the mixture of pooled and single worm sample types, broad-scale genetic relatedness between samples and populations was explored using allele frequencies rather than genotypes. Allele frequencies were extracted using the R package vcfr (Knaus and Grünwald, 2017) from the AD (allele depth) parameter in the multisample VCF file as:

$$\text{allele freq} = \text{depth of alternative allele} / (\text{depth of alternative allele} + \text{depth of reference allele}) * 100$$

Principal Component Analysis (PCA) on allele frequencies was performed using the R function prcomp(). PCA of mitochondrial and *Wolbachia* variants was performed using the R package SNPReLATE (Zheng et al., 2012) instead of allele frequencies, which allowed us to incorporate missing SNPs in the PCA and thereby use more variants in the analysis.

Other parameters and technical aspects potentially driving the observed clustering of USA samples were assessed to explore the PCAs of nuclear variants. The depth per sample was obtained using the R package vcfr (Knaus and Grünwald, 2017), whereas the missingness and the excess of heterozygosity (inbreeding coefficient “F”) were estimated using vcftools on nuclear variants.

2.4.2. Genetic diversity within and between populations

Genome-wide nucleotide diversity (π) within populations and absolute nucleotide divergence (D_{xy}) between pairs of populations were determined in 100 kb non-overlapping sliding windows using pixy v.1.2.7.beta1 (Korunes and Samuk, 2021). Pixy is a command line tool for calculating genetic diversity while accounting for invariant sites, which are essential for the correct computation of π and D_{xy} . Briefly, all individual GVCF files were genotyped using the tool GenotypeGVCFs with the --all-sites flag, which allows the inclusion of invariant sites during genotyping. First, invariant sites were selected using the flag --select-type-to-include 'NO_VARIATION' in the GATK tool SelectVariants and then merged with the previously filtered nuclear SNP set. As mentioned above, only nuclear positions in the main chromosomes were selected. SNPs and invariants were merged using the GATK MergeVcfs function and indexed using samtools.

2.4.3. Mapping of proposed genetic markers for macrocyclic lactone resistance

Markers for anthelmintic resistance were originally developed using the fragmented nDi.2.2 genome. These markers were distributed on many different contigs and scaffolds with little understanding of how they related to each other in the genome. To address this, SNP coordinates and 100 bp flanking region were extracted from the nDi.2.2 using bedtools get_fasta and mapped to the dimmitis_WSL.2.2 genome using nucmer v.3.1 (Kurtz et al., 2004). Of the 42 original markers, two markers – NODE_42411 (nDi.2.2.scaf00001:466197) and NODE_9400 (nDi.2.2.scaf00005:662854) – were not mapped. The above mapping process was repeated, but this time, using a 10,000 bp flanking region to increase the likelihood of finding the region of the genome; however, the sites still could not be found, leading us to

conclude that they were redundant sequences in the original assembly that were removed during curation. SNP positions in the curated genome were confirmed by identifying variants in the VCF file from the population genomics data, from which allele frequencies per population were calculated.

3. Results and discussion

3.1. Genome structure of *D. immitis*

Comparative karyotype studies from male and female parasites indicate the *D. immitis* genome is composed of four autosomes, with heterogametic males containing additional X and Y sex chromosomes and homogametic females containing two X chromosomes ($2n = 10$) (Sakaguchi et al., 1980; Delves et al., 1986). This karyotype is broadly consistent with the related filarial nematodes *B. malayi*, *Wuchereria bancrofti*, and some *Onchocerca* spp. (excluding *O. volvulus* and *Onchocerca gibsoni*, which have undergone an additional chromosomal fusion to produce $3A + XY$) (Post, 2005).

Recently, a high quality de novo assembly (ICBAS_JMDir_1.0) for *D. immitis* was generated using long-read Oxford Nanopore sequencing of DNA derived from a single parasite (Gomes-de-Sá et al., 2022), representing a significant improvement in genome contiguity relative to the original draft assembly, nDi.2.2, published in 2012 (Godel et al., 2012) (Table 1). To provide greater insight into the genome structure of *D. immitis* and to better understand genome-wide genetic variation among globally diverse isolates, we guided scaffolding of the ICBAS_JMDir_1.0 assembly against the chromosomal reference genomes of *B. malayi* and *O. volvulus* (Fig. 1A and B, respectively). This approach achieved an assembly containing 99.7% of sequence data in four autosomal scaffolds of approximately equal length and one sex-linked chromosomal-scale scaffold approximately twice the length of the autosomes, consistent with the expected female karyotype and distribution of conserved Nigon units of chromosome structure (Gonzalez de la Rosa et al., 2021). Although not all contigs were incorporated into chromosomal scaffolds and gaps have been introduced by scaffolding, relative to previous assemblies, this curated assembly is highly contiguous (Supplementary Fig. S1) and is more complete based on a comparison of conserved BUSCO sequences (Table 1). We note that the new assembly has a higher BUSCO duplication rate than ICBAS_JMDir_1.0, due to the incorporation of recovered contigs containing single-copy orthologs that inadvertently also contained existing orthologs in the assembly. We conservatively chose to keep these duplicated orthologs rather than lose new single-copy orthologs; considering these are primarily located in the extra-chromosomal scaffolds (0.3% of the assembly), their inclusion should have minimal impact on population genetic (or further) studies and will likely be made redundant when the genome is completed. The X chromosome was identified based on synteny with the *O. volvulus* X chromosome and half coverage on the *D. immitis* X chromosome from sequencing of male parasites (Fig. 1C). The presence of both half and full coverage on the X chromosome is consistent with a shared pseudoautosomal region of the X and Y chromosomes, with the half-coverage region representing the X-chromosome-specific region; we could not resolve the Y-chromosome-specific region, which may be achieved by further long-read sequencing of male parasites. Defining the sex-specific region on X enabled the delineation of male (X-to-autosome [X:A] ratio ≈ 0.5) and female samples (X:A ≈ 1.0) from mixed-sex populations, i.e., pooled microfilaria and adult females with gametes (X:A ≈ 0.75), based on sequencing data alone.

Although we identified regions of conserved synteny with both *B. malayi* and *O. volvulus* genomes, considerable rearrangements, particularly toward the ends of the chromosomes, were also identified

Table 1
Genome assemblies of *Dirofilaria immitis* and comparators with other filarial genome assemblies.

	<i>D. immitis</i>	<i>D. immitis</i>	<i>D. immitis</i>	<i>Brugia malayi</i>	<i>Onchocerca volvulus</i>
Version	dimmitis_WSI_2.2; this study	ICBAS_JMDir_1.0; (Gomes-de-Sá et al., 2022)	nDi.2.2; (Godel et al., 2012), WBP17	WBP17; (Foster et al., 2020)	V4; WBP17 (Cotton et al., 2016)
Assembly size (bp)	89,475,811	86,813,779	88,309,529	88,235,797	96,427,137
Number of scaffolds (n)	27	110	16,061	197	708
Average length (bp)	3,313,918.93	789,216.17	5,498.38	447,897.45	136,196.52
Largest scaffold (bp)	28,232,375	12,535,412	1,085,577	24,943,668	28,345,163
Scaffold N50 (bp)	15,199,470	4,253,153	71,281	14,214,749	25,485,961
Scaffold N50 (n)	3	7	219	3	2
Number of gaps (n)	156	0	13,431	8	581
Number of Ns (bp)	40,261	0	3,430,609	277,365	3,074,367
BUSCO completeness ^a	C:94.3 [S:93.0, D:1.3], F:1.7, M:4.0	C:94.1 [S:93.7, D:0.4], F:1.6, M:4.3	C:93.7 [S:91.5, D:2.2], F:1.8, M:4.5	C:98.5 [S:97.9, D:0.6], F:0.6, M:0.9	C:97.9 [S:97.3, D:0.6], F:1.0, M:1.1

^a BUSCO v.5.4.3 was run using nematoda_odb10 lineage (n = 3,131); percentage of total BUSCOs shown. C, complete; S, complete, single-copy; D, complete, duplicated; F, fragmented; M, missing.

(Supplementary Fig. S2). We note that without independent evidence derived from *D. immitis* to order and orientate the assembly, these scaffolds should be treated cautiously and may contain assembly errors from the original nanopore assembly and subsequent reference-based scaffolding. Nonetheless, these chromosome-scale scaffolds represent conserved linkage groups based on synteny against both *O. volvulus* and *B. malayi* chromosomal assemblies, delineating autosome and sex chromosomes and providing a means to differentiate chromosome-specific genetic markers.

3.2. Genome-wide genetic variation within and between broadly sampled populations

The genome-wide distribution of genetic variation from 32 *D. immitis* datasets was assessed (see Supplementary Table S1 for details). The samples from which these data were derived were heterogeneous regarding biological stage and sample type: seven came from single adult worms, five from single microfilaria and 19 from pooled microfilariae. Considering the limited sample availability, the geographic range of sampling was also limited to three regions (Fig. 2A): 17 samples were collected in Australia (12 from QLD and five from NSW), one sample was collected in Pavia (PAV), Italy, and the remaining 13 samples were collected in the USA (one sample in Michigan, Illinois, Tennessee, Arkansas, Mississippi and Texas; five samples in Georgia and two samples in Louisiana).

Genetic diversity was assessed in the three genomes of *D. immitis*: the parasite's nuclear and mitochondrial genomes and its intracellular endosymbiont, *Wolbachia*. In total, we identified 422,935 nuclear single nucleotide variants (of which 96,313 were indels), 49 mitochondrial variants (including 10 indels) and 447 *Wolbachia* variants (including 198 indels). Given the variable amount of sequencing data, mapped read coverage and levels of contamination (i.e. dog and bacterial DNA; Supplementary Fig. S3) per sample, we subset groups of samples for each analysis. For example, due to per-sample missingness, all samples from QLD had to be removed from the nuclear variant set (Supplementary Fig. S4A), and two samples from QLD were removed from the mitochondrial variant sets (Supplementary Fig. S4B), whereas all samples from QLD and three from the USA (ARK, TEN and TEX) were removed from the *Wolbachia* subset of variants (Supplementary Fig. S4C). Therefore, 19 samples were assessed using nuclear variants, 29 with mitochondrial variants, and 18 with *Wolbachia* variants. A per-site missingness threshold of 0.8 for nuclear and mitochondrial sets and 0.7 for the *Wolbachia* variant subset was applied.

Analyses of broad-scale genetic relationships between samples using PCA revealed only weak genetic structure among samples using either mitochondrial or *Wolbachia* variants alone (Supplementary Fig. S5A and B, respectively). The lack of genetic structure

was likely due to the low number of mitochondrial variants and the high and variable levels of missing data in *Wolbachia* variants (Supplementary Fig. S4C). Conversely, PCA using nuclear variants showed a broad-scale population structure clearly separating samples belonging to the three sampling countries (Australia, Italy and the USA) (Fig. 2B) with ~70% of the genetic variance explained by the first two principal components. Furthermore, two distinct clusters of samples were observed among the samples from the USA. However, the two clusters of samples were not divided by sampling location or other geographic variables, as might be expected.

To understand these two USA clusters and differentiate potential technical from biological factors that might be driving this signal in the PCA, we assessed additional variables, including the sample type (pooled versus single worm), sequencing depth, per sample missingness, and heterozygosity estimated by inbreeding coefficient F in the PCA. These analyses highlighted that sample type, whether the sample was obtained from a single worm or a pooled population of worms, i.e. pooled microfilariae, more obviously explained the two clusters than geography. Only a single pooled sample (TEX, strong red colour in Supplementary Fig. S6A) is grouped with data from single individuals. This sample had lower coverage than the other pooled samples (Supplementary Table S2), which may have resulted in its grouping with other low-coverage, single-worm samples. The pooled-versus-single worm effect is correlated with sequencing depth (higher depth in pooled samples; Supplementary Fig. S6B), missingness (less missing data in pooled samples; Supplementary Fig. S6C), and heterozygosity (excess heterozygotes in pooled samples and excess homozygotes in single worm samples; Supplementary Fig. S6D). These data highlight the challenge of working with mixed sample types and that differences in sample type (single/pooled, microfilariae/adults) and sample processing (DNA extraction, sequencing platform/depth) will impact the ability to define population genetic structure and genetic associations. Such biases are important to consider when comparing genetic traits such as anthelmintic resistance when sampling has been performed across different hierarchical levels (e.g., life stages, within/between countries) (Doyle and Cotton, 2019).

To explore within and between population variation, we calculated nucleotide diversity (π) and nucleotide divergence between populations (D_{xy}) in 100 kb non-overlapped sliding windows throughout the genome. Genome-wide, π tended to increase towards the ends of chromosomes (Supplementary Fig. S7), which is best demonstrated on chromosomes 1, 3 and X, the latter of which has an intrachromosomal peak in π that likely reflects the ancient chromosome fusion shared with *O. volvulus* (Cotton et al., 2016). π was significantly different between populations ($P < 0.001$; Wilcoxon rank sum test), being highest in samples from

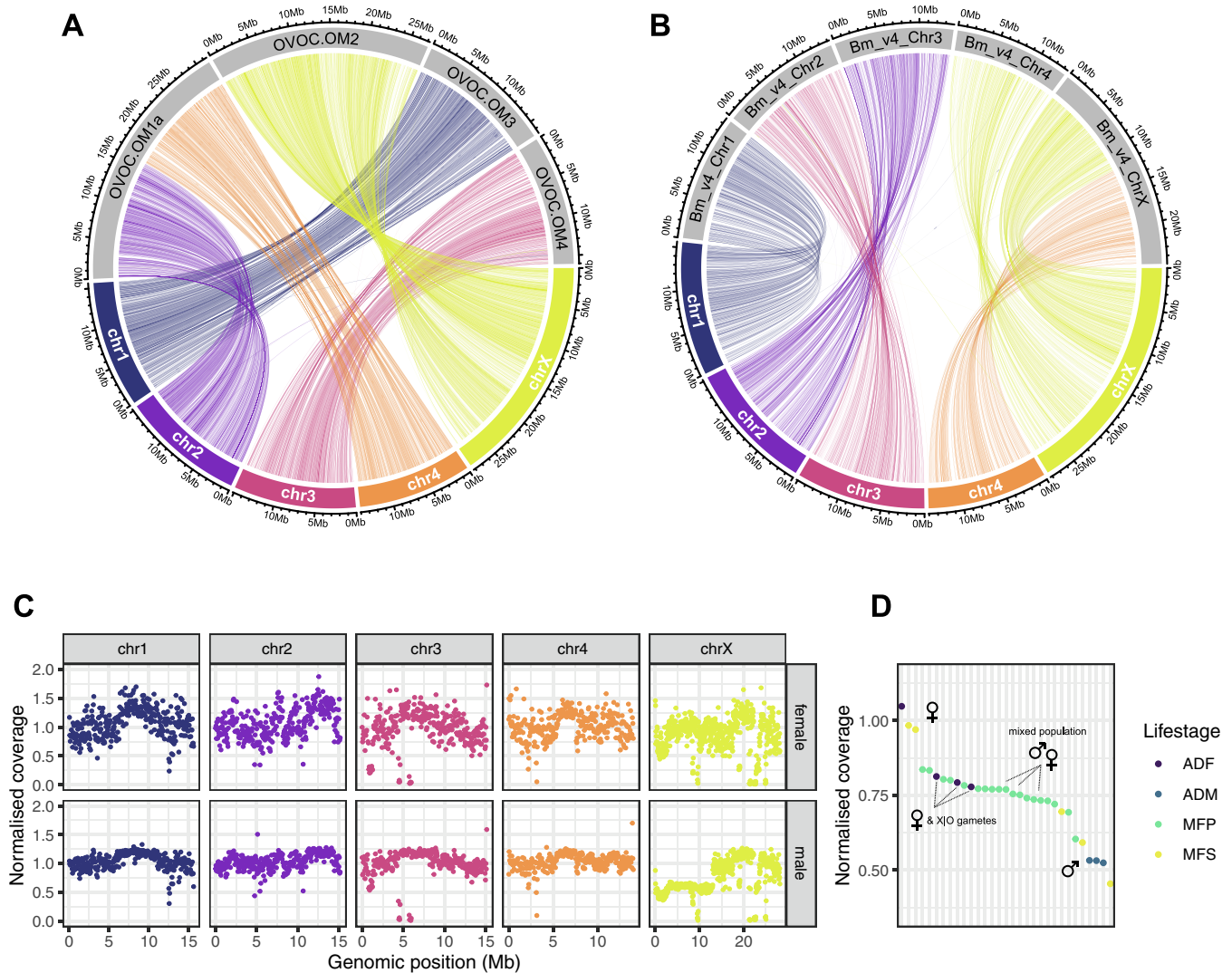


Fig. 1. Genome structure and coverage of *Dirofilaria immitis* chromosomes. Broad-scale chromosome structure and rearrangements between the curated *D. immitis* genome dimmitis_WSL_2.2 and (A) *Onchocerca volvulus* and (B) *Brugia malayi* chromosomal assemblies is shown. Each link between chromosomes represents a minimum 5 kb hit determined by whole genome alignments using minimap2. (C) Normalised sequencing coverage of the *D. immitis* chromosomes comparing a female (top) and male (bottom) sample to highlight the drop in coverage in the first half of the X chromosome in the male sample. Each point represents coverage per 50 kb window throughout the genome, each normalised by the genome-wide median coverage. (D) Comparison of normalised X-to-autosome (X:A) coverage among the sample cohort. ADF, adult female; ADM, adult male; MFP, pooled microfilaria; MFS, single microfilaria.

the USA (mean[Pi] = 7.36×10^{-4}), followed by the Italian sample (mean[Pi] = 8.6×10^{-5}) and then the Australian samples (mean[Pi] = 3.9×10^{-5}) (Fig. 2C). These values of genetic diversity are relatively low compared with other vector-borne filarial parasites such as *O. volvulus* (mean[Pi] = 4.0×10^{-3}) (Choi et al., 2016) and *W. bancrofti* (mean[Pi] = 2.4×10^{-4}) (Small et al., 2016). This low level of nucleotide diversity may limit the rate and likelihood of resistance to treatment developing broadly, or at least relative to some livestock-infective helminths in which resistance is widespread and evolved rapidly (Samson-Himmelstjerna et al., 2021), likely as a consequence of high levels of genetic diversity (Doyle et al., 2020). We also explored diversity within the USA populations, first considering the state where the sample was collected and then the sample type (pooled or single specimens). These data suggest that some populations are more diverse than others in the USA (Supplementary Fig. S8A); however, this interpretation is somewhat confounded by the fact that there are different sample types per population and that pooled samples showed higher diversity than

single samples (Supplementary Fig. S8B). Therefore, these data further support the relevance of the preparation of the samples before sequencing in the assessment of population structure and genetic epidemiology. When more sequencing data becomes available not only for *D. immitis* but also for other helminths, future work must evaluate different approaches to integrate large datasets of specimens with different origins and life stages. Finally, the lack of apparent genetic structure and higher nucleotide diversity among geographically defined samples within the USA suggests a largely panmictic population and widespread transmission zone despite being a vector-transmitted disease, which, if true, entails significant challenges to ongoing control. Divergence in nucleotide diversity, Dxy, between populations showed similar patterns to genome-wide Pi throughout the chromosomes with increased variance toward the ends of chromosomes and small discrete peaks of differentiation throughout (Fig. 2D). There was, however, a subtle trend to suggest AUS_versus_ITL was less divergent and AUS_versus_USA more divergent than USA_versus_ITL.

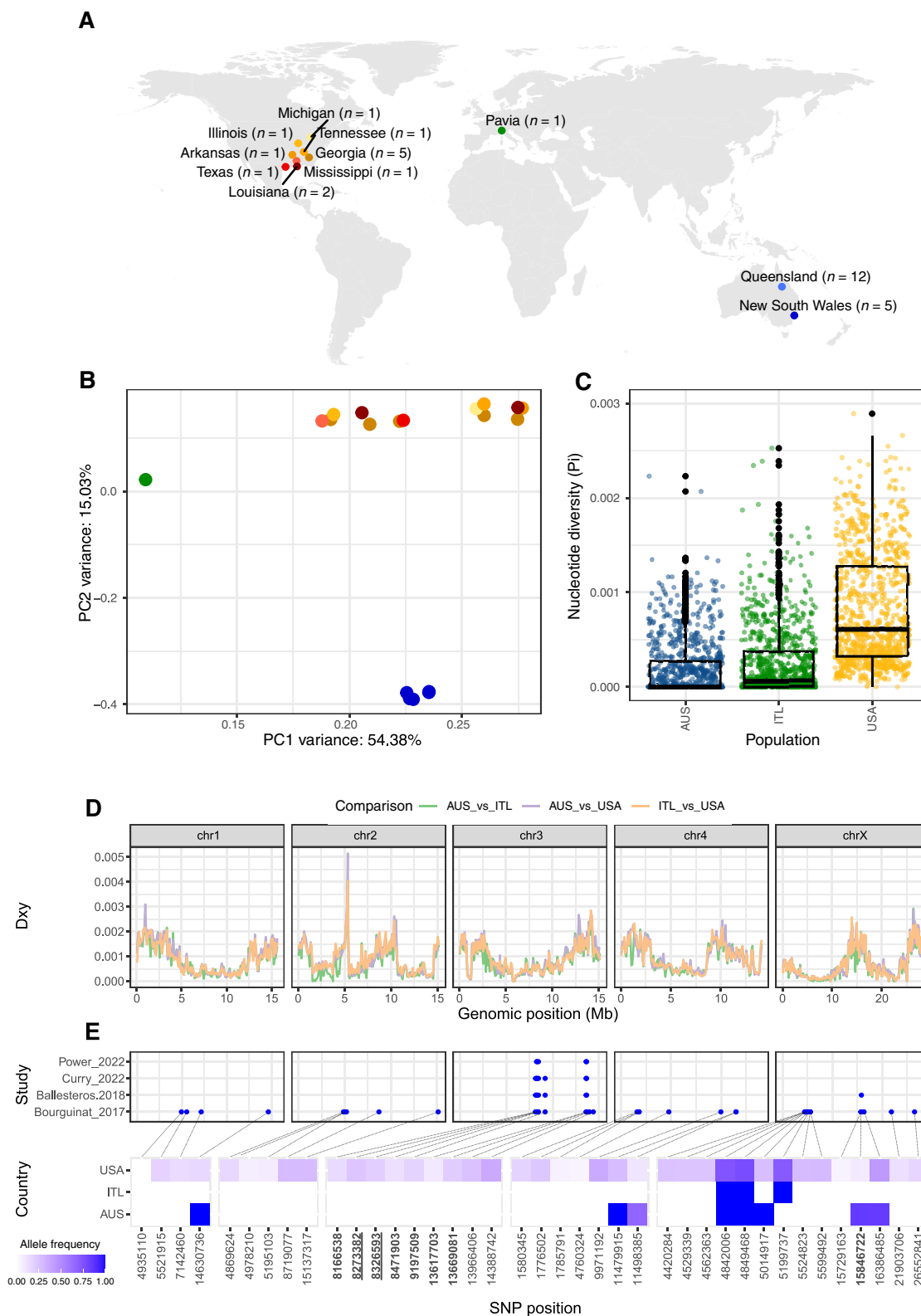


Fig. 2. Population structure and genome-wide analyses of within and between population genetic diversity. (A) A world map shows the broad locations (city/state) from which samples analysed were originally collected. Sample numbers per location are indicated. (B) Principal component analysis (PCA) using 126,728 nuclear single nucleotide polymorphism (SNP) frequencies from 19 samples. PC1 and PC2 are shown, which explain 54.4% and 15.0% of the variation, respectively. Samples are coloured by location as shown in panel (A). (C) Box plots show nucleotide diversity (P_i) distribution per geographic region. Each point represents 100 kb non-overlapping sliding windows in the genome. (D) Comparison of absolute nucleotide divergence (Dxy) genome-wide between pairs of populations. Colours indicate paired comparisons, including Australia (AUS) versus Italy (ITL) (green), AUS versus United States (USA) (purple), and ITL versus USA (orange). Vertical dashed lines indicate chromosome boundaries. (E) Chromosomal placement of genetic markers previously proposed to be associated with macrocyclic lactone resistance (top panel). The original 42 markers were developed using the fragmented assembly nDi.2.2 (Bourguinat_2017); here, we show some of these variants cluster on discrete regions of chromosomes. Subsequent studies that refine (Ballesteros_2018; 10 and two SNP models) and validate these SNPs in independent populations (Curry_2022 and Power_2022) are shown. The allele frequencies of the variants in the study populations are shown (bottom panel), with the 10 SNP model variants in bold and the two SNP model variants in bold and underlined.

3.3. Chromosomal context of genetic markers associated with ivermectin resistance

Resistance to MLs is increasingly recognised as a threat to *D. immitis* control, particularly in the USA, where drug failures and resistant isolates have been defined and characterised (Pulaski et al., 2014; Maclean et al., 2017; Diakou and Prichard, 2021). Understanding the genetic determinants of drug resistance has been challenging, partly due to a lack of high quality genome resources and a poor understanding of how genetic variation is distributed within and between populations under drug pressure. A panel of 42 candidate resistance genetic markers derived from whole genome sequencing data were identified (Bourguinat et al., 2015), which was subsequently refined to a small panel (between two and 10 SNPs) that was predictive of a resistance phenotype (Bourguinat et al., 2017; Ballesteros et al., 2018; Curry et al., 2022b). However, these genetic markers were identified and developed using the fragmented draft genome nDi.2.2, and, therefore, their positions in the genome relative to each other – which may provide insight into the mode of drug selection and degree of linked selection – were largely undefined.

We have mapped 40 of 42 SNP positions on the chromosomes, revealing hits to all five chromosomes (Fig. 2E; top panel). Surprisingly, some variants were found in discrete clusters, including those proposed to be most predictive of resistance; notably, on chromosome 3, six SNPs from three distinct nDi.2.2 scaffolds were found in one cluster (range: 8,166,538–9,197,509 bp), and in another cluster, four SNPs from three distinct nDi.2.2 scaffolds (range: 13,617,703–14,388,742 bp) were mapped (Fig. 2E). These two clusters on chromosome 3 contained all but one mapped SNP from the two and 10 SNP predictive models defined by Ballesteros and colleagues (Ballesteros et al., 2018). Therefore, the chromosomal context of the new genome has provided insight into the physical linkage between these specific variants, which could not be achieved using the draft genome alone. A description of the 42 ML resistance SNPs and their relative positions in the nDi.2.2 and dimmitis_WSI_2.2 genome assemblies can be found in Supplementary Table S3.

Analysis of allele frequency in our studied populations showed that these variants are present at a moderate frequency in the USA but are largely absent or are fixed variants unlikely to be associated with resistance from Italian and Australian samples (Fig. 2E; bottom panel), consistent with previous studies (Curry et al., 2022b; Power and Šlapeta, 2022). The relevance of these predictive variants outside of the USA is unknown and requires further validation with phenotypically defined non-USA isolates. We are underpowered to further resolve the genetics of resistance based on low sample numbers and limited drug susceptibility data among the available samples analysed here. However, these results demonstrate the value of a curated chromosomal genome assembly (Doyle, 2022), enabling a more precise differentiation between genetic variants associated with drug selection from unlinked background variation. Furthermore, it provides a scaffold for genetic mapping and genome-wide association experiments in *D. immitis*, which has been essential for understanding the genetics of anthelmintic resistance in other helminth species of humans (Doyle et al., 2017; Le Clec'h et al., 2021) and veterinary importance (Doyle et al., 2022; Beesley et al., 2023).

4. Conclusion

We have curated a contiguous and largely complete genome assembly for *D. immitis*, revealing the chromosome structure and genomic rearrangements for the first known time. Further validation of assembly joins and structural variation using orthogonal

sequencing technologies, for example, HiC chromatin conformation capture, will ensure this chromosomal assembly remains a valuable resource for the parasitology community for some time. Using publicly available genome data supplemented with new sequence data, we have described broad-scale within- and between-population genetic diversity among geographically diverse isolates. Available data limit our resolution toward understanding genome-wide diversity and differentiation between populations but will improve by denser sampling and sequencing of parasites throughout their natural range. Finally, a chromosomal assembly now allows a more precise investigation of selection on genome-wide genetic variation and will enhance our understanding of parasite transmission and the spread of genetic variants responsible for resistance to treatment.

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Dog sampling in Australia was obtained after receiving written consent from the owners. Research performed at the University of Sydney was approved by the Animal Research Ethics Committee, which follows the NSW Animal Research Act 1985 and the Australian code for the care and use of animals for scientific purposes 8th edition (2013). Raw sequencing data used in this study are publicly available from the European Nucleotide Archive (ENA) and are described in Supplementary Table S1. The code used to generate and analyse data and plot figures can be found at https://github.com/Gandasegui/pop_genomics_dirofiliaria. The dimmitis_WSI_2.2 genome is available at: ftp://ngs.sanger.ac.uk/production/pathogens/sd21/dimmitis_genome/dimmitis_WSI_2.2.fa. ISGlobal receives support from the Spanish Ministry of Science and Innovation through the “Centro de Excelencia Severo Ochoa 2019–2023” Program (CEX2018–000806–S) and support from the Generalitat de Catalunya through the CERCA Program. SRD is supported by a UKRI Future Leaders Fellowship [MR/T020733/1] and the Wellcome Trust (UK) through core funding to the Wellcome Sanger Institute (UK) [206194]. The Canine Research Foundation and Dogs Victoria, Australia, supported the research in Australia. We gratefully acknowledge the Pathogen Informatics group at the Wellcome Sanger Institute for informatics support, the Doyle group for constructive feedback on the analyses and manuscript, and the parasitology community for their open access data and sharing of genome resources. For the purpose of Open Access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

Appendix A. Supplementary material

Supplementary material to this article can be found online at <https://doi.org/10.1016/j.ijpara.2023.07.006>.

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