LOVE IN THE TIME OF *ONCHOCERCA*

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ABSTRACT

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This dissertation investigated the prevalence rate and vector species of the under-studied, vector-borne parasite *Onchocerca lupi.* It also develops and applies methods that provided the opportunity to conduct whole-genome sequencing (WGS) of this newly emerging cryptic nematode to characterize the genomic diversity in the southwestern United States (U.S.). Chapter One is a review of the literature on elimination campaigns against important and well-studied filarial nematodes infecting humans. It also proposes the necessary steps critical for *Onchocerca lupi* elimination strategies. Chapter Two details the first large-scale prevalence study of *O. lupi* in companion animals in an endemic area of the U.S. as well as the discovery and identification of putative vector species in the Southwestern U.S. Chapter Three presents the first complete mitochondrial genome for *O. lupi.* Chapter Four details the design and validation of a novel, real-time PCR assay for quantifying the host-to-parasite DNA ratio (LupiQuant) from complex onchocercosis samples. This assay utilizes single copy genes from the canine host and *O. lupi* that were inserted into plasmid constructs, cloned, and serially diluted to serve as gene copy number controls. This chapter also describes the first draft genome of *O. lupi.* Chapter Five expounds on the methods produced in Chapter Four and provides informed sample selection for

WGS of 16 adult worms from the US, Turkey, and Romania. This chapter also defines the genomic diversity of *O. lupi* and its *Wolbachia* endosymbiont within the US. More broadly, this dissertation provides the necessary forward momentum toward *O. lupi* control and eventually, elimination.

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To numerous colleagues and friends, thank you for your many words of encouragement along the way. It has meant so much. And last but not least, to my family, who have never stopped believing in me and telling me how proud they are of my strength and persistence, and my beloved Wyatt, for whom I would do it all over again.

TABLE OF CONTENTS

.

LIST OF TABLES

CHAPTER TWO:

CHAPTER FOUR:

CHAPTER FIVE:

LIST OF FIGURES

DEDICATION

For my beautiful baby boy, Wyatt. Words cannot begin to convey the depth of my love and devotion to you, and you were the sole reason I delved into the parasitic realm. Because of you, I became obsessed with *Onchocerca lupi*, and I am no less passionate in my determination to lessen this parasite's impact. It is because of you, beloved Wyatt, that I have come so far on this journey, and it is your legacy that compelled me to attainment. I am forever humbled and most grateful. Sweet dreams, little man.

PREFACE

Wyatt, my dog and soulmate, was the catalyst for this research and my dissertation. It took countless veterinary appointments over several months and eventually exploratory surgery to receive Wyatt's diagnosis of the filarial nematode *Onchocerca lupi*. The utter dearth of literature regarding the life cycle and vector species along with the lack of commercial diagnostic and evidence-based therapeutics was motivation for the research within this dissertation. Wyatt's lifelong fight with this parasite and the long-term effects it had on him for 12 years set my resolve to work toward better diagnostics, treatments, and outcomes for this cryptic disease.

Herein, the chapters of this dissertation were written according to the guidelines of targeted journals. Chapter One is a review of literature regarding successful nematode elimination programs and was not written for peer-reviewed publication. Chapter Two is formatted according to the Journal of Veterinary Sciences: Parasitology and is currently under review. Chapter Three has been published in the journal Mitochondrial DNA Part B. Chapter Four has been published in PLOs ONE. Chapter Five is in preparation for submission, but a target journal has not been selected. There will be some redundancy in the introduction sections of each chapter as a result of combining these articles within Northern Arizona University formatting requirements.

CHAPTER ONE

LESSONS FROM SUCCESSFUL NEMATODE ELIMINATION STRATEGIES

INTRODUCTION

Filaria are defined as tissue-dwelling parasitic nematodes that infect vertebrates and are spread through hematophagous arthropods (1). While these parasitic nematodes are most commonly observed in developing countries, at any given time, more than one third of mankind is infected with a parasitic nematode (2). Astoundingly, the World Health Organization (WHO) estimates more than 1.5 billion people are at risk of developing filarial nematode infections worldwide (3). Unfortunately, current parasitic nematode control programs are universally based on mass drug administration, a useful but inadequate method in terms of eliminating lymphatic filariae (4). While filarial parasites cause the most severe pathology associated with parasitic infection in humans (4), unique technical challenges (inability to culture, variable presence of host DNA, metagenomic analyses) associated with the filarial nematode biology has retarded progress in the development of experimental tools and resources. With the advent of next generation sequencing and continuously diminishing associated costs, the past 12 years has heralded helminth research into the 'omics' era (5) with the promise of contributing to a better understanding of the biology of these parasites needed to support elimination goals (6). Furthermore, current helminth genomics research has shown an improved understanding of

disease dynamics and epidemiology with the use of whole genome sequencing(WGS) (7–9). Regardless of the astonishing morbidity these parasitic nematode infections cause, comparatively few studies have applied population genomics to these truly neglected tropical diseases (10).

Given the biological complexity of these organisms, a multitude of technical challenges can hinder genomics studies and elimination progress. The aim of this chapter is to provide a framework which outlines the most important advances needed in the context of successful elimination and control strategies of filarial nematode research. One major obstacle in studying these important pathogens is the inability to maintain filarial nematodes outside of the host system; samples must be extracted directly from host blood and tissue, procedures which can be both costly and invasive (4). This inability to rapidly generate parasites for genomic studies has severely hindered efforts to generate high-quality, fully annotated genomes and subsequently impedes the development of nematode vaccines, novel therapeutics, and effective mitigation strategies. Since the advancement of helminth genomics since the year 2000, scientists have generated 262 draft genomes within 29 Families belonging to the Order Rhabditida (including free-living nematodes). This includes the filarial nematodes that represent the leading cause of morbidity in humans: *Loa loa*, *Wuchereria bancrofti*, *Onchocerca volvulus,* and *Brugia malayi* as well as of veterinary concern: *Dirofilaria immitis* and *Onchocerca ochengi* (4). Given the staggering numbers of filarial infections worldwide, coupled with the millions of at-risk populations for developing these diseases, the utter dearth of available genomics data for these parasites is distressing and requires immediate public health attention.

Further complicating WGS studies on these filarial parasites is the mutable presence of host DNA within a sample. Research investigating the genomic relationships of the cattle parasite *Onchocerca ochengi* and a potential novel *Onchocerca* species demonstrated a

biological challenge when sequencing these parasites; of the 20 whole worms they sequenced, 10 were essentially unusable as the sequence data was entirely host bovine DNA (11). To circumvent the variable host DNA in a given parasite sample, scientists studying blood parasites in cattle, demonstrated the utility of dual, single copy gene targets of both the host and parasite using quantitative PCR (qPCR) to calculate the DNA ratio (12,13). While currently seldom applied in filarial nematode research, this approach is critical for efficient high-throughput sequencing by informed sample collection.

The advancement of tools and resources for combatting filarial nematodes through genomics research is vital for improved elimination strategies (14), however, mass drug administration (MDA) and effective vector control measures are the cornerstones of filarial disease control. Research has shown successful elimination through integration of MDA and vector control (15,16). Implementation of vector control measures has three critical benefits: 1) filarial transmission is suppressed without the need to identify all that are infected, 2) the risk of re-establishment of transmission from imported microfilaria positive animals, humans, or arthropods is greatly reduced, 3) the risk of parasite transmission is minimized in locations with both vector species and reports of parasite disease (15). However, to implement these effective vector control measures, an in-depth understanding of the vector species' ecology and biology is imperative.

While the majority of human filarial nematode infections occur within the developing world, a zoonotic canine parasite, *Onchocerca lupi*, has been reported in dogs with increasing incidence in both the United States, Europe, the Middle East, and Northern Africa (19–27). Reports of increasing incidence of this vector-borne disease in canine populations highlight the increasing urgency to develop effective mitigation strategies (23,24,28–30). In addition to the

increase in canine onchocercosis, there have been ~18 confirmed human *O. lupi* cases globally (31–36) with seven occurring in the southwestern United States (22,29–31). To date, we have a severely limited understanding of this parasite in terms of biology, vector ecology, population structure, phylogenetic relatedness, and gene content. Currently, disease control and treatment relies entirely upon administration of the combination of the antibiotic doxycycline in conjunction with the antiparasitic ivermectin, which is solely microfilaricidal (1); there is no cure for *O. lupi* infection. As adult *Onchocerca* species are known to live up to 15 years within the host species, ivermectin must be administered for decades in humans (1) and the lifespan of dogs. However, canines that harbor a mutation within the *MDR1* gene cannot tolerate the drug ivermectin and subsequently have no treatment options for *O. lupi* infection (38). Considering previous research regarding successful filarial nematode control strategies, two central approaches that impact parasitic control measures are:

1) The most effective strategy for breaking the disease transmission cycle is implementing a combination of MDA and vector control.
2) The integration of whole genome sequencing of parasitic nematodes into public health policy allows for improved surveillance tools, diagnostic and vaccine development, and novel drug target identification.

Tangible scientific goals gleaned from more heavily studied human parasites and applied toward *O. lupi* control and elimination efforts include both vector species identification and genomic sequencing. Genomic datasets provide the research community the opportunity to develop novel surveillance tools and resources, identify novel drug targets, create vaccines, and understand disease epidemiology all in the context of parasite biology; this knowledge can be directly applied to control strategies and elimination efforts. We currently have a unique opportunity to integrate comparative genomics and population genetics into *Onchocerca lupi*

elimination strategies. To date, studies that have investigated the genetic diversity and genetic differentiation between populations of nematodes have typically used the mitochondrial gene *cytochrome oxidase I* (COI) (17,39–42). For instance, a recent study investigating the COI gene of the filarial nematode *Onchocerca lupi* in coyote populations reported low diversity and could not infer relatedness (17). This gene target generally produces low resolution in terms of population diversity and structure. Designing sensitive, specific, and effective pathogen surveillance tools requires accurate population structure knowledge that is achievable using whole-genome sequencing. This integration of *O. lupi* population genomics data into public health policy will allow for improved surveillance of both vector and host. Understanding the vector species and gaining invaluable insight and knowledge of the ecology and complicated molecular biology will further the overarching goal of *O. lupi* elimination. Herein, we build on existing knowledge by focusing our efforts on vector species identification as well as genomic sequencing to inform future elimination program decisions.

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CHAPTER TWO

BITING MIDGES (DIPTERA: CERATOPOGONIDAE) AS PUTATIVE VECTORS OF ZOONOTIC ONCHOCERCA LUPI (NEMATODA: ONCHOCERCIDAE) IN NORTHERN ARIZONA AND NEW MEXICO, SOUTHWESTERN UNITED STATES

ABSTRACT

Onchocerca lupi (Rodonaja, 1967) is an understudied, vector-borne, filarioid nematode that causes ocular onchocercosis in dogs, cats, coyotes, wolves, and is also capable of infecting humans. Onchocercosis in dogs has been reported with increasing incidence worldwide. However, despite the growing number of reports describing canine *O. lupi* cases as well as zoonotic infections globally, the disease prevalence in endemic areas and vector species of this parasite remains largely unknown. Here, our study aimed to identify the occurrence of *O. lupi* infected dogs in northern Arizona, New Mexico, and Utah, USA and identify the vector of this nematode. A total of 532 skin samples from randomly selected companion animals with known geographic locations within the Navajo Reservation were collected and molecularly surveyed by PCR for the presence of *O. lupi* DNA (September 2019 – June 2022) using previously published nematode primers (COI) and DNA sequencing. *O. lupi* DNA was detected in 50 (9.4%) sampled animals throughout the reservation. Using positive animal samples to target geographic locations, pointed hematophagous insect trapping was performed to identify potential *O. lupi* vectors. Out of 1,922 insects screened, 38 individual insects and 19 insect pools tested positive

for the presence of *O. lupi*, all of which belong to the Diptera family. This increased surveillance of definitive host and biological vector/intermediate host is the first large scale prevalence study of *O. lupi* in companion animals in an endemic area of the U.S. and identified an overall prevalence of 9.4% in companion animals as well as multiple likely biological vector and putative vector species in the southwestern United States. Furthermore, the identification of these putative vectors near human populations coupled with multiple, local zoonotic cases highlight the One Health importance of *O. lupi*.

INTRODUCTION

Onchocerca lupi (Nematoda: Onchocercidae) is an emerging zoonotic filarial nematode that was first described in a grey wolf from Caucasia in the Republic of Georgia in 1967 (1). Within the last decade *O. lupi* has shown to be endemic to regions of the United States (US), Europe, Northern Africa, and the Middle East (2–13). The most important definitive hosts of *O. lupi* are domestic dogs (*Canis lupus familiaris);* however, this parasite has also been reported to infect humans (1,10,14–16) and wild carnivores like coyotes (*Canis latrans*) and more rarely, wolves (*Canis lupus*), and cats (*Felis catus*). While the increase in incidence of this parasite has renewed interest world-wide, low occurrence of disease within human populations coupled with *O. lupi* classified as a non-reportable veterinary disease in the United States and the current lack of a commercial diagnostic severely impedes our understanding of disease prevalence and geographic distribution. Due to the implications for both animal and human populations, the purpose of this study was to determine the occurrence of *O. lupi* in companion animal populations on the Navajo Reservation, located in the southwestern US, as it has overlapping reports of *O. lupi* in humans, dogs, and coyotes (15,17–19) and determine potential vector species.

While *O. lupi* is considered a vector-borne parasite that is putatively transmitted by dipteran hematophagous insects, as are other congeneric species (20), demonstration of active transmission by a suspected vector species remains absent for this parasite. There is limited knowledge on the life cycle of *O. lupi;* the current dogma is grounded in knowledge from more heavily studied *Onchocerca* species (e.g., *Onchocerca volvulus, Onchocerca ochengi*) which suggest the blackfly *Simulium* spp. (Diptera, Simuliidae) as the putative intermediate host of *O. lupi* (21). Recent research has identified *Simulium tribulatum* as a putative intermediate host for this parasite along the San Gabriel River watershed in southern California (USA), but vector competency has yet to be explored (22). An additional study has identified *O. lupi* DNA sequences acquired from head and bodies of *Simulium griseum* but has not yet been published in peer-reviewed literature. Subsequent black fly sampling in areas of California failed to detect *O. lupi* DNA but identified DNA of other *Onchocerca* genetic lineages likely associated with wild ungulates (24,25).

Blackflies, such as *Simulium* spp., are the most widespread of the small biting Diptera (26). The genus *Simulium* can thrive in a wide range of climates from tropical to temperate conditions, but the presence of a running water habitat is a necessary component of their life cycle (26). However, reports of autochthonous *O. lupi* in canines from regions lacking the necessary water habitats required for blackfly breeding suggests the possibility of an *O. lupi* vector outside the Simuliidae family. The principal method for control of vector-borne diseases, such as lymphatic filariasis and malaria, is through vector control (e.g., targeted deployment of insecticides) and requires extensive knowledge of the vector species life cycle (27). There is an urgent need to extend the insect sampling spectrum beyond urban waterways to explain increasing endemic disease in anhydrous regions such as Navajo Reservation (43,452km^2) in the

southwestern US; this is essential for implementing effective vector control strategies to mitigate *O. lupi* transmission.

The dogma around *O. lupi* considers canid species as definitive hosts and an unknown arthropod species as intermediate host. Sexually mature nematode develop in the sclera of the eye within the definitive host where females release microfilariae into canid skin, predominantly in the head, ears, interscapular, and lumbar regions (21). While the putative simuliid intermediate host takes a blood meal, unsheathed microfilariae are ingested and migrate to the midgut followed by the thoracic muscles and develop from L1 (non-infectious) to L3 (infectious) larvae. Once infectious, the L3 migrates to the mouthparts of the intermediate host, ready to infect a vertebrate host during its next blood meal. It is important to consider that, to date, L3 larvae have not been identified in the head of an intermediate host for *O. lupi*, which would serve as unequivocal biological proof for vector suitability. However, current research from closely related *Onchocerca* species suggests L3 larvae are the only developmental stage present in the head of intermediate hosts (28,29). Current PCR-based methods for *O. lupi* identification alone cannot differentiate viable from nonviable or immature (L1 and L2) from infective (L3) larvae within wild-caught insects and are therefore insufficient when screening individuals or pools of whole insects as potential intermediate hosts. Therefore, the identification of *O. lupi* DNA within the heads of suspected vectors suggests active transmission as only infectious L3 larva DNA should be present in the head of the arthropod, serving as a molecular confirmation of vector suitability.

Current knowledge regarding the patterns of epidemiology and vector-parasite interactions of *O. lupi* is lacking, which is unfortunate as reported incidence in dogs as well as human cases seem to be rising (2–4,13,17). *O. lupi* is considered endemic in canids within the

southwestern US, which includes California, Arizona, New Mexico, and Texas (3,4,15,22). Worldwide, there have been 18 reported human cases of zoonotic *O. lupi*, five of which were in the southwestern United States (9,10,13,17,19) within the past 12 years (21). These clinical reports coupled with the presence of positive *O. lupi* coyotes in northern Arizona and southern New Mexico (15) indicate a need for increased surveillance of hosts and vectors in regions with overlapping *O. lupi* infections in human, dog, and wildlife populations. Furthermore, identifying where *O. lupi* occurs in the environment is crucial for determining the risk of spread to non-endemic regions as well as determining risk for human populations. In the present study, we conducted surveillance of *O. lupi* in canine populations on the Navajo Reservation, located in the southwestern US (Arizona, New Mexico, and Utah), and used these data to target geographic locations for collection and molecular screening of hematophagous insects to identify potential vectors of the parasite in this region.

MATERIALS AND METHODS

Ethics Statement

This study was approved by IACUC of Northern Arizona University (Approved protocol 19-016). Written informed consent was obtained from owners for animal participation. Geographic coordinates for positive dog and insect trap site locations were excluded from this manuscript for privacy purposes.

Geographic/Sampling location(s)

The Navajo Reservation is the largest federally recognized sovereign region retained by the Navajo Nation within the southwestern US and is located largely on the Colorado Plateau. The Reservation covers 43,452km^2 spanning the states of Arizona, New Mexico, Colorado, and Utah. The Navajo Reservation varies in altitude (940 m to 3153 m) and encompasses several distinct

landscapes with varying climates (30). Regardless of the distinct terrains, water is sparse throughout most of the Reservation (31). Three distinct topographies, each with a different climate and vegetation, makeup the Navajo Reservation: the cold, subhumid climate of the mountains, the intermediate steppe climate, and the warm, arid desert climate. Ponderosa pine-covered high plateaus account for 8% of the region with a reported cold and subhumid climate with lows and highs between -15.6°C and 26.7°C and an annual rainfall of 40.64-68.58 cm (32). With a described climate as cold and dry, the mesas and high plains account for 37% of the region with temperatures ranging between -12.22°C and 31.11°C and annual rainfall between 30.48-40.64 cm and reported vegetation is listed as grasses, sagebrush, and pinyon-juniper (30,33). Lastly, the warm, arid desert landscape accounts for 55% of Navajo land with temperatures ranging between -11.67°C to 43.33°C and annual rainfall between 17.78-27.96cm (32). Desert vegetation such as grasses and browse plants are limited (30). A 2010 census reported the total population on the Navajo Reservation as 173,637 people (34) whom are widely dispersed across the reservation in part because of the scarce availability of water (35). This study conducted companion animal and insect sampling within all three climates on the Navajo Reservation.

Animals and samples

From September 2019 through July 2022, we collected 532 skin tissue samples from companion animals (including both domestic and stray dogs and cats) throughout the Navajo Reservation (Arizona, New Mexico, and Utah) to gain thorough surveillance data. Samples were obtained primarily through spay and neuter clinics as well as from any animal undergoing a surgical procedure with owner consent at three veterinary clinics and a mobile unit. Selection criteria included all animals with a minimum estimated age of 6 months. Upon sample collection, animals in this study did not display clinical disease symptoms typical of canine ocular

onchocercosis such as nodule formation. However, animals were selected for this study regardless of their health status. All animal handling and sample collection was in accordance with IACUC regulations. Skin samples were obtained using a disposable 0.2cm skin punch from the inter ocular frontal area of the head, which was previously identified as one of the predilection areas for presence of *O. lupi* microfilariae on the canine host (36), and immediately fixed and stored in 80% ethanol.

Molecular screening

Upon receipt in the laboratory, skin samples were stored at 4^{o}C until processing. DNA was extracted from 532 skin samples using the Qiagen Dneasy Blood and Tissue kit with a preliminary overnight incubation at 56^{o}C following manufacturer's recommendations within the tissue lysis protocol (Qiagen). The conventional PCR (cPCR) screening for *O. lupi* from skin samples was based on the partial sequence of the cytochrome c oxidase gene (COI; COIF: 5'-TGATTGGTGGTTTTGGTAA-3', COIR: 5'-CATAAGTACGAGTATCAATATC-3') as described previously (37). The cPCR amplification was carried out with a final 25µL reaction volume consisting of 1X KAPA 2G Master Mix, 8µL of genomic DNA, and 100nM forward and reverse COI primers. The following thermocycler conditions were used: 95 ^{o}C x 3 min, 35 cycles of (95 ^{o}C x 15s, 60 ^{o}C x 30s, 72 ^{o}C x 1.5 min), followed by 72 ^{o}C x 1 min. The presence of PCR product was determined by visualization using gel electrophoresis with a 2.0% TAE agarose gel and sequenced directly with capillary electrophoresis using a BigDye Terminator v3.1 Cycle Sequencing Kit on the 3130 Genetic Analyzer platform (Applied Biosystems). Fifty-two Forward and reverse sequences were assembled using SeqMan v17 (DNAStar) and were queried with BLASTN (38) against the NCBI Nucleotide database (nt) to confirm their identity as *O. lupi*.

Insect collection

Insects were collected between May and August 2021 from 15 individual sites spanning Arizona and New Mexico on the Navajo Reservation using BG-Sentinel mosquito traps (Biogents) baited with both CO_2 and the BG-Lure chemical attractant (Biogents). Funding dictated a single insect trapping season. Twelve of the fifteen trap sites were targeted collection points; COI cPCR positive dogs identified from phase 1 of this study informed these locations. Three additional non-informed trap sites were included based on accessibility, permissions, and close proximity to dry creek beds. Due to remote trapping locations, traps generally collected insects continuously for seven days with nonstop CO_2 emittance. At the end of seven days, insects were collected and flash frozen for transport to the laboratory at Northern Arizona University. Traps were moved between the 15 locations throughout the insect trapping season depending on accessibility and permissions. Generally, each trap site collected insects during a single week only, however, when technical difficulties (CO2 emittance issues) arose, traps were left at the same site an additional week. Insects were stored at -20^{o}C until further processing. A Leica S8 AP0 dissecting microscope with an attached Canon EOS Rebel T3 camera was used to process all insects which included morphological examination, counting, photographing, and sorting. The small/minute size and great abundance of biting midges at most locations informed insect pooling from the same trap in sets of 50 biting midges. Biting midges were pooled into pools between 2-50 insects based on the following criteria: the same species, the same site, the same collection week. Seventy biting midge pools were initially processed as whole insects, however, once positive pools were identified, the remaining fifteen pools had DNA extracted from the heads and bodies separately.

DNA extraction, cPCR, and sequencing

Total DNA was extracted from heads and bodies of Dipteran insects using the Dneasy Blood and Tissue Kit (Qiagen) with the addition of 5 min bead beating at 1500 Hz followed by an overnight proteinase-K digestion at 56ºC following their tissue lysis protocol. Extracted DNA was screened for parasite presence using previously published mitochondrial COI gene primers as described above. All samples were evaluated by gel electrophoresis and samples displaying a band were sequenced directly using Sanger sequencing and sequence data was assembled, queried, and deposited using the same methods listed above.

To molecularly determine Diptera species identification where parasite presence was detected, partial sequence using Diptera universal primers (39) for the COI gene (LCO1490F: 5'-GGTCAACAAATCATAAAGATATTGG-3' and HC02198R: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' and were amplified as with the same parameters of the parasite screening, but with modified cPCR conditions: 95ºC x 3 min, 35 cycles of (95ºC x 15s, 55ºC x 30s, 72ºC x 1.5 min), followed by 72ºC x 1 min. Results were visualized on a 2.0% TAE gel and subsequently sequenced, assembled, and examined using the same methods as listed above. The target amplicon size was 710bp. Sequences were queried against the NCBI nt database using BLASTN. To further confirm and identify insect species, a database of *cytochrome oxidase subunit* I sequences downloaded from NCBI was generated with the RESCRIPt QIIME 2 plugin version 2021.11.0+3.g8aa880e *(40)* using the taxonomy ids 7147 (Diptera), 6231 (Nematoda), and 2 (Bacteria). Sequences were removed from the database if they contained 5 or more degenerate bases, 10 or more homopolymers, were <500 or >1600 nt in length, or did not have a top BLASTX hit to a COI sequence in the Uniprot Reviewed (Swiss-Prot) database (41) downloaded May 18, 2022. Taxonomy was assigned to sequences using this database and the q2-feature-classifier with the classify-sklearn naïve Bayes approach (42) within

QIIME 2 version qiime2-2022.2 *(43)*. Diptera COI DNA sequences from positive *O. lupi* insects were deposited to the GenBank sequence database under BioProject PRJNA890670. Additionally, Diptera COI gene sequences were aligned using MUSCLE (44) along with 12 publicly available Diptera COI gene sequences (Supplemental Table 1). A maximum likelihood phylogenetic tree was generated using MEGAX (45) and the Tamura-Nei model of substitution and 1,000 bootstrap pseudoreplicates. Tipulidae sp. COI gene (accession number MF829076.1) was included as an outgroup. Sequencing data was deposited in GenBank under the BioProject PRJNA890670.

RESULTS

Of the 532 animals screened in this study, the initial partial COI cPCR results identified 52 skin samples as positive for the presence filarioid nematode DNA. As the COI cPCR primers used in this study are not species specific for *O. lupi*, these 52 samples of interest were directly sequenced using Sanger sequencing technology to confirm species identity. BLAST results showed 50 of the 52 COI sequences had the closest sequence similarity with 100% identity over a minimum of 411 bases) with *Onchocerca lupi* (accession number YP_010142643.1). All 50 *O. lupi* sequences were identical. The overall occurrence of *O. lupi* in companion animals on the Navajo Reservation was 9.4%. Two of the 52 samples had BLAST results with the closest sequence similarity (99.42-100%) to the canine heartworm, *Dirofilaria immitis* (accession number MT027229.1). Each *O. lupi* positive animal was considered as a local hotspot for insect trapping.

Insect trapping resulted in more than 5,000 insects collected from May 2021 through August 2021. Of these, 1,922 insects (38.44%, 70 pools ranging from 2-50 whole insects along with 15 pools and 205 individual insects with heads and bodies processed separately) were

extracted and screened for parasite presence. The remaining insects were identified morphologically either as non-biting insects (e.g., fruit flies, etc.) or redundant pools of biting midges from trap sites where *O. lupi* positive insects were already identified. A total of 38 insects (29 bodies and 9 heads) and 19 pools were positive for *O. lupi* from 12 of the 15 trap sites (Table 1). These insects were then molecularly identified using Diptera COI gene primers. Unknown Ceratopogonidae sp. (biting midges) were the most abundant insect trapped and had the highest rates of presence of *O. lupi* DNA*;* 15 pools across four trap sites were *O. lupi* positive (Table 2). Initially, the size and profusion of biting midge pools imposed pool processing as whole insects; however, once *O. lupi* positive biting midge pools were identified, remaining pools were processed with heads and bodies separated. *O. lupi* DNA was identified in both the head and body in biting midge pools. Three pools of biting midges *Culicoides sonorensis* were identified as *O. lupi*-positive at 3 separate trap sites as well as 1 pool of *Culicoides variipennis* at a single trap site. *O. lupi* DNA was also found in the heads of *Culicoides sonorensis* but was detected at lower concentrations than in the pools of the unknown Ceratopogonidae. A total of 19 stable flies (11 bodies and 8 heads), *Stomoxys calcitrans*, were positive for *O. lupi* DNA at 3 different trap sites. Single insect positives from individual sites included eye gnats of the genus *Hippelates* sp., Tachinidae sp., Anthomyiidae *sp., Coenosia attenuata, Lucilia sericata,* Oscinelinae sp., *Delia platura, Ravinia errabunda,* and seven single fly, single site undetermined species (Figure 1). Two Ceratopogonidae sp. biting midges from a single site were positive for an undescribed species of *Onchocerca.* The highest partial COI identity for this parasite was to *Onchocerca lienalis* (94.4%, accession number KX853325) (Supplemental Figure 1). Additionally, DNA of the horse stomach nematode *Habronema muscae* was identified in a single house fly, *Musca domestica,* at a single site.

DISCUSSION

This study conducted molecular screening of companion animals to identify the occurrence of the filarial nematode *O. lupi* and field surveillance of hematophagous insects to demonstrate possible intermediate hosts *of O. lupi*. This study identified 9.4% occurrence of *O. lupi* in asymptomatic companion animals on the Navajo Reservation in Arizona, New Mexico, and Utah in the southwestern United States from 2019-2022. This is the first large-scale study on the prevalence of *O. lupi* in dogs and cats in the US, as most published reports focus on clinical cases, diagnostics, and new geographic records (3,4,46–48). Only a few epidemiological studies have been conducted for determining the prevalence of *O. lupi* in canine populations, none of these in North America. Previous studies in Greece, Portugal, and Spain reported positivity rates in dogs ranging from 4.8-8% (46,47). Interestingly, studies investigating prevalence rates of other *Onchocerca* species demonstrated a correlation between statistically higher prevalence rates and host age (49). Many of our samples were collected from spay and neuter clinics focusing on young animals; because of the heavy sampling of these younger animals, it is possible we are under-estimating the prevalence rate of *O. lupi*, particularly because we lack detailed knowledge of various biological parameters of its life cycle, including pre-patent period and patency. Furthermore, it is known that various *Onchocerca* species have an uneven distribution of microfilariae in the dermis of their hosts (50), which may have generated some false negative results in this study as our *O. lupi* detection relied solely upon microfilariae presence within skin snips from a single anatomic location of companion animal hosts. It is important to consider that the animals that were positive for *O. lupi* appeared to be healthy and did not display symptoms of disease at the time of sample collection. This could be due to their young age combined with *Onchocerca'*s long development period into gravid adults. It is also

feasible to consider canines with varying levels of tolerance of *O. lupi* infections. The absence of ocular and skin symptoms in conjunction with cPCR positive tests brings into question the pathogenic role of *O. lupi* within its definitive hosts.

All *O. lupi* positive insects belonged to the Order Diptera; we identified 13 different species (Table 1). Among the 38 positive flies, seven could not be identified to species-level morphologically or molecularly. It is not surprising that this study identified unknown Diptera species on the Navajo Reservation, as to date, no comprehensive seasonal entomological survey has been conducted; however, there have been over 150,000 species described within the Order Diptera (51). It is estimated that 5 million morphologically distinct dipteran species exist but have not yet been described (52). Furthermore, the Sanger sequencing data for four of these unknown flies shows mixed calls across the COI gene indicating mixed samples or contamination. Given that these unknown flies were identified as singletons at single locations suggests it's unlikely any of these unknown non-biting flies are true vectors of *O. lupi*. Additionally, it is unlikely that the eight *O. lupi* positive singleton non-biting flies (Tachinidae sp., Anthomyiidae sp., *Coenosia attenuata, Lucilia sericata,* Oscinelinae sp., *Ravinia errabunda, Delia platura,* Hippelates sp.) found at single sites are the vector species responsible for disease transmission on the Navajo Reservation. We hypothesize these flies (Tachinidae sp., *Coenosia attenuate,* Oscinelinae sp.) are either predators of smaller insects and acquired *O. lupi* DNA by feeding on insects that took blood meals from an infected host or may have only fed on serosanguinolent secretions of an infected definitive host (*Lucilia sericata, Ravinia errabunda,* Anthomyiidae sp., *Delia* platura, Hippelates sp.). The four Fanniidae sp. found at a single site have potential as *O. lupi* vectors. Three species of Fanniidae, *Fannia canicularis, Fannia benjamini,* and *Fannia thelaziae,* have been implicated as the vector of the eye worm *Thelazia*

californiensis by ingesting L1 larvae while feeding on lacrimal secretions. These larvae mature into L3 infectious larvae within the fly and are transmitted to a new host while feeding on lacrimal secretions (53). However, we hypothesize it is unlikely these serve as competent *O. lupi* vectors as they were present only at a single trap site. Seven cryptic fly species found at two different trap sites were unable to be morphologically or molecularly identified. Morphological identification determined these flies were seven different species and unlikely true vectors of *O. lupi* given their infrequency and non-biting appearance.

In this study we identified the stable fly, *S. calcitrans,* (Diptera: Muscidae) containing *O. lupi* DNA, but its role as a suitable vector remains unclear. Stable flies are known to feed on dogs, particularly dog's ears causing bleeding, lesions/crusts. This would likely attract other non-blood feeding dipteran for an opportunistic meal of organic matter/nutrients. While stable flies are a disease vector (*Habronema microstoma;* horse stomach worm), they are largely considered deficient biological vectors of disease in comparison to other blood feeding flies (54). To date, pathogen/parasite development or reproduction has not been demonstrated within the stable fly other than the horse stomach worm. However, despite being inefficient biological vectors, as a mechanical vector, stable flies are still culpable for pathogen/parasite transmission. In this study, we identified 19 stable flies, including 11 bodies and 8 heads, as positive for *O. lupi* DNA. Positive stable flies originated from 3 different locations on the Navajo Reservation. We hypothesize the *O. lupi* positive stable flies likely fed on an infected mammal and while they are not biological vectors, it is still plausible they are mechanical vectors. Even so, we expect both human populations and companion animals are at low risk of *O. lupi* transmission from stable flies.

Multiple *Ceratopogonidae* sp. (biting midges) were identified as the most abundant *O. lupi* positive insect in our study (*O. lupi* was present in the heads and bodies) and likely an intermediate host in Northern Arizona and New Mexico. The Ceratopogonidae (Diptera) family, which includes the genus *Culicoides*, commonly called biting midges or “no-see-ums", are considered hematophagous pests and vector several infectious agents including viruses, protozoans, and filarioid nematodes worldwide (56–58). Biting midges are most notable as vectors of several arbovirus livestock diseases such as bluetongue virus (BTV), African horse sickness virus (AHSV), epizootic hemorrhagic disease virus (EHDV), and Schmallenberg virus (SBV), all of which are of considerable veterinary and economic relevance (59). Importantly, biting midges have been implicated as vectors for several filarial nematodes including *Mansonella ozzardi* (Nematoda: Onchocercidae) with humans as the primary host (60), as well as seven reported species of *Onchocerca* infecting a range of hosts including mammals from the biological families Bovidae, Cervidae, and Equidae (20,58). The Ceratopogonidae biting midges, which we identify here as potential vectors for *O. lupi*, are widespread throughout the U.S. (61). When considering the potential of vector-borne disease dissemination into non-endemic regions, it is imperative to examine the potential range expansion of the vector species. In terms of range expansion, there are two aspects of vector dispersal: 1) wing-propelled flight of females to find nearby blood-meals (upwards of 5 km) (62), and 2) wind-borne dispersal from wind streams with potential to spread these vector-borne diseases into non-endemic regions upwards of 200 km away (63). Research has demonstrated recent range expansion of *Culicoides* spp. in the southeastern US (64), and when coupled with wind expansion potential, highlights the immediate risk for vector establishment, and dissemination of *O. lupi* into non-endemic regions.

We hypothesize that *O. lupi* has adapted to two families of hematophagous Diptera as competent

vectors depending on the variable geographic area – Simuliidae and Ceratopogonidae. The Navajo Reservation is bordered by the San Juan River in the north, the Little Colorado River (flow in much of this river is ephemeral) to the south, and the Colorado River to the west (65). Despite these major waterways surrounding the Navajo Reservation, the Reservation itself is vast, with large regions lacking moving water habitats necessary for blackfly development. Transmission of the filarial nematode *M. ozzardi*, first described in 1897, further supports our hypothesis of transmission of *O. lupi* by two families of dipteran vectors (66). *M. ozzardi* is a human parasite transmitted by biting midges (mostly members of the genus *Culicoides*) and blackflies (genus *Simulium*) depending on the endemic location. For example, blackflies of the *Simulium* genus were identified as *M. ozzardi* vectors in Central and South America while *Culicoides* spp. were implicated in transmission in Mexico and several islands (66). Additionally, an important factor that further supports our hypothesis is that biting midges are superficial blood feeders, as are black flies, which allows for involvement in transmitting skin-dwelling filarioid nematodes as opposed to solenophagy Diptera that feed directly from blood capillaries of vertebrate hosts (e.g., mosquitoes).

Additionally, a pool consisting of two biting midges from a single site (site 9) showed the presence of parasite DNA in the initial COI cPCR. The COI DNA sequence belongs within the *Onchocerca* genus, however, the species has yet to be described (Supplemental Figure 1). Blast results showed the closest match to this unknown sample was *Onchocerca lienalis* with 94.56% identity. Little is known regarding unknown *Onchocerca* species in wildlife and arthropods (24), however, a more comprehensive examination of unknown *Onchocerca* species within North America is needed to catalog the diversity of both *Onchocerca* and the arthropods vectoring them.

Current *O. lupi* surveillance tools targeting the COI mitochondrial gene are limited to detection and cannot provide insight into population structure, at least within the United States. Two recent studies published the first mitochondrial genome as well the first draft genome for *O. lupi* (18,67). These studies should facilitate the development of high-resolution surveillance tools for *O. lupi* to understand the genetic diversity and relationships among isolates globally. Further research regarding vector competency of Ceratopogonidae and *Culicoides* species is necessary to confirm their role as intermediate hosts for *O. lupi*. Subsequent knowledge of the vector life cycle is paramount to break the cycle of *O. lupi* transmission on the Navajo Reservation. Mitigation efforts to control the vector species will likely decrease and prevent *O. lupi* disease in companion animals thereby reducing the risk of human infection.

CONCLUSIONS

Our increased surveillance of definitive hosts represents the first large scale study of *O. lupi* in companion animals from an endemic area of the US. We identified a prevalence rate of 9.4% and, from these data points, conducted targeted insect trapping to define putative vectors in the southwestern US. We found strong evidence that biting midge species may be putative vectors in Northern Arizona and New Mexico. We hypothesize that *O. lupi* has adapted to two families of hematophagous Diptera as competent intermediate hosts depending on the geographic area, Simuliidae and Ceratopogonidae. Further research regarding vector competency of Ceratopogonidae and *Culicoides* species is necessary to confirm their role as intermediate hosts for *O. lupi*.

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Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the US Government.

FIGURE LEGENDS

Figure 1. Maximum likelihood tree of *O. lupi* positive Diptera species including 12 publicly available COI genes. The phylogenetic tree was rooted using the outgroup Tipulidae sp. (accession number MF829076.1). Accession numbers are included on the tree for all included publicly available COI genes. The ** denotes COI genes generated in this study (Supplemental Table 1).

Supplemental Figure 1. Maximum likelihood tree of the COI gene from *Onchocerca* species using *Onchocerca lupi* as the reference gene. The unknown *Onchocerca* species found at site 9 in two biting midges is shown in red. This analysis included 533 bases and had a total of 135 SNPs. Positive *O. lupi* insects from this study are denoted with an *.

TABLES

Table 1. Insect trapping site descriptions that includes water sources and species of positive insects. Informed insect trap sites were based on close proximity to an *O. lupi* positive companion animal; non-informed insect trap sites refer to locations with no *O. lupi* positive companion animal near-by and were based solely on permissions to trap on the land. *O. lupi* was found in the heads of Ceratopogonidae sp, *Culicoides sonorensis*, *Stomoyxs calcitrans*, and a single Fanniidae sp. Locations with * denote *O. lupi* positive insect trap sites.

Location	Informed	Water Source	*O. lupi* Positive Species
Site 1*	Yes	Lake, creek, farm line, community reservoir	Ceratopogonidae sp.
Site 2*	No	Dry Creek Bed	Ceratopogonidae sp., *Culicoides sonorensis*,
Site 3	No	No	-
Site 4*	Yes	Large Animal Water Trough	*Stomoxys calcitrans,* Tachinidae sp.
Site 5*	Yes	Dry Creek Bed	Unknown sp.
Site 6*	Yes	Large Animal Water Trough	*Stomoxys calcitrans*
Site 7	Yes	Large Animal Water Trough	-
Site 8*	Yes	No	Faniidae sp., *Anthomyiidae sp.*,
Site 9*	Yes	No	*Stomoxys calcitrans, Lucilla sericata*
Site 10*	Yes	Standing Rainwater	*Ravinia errabunda,* Unknown sp.
Site 11*	Yes	No	*Hippelates* sp.
Site 12*	Yes	No	Ceratopogonidae sp., *Culicoides variipennis, Delia platura, Coenosia, paradidyma*
Site 13	No	No	-
Site 14*	Yes	Dry Creek Bed	*Culicoides soroensis*
Site 15*	Yes	Dry Creek Bed	Ceratopogonidae sp., *Culicoides soroensis,* Oscinellinae sp.

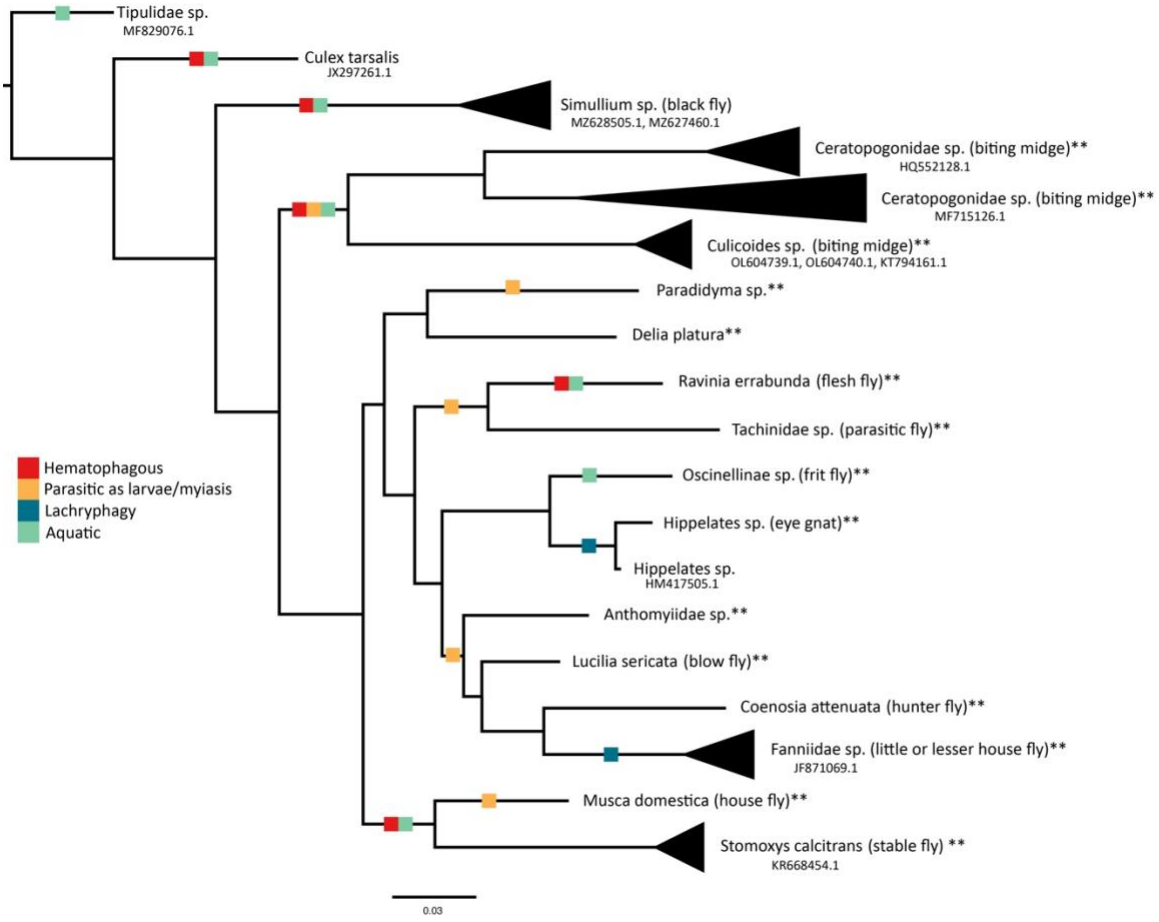
Table 2. Insects positive for the presence of *O. lupi*. Asterisk* indicates an insect pool containing 2-50 insects. Biting column refers to the insect's known ability to bite.

Species	**Number of Insects**	**Number of Sites**	**Family**	**Biting**
Ceratopogonidae **sp.**	15*	4	Ceratopogonidae	Yes
Culicoides sonorensis	3*	3	Ceratopogonidae	Yes
Culicoides variipennis	1*	1	Ceratopogonidae	Yes
Stomoxys calcitrans	19	3	Muscidae	Yes
Fanniidae sp.	4	1	Faniidae	No
Hippelates sp.	1	1	Chloropidae	No
Tachinidae sp.	2	2	Tachinidae	No
Anthomyiidae sp.	1	1	Anthomyiidae	No
Coenosia attenuata	1	1	Tachinidae	No
Lucilia sericata	1	1	Calliphoridae	No
Oscinellinae sp.	1	1	Chloropidae	No
Delia platura	1	1	Anthomyiidae	No
Ravinia errabunda	1	1	Sarcophagidae	No
Unknown	7	2	-	-

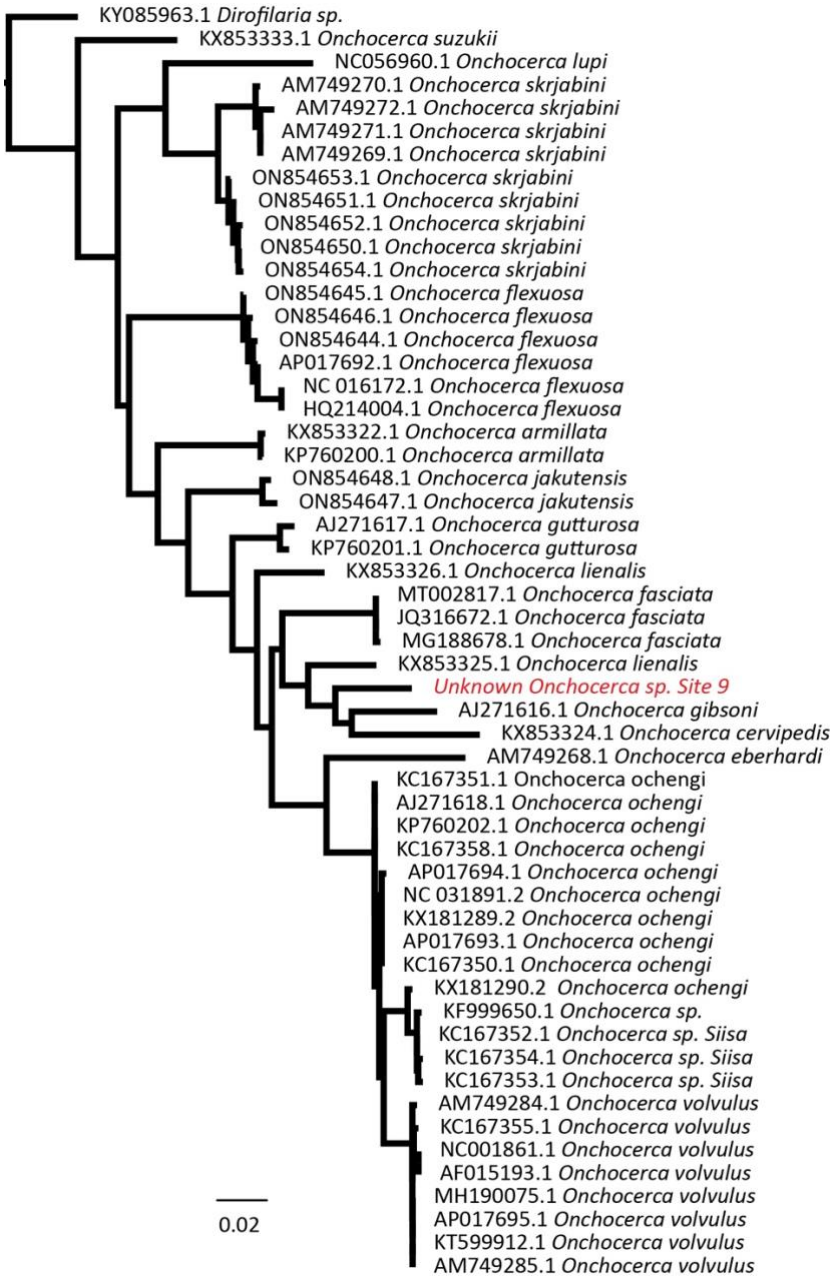
Supplemental Table 1. Gene sequences included from both this study and publicly available genes included in Figure 1, the Diptera COI gene tree.

SPECIES	GENBANK ACCESSION NUMBER	SOURCE
Simulium ornatum	MZ628505.1	publicly available
Simulium intermedium	MZ627460.1	publicly available
Simulium vanluni	MN514760.1	publicly available
Ceratopogonidae sp.	HQ552128.1	publicly available
Fanniidae sp	JF871069.1	publicly available
Hippelates sp	HM417505.1	publicly available
Stomoxys calcitrans	KR668454.1	publicly available
Ceratopogonidae sp.	MF715126.1	publicly available
Culicoides sonorensis	OL604740.1	publicly available
Culex tarsalis	JX297261.1	publicly available
Culicoides variipennis	KT94161.1	publicly available
Culicoides sonorensis	OL604739.1	publicly available
Fanniidae sp rep1	OQ720968	This study
Fanniidae sp rep2	OQ720969	This study
Fanniidae sp rep3	OQ720970	This study
Fanniidae sp rep4	OQ720971	This study
Hippelates sp	OQ720972	This study
Paradidyma sp	OQ720973	This study
Stomoxys calcitrans	OQ720974	This study
Ceratopogonidae sp rep1	OQ720975	This study
Ceratopogonidae sp rep2	OQ720976	This study
Ceratopogonidae sp rep3	OQ720977	This study
Culicoides sonorensis	OQ720978	This study
Culicoides variipennis	OQ720979	This study
Lucilia sericata	OQ720980	This study
Anthomyiidae	OQ720981	This study
Ravinia errabunda	OQ720982	This study
Delia platura	OQ720983	This study
Oscinellinae	OQ720984	This study
Tachinidae sp rep1	OQ720985	This study
Coenosia attenuata	OQ720986	This study
COI Olupi from canine	OQ716812	This study
COI Olupi from biting midge	OQ509788	This study

Figure 1. Maximum likelihood phylogenetic analysis of *O. lupi* positive Diptera species including 12 publicly available COI genes.



Supplemental Figure 1. Maximum likelihood phylogenetic analysis of the COI gene from Onchocerca species using *Onchocerca lupi* as the reference gene. An unknown *Onchocerca* species found in this study is highlighted in red.



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CHAPTER THREE

COMPLETE MITOCHONDRIAL GENOME OF ONCHOCERCA LUPI (Nematoda, Onchocercidae)

ABSTRACT

Onchocerca lupi, Rodonaja 1967, is an emerging, zoonotic filarial nematode parasite that causes ocular disease in dogs, cats, wild canids, and humans. It is the causative agent of ocular onchocercosis in canines with increasing incidence in both North America and the Old World during the early 21st century. We report the complete mitochondrial genome of an *O. lupi* isolate from a dog from Arizona, southwestern USA, and its genetic differentiation from related *Onchocerca* species. The whole mitochondrial genome was obtained from whole genome sequencing of genomic DNA isolated from an adult worm. This mitogenome is 13,766bp in size and contains 36 genes and a control region. This mitogenome provides a valuable resource for future studies involving epidemiological surveillance, population genetics, phylogeography, and comparative mitogenomics of this emerging pathogen and other parasitic nematodes.

Nematodes of the genus *Onchocerca* are found world-wide and infect a range of hosts such as humans, wild and domestic ungulates and carnivores, and comprise in excess of 30 valid species[1]. *Onchocerca lupi* is a tissue-dwelling zoonotic filarial parasite that causes ocular onchocercosis in dogs, cats, coyotes, wolves, and recently, humans[2]. The putative biological vector of *O. lupi* are black flies (Diptera: Simuliidae), which transfer the infective third-stage larva to the

mammal definitive host. Infection of the definitive host is usually associated with ocular disease and may lead to blindness. Since 2010, there have been increasing reports of onchocercosis in domestic dogs and cats in North America and areas of Europe, North Africa and the Middle East[3–5], recent reports in coyotes[2], and zoonotic infections[6,7]. There is currently a knowledge gap regarding the genomics, population structure, and phylogeography of *O. lupi*. Previous research has utilized single mitochondrial genes (COI, ND5) for phylogenetic analyses providing low resolution relationships from global samples[2,8]; however, recent research of other zoonotic parasites identified phylogenetic relationships using whole mitochondrial sequencing that were indiscernible when examining short gene sequences[9,10]. Here, we report the complete mitochondrial genome of a single *O. lupi* nematode from the United States.

One adult nematode was collected by a local veterinarian from a privately-owned, infected dog from Flagstaff, AZ, USA (35° 11' 53.05" N, -111° 39' 4.57" W) in 2010. The specimen was pulverized by reciprocal shaking with steel beads at 30 Hz and DNA was extracted using a phenol-chloroform protocol[11] modified with prolonged heated lysis and stored at -20°C. Taxonomic identity was determined by Sanger sequencing using the previously published COI mitochondrial gene primers[12]. Genomic DNA was prepared for sequencing using previously published methods[13] and sequenced on an Illumina HiSeq 2500 platform. An *O. lupi* specimen was deposited at the Pathogen and Microbiome Institute (https://in.nau.edu/pmi/, Roxanne Nottingham, Roxanne.nottingham@nau.edu) under voucher number OL-202101.

Due to the presence of both nuclear parasite as well as host DNA, mitochondrial reads were extracted bioinformatically through alignment to *Onchocerca volvulus* reference mitogenome (AF015193; 13,747bp); reads that mapped to the *O. volvulus* mitochondrial genome were separated using SAMtools v1.9[14], assembled using SPAdes[15] to an average depth of 303X, and

examined for circularity using Circlator[16]. The entire circularized *O. lupi* mitogenome is 13,766 bases (GenBank accession MW266120). The mitogenome consists of twelve protein-coding genes (PCG), twenty-two transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes and one noncoding region (NCR). One DNA strand serves as the template strand for all genes. All PCGs are syntenic with the *O. volvulus* mitogenome; however, total synteny differs by the placement of one tRNA-Lys. The nucleotide distribution of the mitogenome was biased toward A+T (68%) which is similar to other reported filarial nematodes[17].

Bayesian phylogenetic analyses of seven *Onchocerca* samples were implemented with the program ExaBayes v1.5.1[18] using the concatenated, shared SNP loci produced by the SNP pipeline NASP[19], which spanned 12,259 shared positions (89%) across four *Onchocerca* species with the canine heartworm, *Dirofilaria immitis*, serving as the outgroup. This analysis revealed a total of 2,260 SNPs; 852 SNPs were parsimony-informative positions revealing *O. lupi* as the most basal species within the genus *Onchocerca*, considering the limited number of species with characterized mitogenomes. We report the completed mitogenome of *O. lupi* by next-generation sequencing and molecular phylogenetic placement within the genus *Onchocerca*. This mitogenome will provide a novel, strong foundation for further studies involving phylogenetic relationships among *Onchocerca* species and observations of the origin and range expansion of *O. lupi*.

Data Availability.

The genome sequence data that support the findings of this study are openly available in GenBank of NCBI at https://www.ncbi.nlm.nih.gov/nuccore/MW266120.1 under the accession no. MW266120. The associated BioProject and Bio-Sample numbers are PRJNA733160 and SAMN19369283.

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FIGURE LEGENDS

Figure 1. Bayesian phylogenetic relationships of *Onchocerca lupi* and 3 additional *Onchocerca* species based on concatenated SNPs that covered 89% total bases of the reference *O. lupi* mitogenome. Bayesian posterior probability values are shown below branches while SNP numbers are shown above corresponding branches. A "//" indicates a broken branch length that was shortened for visual purposes.

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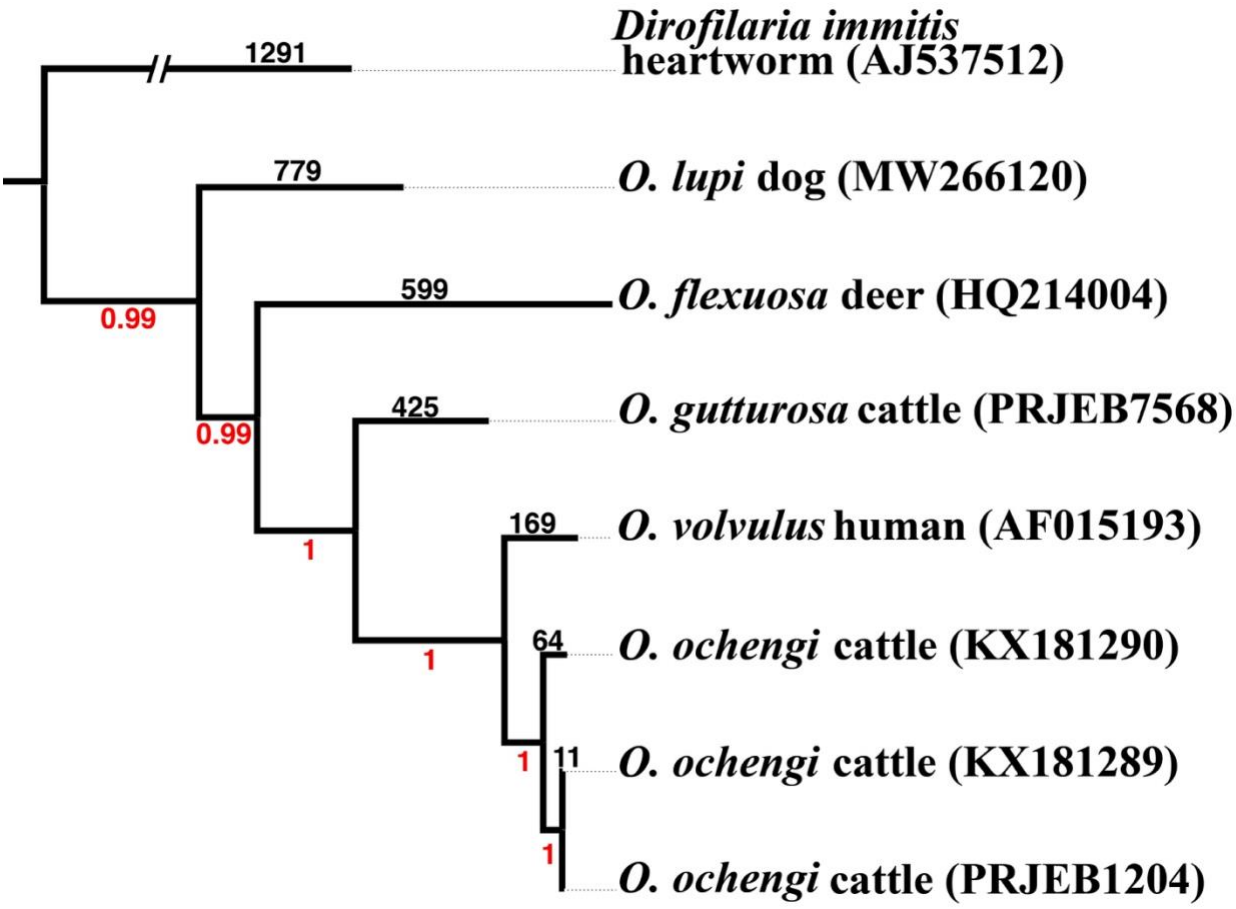

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CHAPTER FOUR

LUPIQUANT: A REAL-TIME PCR BASED ASSAY FOR DETERMINING HOST-TO-PARASITE DNA RATIOS OF ONCHOCERCA LUPI AND HOST CANIS LUPUS FROM ONCHOCERCOSIS SAMPLES

ABSTRACT

Onchocerca lupi is a filarial nematode that causes ocular onchocercosis in canines globally including North America and areas of Europe, North Africa, and the Middle East. Reported incidence of this parasite in canines has continued to steadily escalate since the early 21st century and was more recently documented in humans. Whole genome sequencing (WGS) of this parasite can provide insight into gene content, provide novel surveillance targets, and elucidate the origin and range expansion. However, past attempts of whole genome sequencing of other *Onchocerca* species reported a substantial portion of their data unusable due to the variable over-abundance of host DNA in samples. Here, we have developed a method to determine the host-to-parasite DNA ratio using a quantitative PCR (qPCR) approach that relies on two standard plasmids each of which contains a single copy gene specific to the parasite genus *Onchocerca* (major body wall myosin gene, myosin) or a single copy gene specific to the canine host (polycystin-1 precursor, *pkd1*). These plasmid standards were used to determine the copy number of the *myosin* and *pkd1* genes within a sample to calculate the ratio of parasite and host DNA. Furthermore, whole genome sequence (WGS) data for three *O. lupi* isolates were consistent with our host-to-parasite DNA ratio

results. Our study demonstrates, despite unified DNA extraction methods, variable quantities of host DNA within any one sample which will likely affect downstream WGS applications. Our quantification assay of host-to-parasite genome copy number provides a robust and accurate method of assessing canine host DNA load in an *O. lupi* specimen that will allow informed sample selection for WGS. This study has also provided the first whole genome draft sequence for this species. This approach is also useful for future focused WGS studies of other parasites.

INTRODUCTION

Onchocerca lupi is a filarial nematode that represents an emerging threat to wildlife, companion animals, and humans (1). First described in the Republic of Georgia in the periocular tissues of a wolf (*Canis lupus lupus)* in 1967 (2), it was only recently detected in domesticated canines and felines in North America and the Old World (3–5). As there is no current commercial diagnostic test for this parasite, *O. lupi* infections are confirmed based on ocular nodules on eyelids, conjunctiva, and sclera (6–8). If nodules are not present but *O. lupi* infection is suspected, the only diagnostic tool currently available is through the detection of microfilariae in skin (9). However, this invasive skin biopsy is heavily dependent on the biopsy location and the density of microfilaria (10) making this tool highly unreliable.

Onchocerca lupi poses a new public health and veterinary threat, but the genomic mechanisms that drive the evolution of pathogenicity are largely unexplored. Because of the growing number of both canine and human cases of *O. lupi*, it is imperative to understand the genomic content of this parasite to identify appropriate and *O. lupi* specific biomarker targets, mitigation strategies, and effective treatments. To date, there are no evidence-based treatment protocols for adult *O. lupi* nematode infections. Current treatment methods are based mostly on *O. volvulus* and involve the anti-microfilaricidal drug ivermectin concurrently given with the

antibiotic doxycycline (11,12). However, there is no cure for this filarial nematode, highlighting the dire need for novel treatment therapies for this emerging parasite; one way to achieve this is by characterizing the whole genome of the parasite itself. Previous research has shown the production of draft genomes for filarial nematodes has significantly contributed to the identification of potential new drug treatment options (13,14). To date, there are no studies investigating the genomic landscape of *O. lupi* beyond mitochondrial genes. However, a recent study involving the closely related *Onchocerca ochengi*, a cattle parasite, described the sequencing of 20 whole genome samples and subsequently reported that data from 10 of those samples were majority host (cattle) DNA and therefore unusable (15). To circumvent costly sequencing of majority host DNA in *O. lupi* samples, we have designed a qPCR to quantify the ratio of *O. lupi* parasite DNA and *Canis lupus familiaris* host DNA within a parasite sample. Here, we implemented a previously published (16) approach based on single copy genes unique to the parasite (*myosin*) or the host *(*polycystin-1 precursor, *pkd1)* (17). Additionally, the highly conserved *pkd1* gene target can be used to quantify DNA ratios from coyote, wolf, and dingo samples in addition to canine hosts. This approach is crucial for informed sample selection for whole genome sequencing of parasitic nematode samples that will allow for the development of novel, species-specific biomarkers for pathogen tracking, identify potential treatment targets, and determine population structure and evolution of this newly emerging zoonotic parasite. Additionally, this study produced the first draft genome for this species using this approach.

MATERIALS AND METHODS

Single Copy Gene Target Selection

The polycystin-1 precursor (*pkd1*) canine gene (accession no. AF483210) was identified as a conserved, single copy gene in a previous study (17) and was selected for use as the host locus

based on these criteria. Pre-aligned *pkd1* canine gene sequences (n=4) (Supplemental Table 1) were downloaded from the NCBI nucleotide database (18) and used for primer design in the online software Primer3 (19). The parasite locus, major body wall myosin gene, was chosen as it was a highly conserved, single copy gene across the genus *Onchocerca*. Briefly, the genomic sequences for 4 *Onchocerca* species (*O. ochengi, O. flexuosa, O. volvulus,* and *O. lupi*) (Supplemental Table 2) were aligned to predicted coding sequences pulled from *O. ochengi* (accession no. ASM90053720v1) reference genome using BWA v0.7.17-r1188 (20) within the NASP v1.2.0 pipeline (21). Nucmer v3.1 (22) was used to identify single copy coding regions within the reference genome. Coding regions that were highly conserved across all *Onchocerca* genomes were considered for primer design using Primer3 software.

Sample Collection and DNA Isolation

Single copy host and parasite genes were amplified from a pre-established *O. lupi* PCR-positive canine skin biopsy sample collected from northern Arizona, United States under IACUC of Northern Arizona University approved protocol 19-016. Genomic DNA was extracted from a complex, biopsied canine skin sample using the Qiagen Blood and Tissue Kit (Qiagen) following overnight lysis, according to the manufacturer's recommendations. *O. lupi* DNA was confirmed using previously published methods (1). Additionally, four adult *O. lupi* isolates from four dogs in Flagstaff, Arizona; Phoenix, Arizona; and Albuquerque, New Mexico (n=2) were used in this study. Adult worms contained within host tissue nodules were isolated using a 0.3% collagenase enzymatic digestion to remove host tissue and subsequently washed four times with PBS (11). Genomic DNA was extracted using a modified filarial parasite genomic DNA isolation protocol (23) as follows: samples underwent three freeze/thaw cycles consisting of three minutes in liquid nitrogen followed by three minutes at 80ºC. Afterward, samples were transferred to 2mL round

bottom tubes with a single 5mm stainless steel bead, 250µL PBS, and 100µL lysis buffer. Using a vortex mixer with a special adapter, samples were vortexed on max speed for 45 min with rotation of the tubes every 10 minutes. Immediately following bead beating, 30µL of 10% SDS was added to each sample along with 2µL of 2–mercaptoethanol and 60µL of proteinase K (20mg/µL). Samples were incubated overnight at 65ºC followed by an RNase A treatment which consisted of adding 15µl of RNase A (10mg/mL) to each sample and incubated at 37ºC for one hour. The Qiagen DNeasy Blood and Tissue kit (Qiagen) was used following the manufacturer's recommendations with one exception; buffer AL was added at a 1:1 ratio with the sample volume. Extracted DNA was stored at -20ºC until further use.

Cloning of the *pkd1* and the myosin gene

The *pkd1* and myosin plasmid construct genes were amplified from the *O. lupi* PCR-positive canine skin biopsy sample using the thermocycler conditions below:

pkd1: 95ºC for 3 min, followed by 40 cycles of 95ºC for 30 seconds, 60ºC for 30 sec, 72ºC for 1:30 min and a final extension of 72ºC for 1 min.

myosin: 95ºC for 3 min, followed by 35 cycles of 95ºC for 30 seconds, 60ºC for 30 sec, 72ºC for 1:30 min and a final extension of 72ºC for 1 min.

gDNA from a PCR-positive *O. lupi* canine skin sample was used as a template for both reactions and no template controls were included for all PCRs. Primer sequences for both the plasmid construct amplicons as well as the SYBR assay targets are given in Table 1. Non-specific banding was observed in the plasmid construct *pkd1* gene PCR; therefore, the PCR product just below the 1000base marker was extracted from a 2% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Both the *pkd1* and myosin amplified product were ligated into a TOPO TA vector (Invitrogen) according to manufacturer's instructions. Plasmid constructs were

amplified through transformation into One Shot TOP 10 chemically competent *E. coli* (Invitrogen) followed by overnight culturing. To ensure plasmid stability, ten individual colonies were re-streaked on LB containing 50mg/mL kanamycin and incubated overnight at 37ºC. Cells were harvested and the cloned plasmid was extracted using the QIAquick Mini Kit (Qiagen). Gene inserts were confirmed for each plasmid by restriction digest using ECORI and sequenced directly with capillary electrophoresis using BigDye Terminator v3.1 Cycle Sequencing Kit on a 3130 Genetic Analyzer platform (Applied Biosystems) using M13 Forward and M13 Reverse (M13 FR) vector primer sites for all replicates. All sequences were queried with blastn (24) against the NCBI Nucleotide database (nt) to confirm the composition of gene targets within each plasmid.

qPCR SYBR Green Based Assay

Internal primers for use with SYBR dye-based qPCR assays were designed using Primer3 software for both *pkd1* and myosin genes. All qPCR assays were performed on an Applied Biosystems QuantStudio 12. Concentrations for the plasmid preps were measured using the Qubit dsDNA BR assay kit (Invitrogen). A fresh tenfold serial dilution ranging over six logs (10^6 to 10 gene copy number (GCN)) of both the pTOPO-*pkd1* and pTOPO-myosin plasmids were used to generate each standard curve (Table 1). A 10µL qPCR mixture was prepared using the PowerUp SYBR Green Master Mix (Invitrogen): 1X PowerUp SYBR Green Master Mix, 0.3µM forward and reverse primers, and 2µL template DNA or plasmid standards. The thermal cycling protocol was as follows:

95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec, 60ºC for 1 min.

Following amplification, a melting curve analysis was used to confirm reaction specificity; a single and specific peak was generated for each primer pair. Both negative and no-template controls were performed in triplicate.

Estimation of gene copy number

The gene copy number (GCN) of both plasmids used in this study were calculated using the following equation (25):

$$GCN = \frac{6.02\ X\ 10^{23}\ \frac{copy}{mol}\ X\ DNA\ amount\ (g)}{DNA\ length\ (bp) X\ 660(\frac{g}{mol/bp})}$$

DNA length represents the combined length of the plasmid (3,931 bp) and corresponding insert (*pkd1* = 951 bp, *myosin* = 991 bp). The DNA amount represents the plasmid concentration multiplied by the volume used. Standard curves were generated using copy number vs. C_q value for all six plasmid dilutions in triplicate for both pTOPO-*pkd1* and pTOPO-*myosin* plasmids.

Host and parasite DNA ratio calculations

GCNs estimated from the pTOPO-*pkd1* and pTOPO-*myosin* standard curves were used to calculate total host and parasite DNA in four *O. lupi* samples. Given the estimated genome sizes of host (2370Mb) and parasite (150Mb) as well as the estimated gene copy numbers, we used the above equation to solve for the "DNA amount" in grams. The following equation was used to calculate host to parasite DNA ratio:

$$Ratio = [Parasite\ DNA/Total\ DNA\ (Host + Parasite)]\ x\ 100$$

DNA sequencing and analysis

Four *O. lupi* DNA samples extracted from adult nematodes were prepared for paired-end, whole genome sequencing on either a MiSeq, HiSeq, or NextSeq using previously described methods (26). To aide in the creation of a reference genome, sample Olupi_Ro2020_NM was sequenced on both Illumina NextSeq and MiSeq instruments. Raw reads were trimmed for adapter sequences using trimmomatic v0.39 (27). The first draft genome assembly for this species was created using SPAdes v3.15.3 (28). Assembly errors were corrected with eight rounds of pilon

(29). Reads were globally aligned to both the dog reference genome (accession number GCA_008641055.1) and our *O. lupi* draft assembly (BioProject PRJNA802584) using bowtie2 v.2.4.2 (30) default parameters with the addition of -I 125, and -X 1800. The number of aligned reads to each reference genome were calculated using PICARD tools v1.125 (31).

RESULTS

Melting temperature and standard curve analysis

Two plasmid standards each containing a highly conserved gene specific to the canine host (951 bp fragment of the *pkd1* gene) or the parasite (991 bp fragment of the *myosin* gene) were constructed. The amplicon targets used for the qPCR assay are nested within larger gene fragments (Table 1). These nested primers produced a single amplicon for each gene target, *pkd1* (336bp) and *myosin* (339bp) (Supp. 1). Melting curve analysis for both *pkd1* and myosin amplicons revealed single peak temperature at 92^{o}C and 81^{o}C respectively for the standard plasmid DNA and the 3 biological samples.

Standard curve slopes were -3.499 for *pkd1* plasmid and -3.509 for the myosin plasmid. Regression analysis was used to evaluate prediction accuracy which resulted in R^2 values of 0.999 (*pkd1*) and 1.0 (*myosin*) and standard curve efficiencies of 93.11% and 92.74%, respectively. No template controls were included in each run to ensure PCRs were contamination-free.

Host-to-Parasite DNA Ratio Predictions

Using DNA extracted from three *O. lupi* isolates, host and parasite DNA ratios were estimated using LupiQuant. Standard curves were plotted using the *pkd1* and *myosin* plasmid constructs; GCN of the host and parasite DNA per sample were calculated using the C_q values in reference to the standard curves. The host to parasite DNA ratio was calculated using equation 2.

In the three *O. lupi* samples used for quantification, parasite DNA (%) ranged from 0.12% to 46.75% to 99.74% (Figure 1).

Whole genome sequencing and DNA ratios

Aligned read counts were tallied and aligned read percentages were compared with the predicted host-to-parasite DNA ratios (Table 2). The WGS data (BioProject PRJNA802584) for three samples showed the parasite DNA alignment (% of total reads) ranged between 1.98% to 47.15% to 94.45% (Figure 1).

DISCUSSION

The ability to produce high quality sequencing data from zoonotic parasites has direct and immediate implications for public and veterinary health. However, the variable amount of background host DNA in parasitic nematode samples can greatly reduce and sometimes entirely eclipse parasite signal (11) in whole genome sequencing. To provide an estimate of nematode signal in complex samples, we designed a robust and accurate qPCR assay (LupiQuant) with separate amplification and detection of parasite and host markers. The assay consists of cloned plasmid standards, each containing a single copy gene target from either the host or parasite. qPCR can then detect the host/parasite ratio that can be used to guide WGS efforts. Correlating WGS data with LupiQuant results showed a strong correlation (Figure 1), demonstrating the power of our approach.

One potential limitation to our approach is the presence of unexpected DNA in the sample (i.e., other pathogens co-infecting the host, contamination). For example, in one isolate from Flagstaff, Arizona, LupiQuant predicted the host-to-parasite DNA ratio as 53.25% host DNA and 46.75% parasite DNA. When the data were mapped against reference genomes, ~63% of the reads failed to align against dog or *O. lupi* references. When examining the ratio of mapped reads instead

of total reads, the LupiQuant ratio estimate was correct. Blast results of the unaligned reads with the NCBI nt database identified what may be a fungal contaminant with less than 50% homology to any published organism. Additional testing on subsequent samples will determine if this contamination is isolated or widespread. The host-to-parasite ratio approach has been used previously for two tick-transmitted intracellular protozoal parasites, *Theileria annulata* and *Theileria parva*, both affecting cattle, but no report exists for filarial nematodes of the genus *Onchocerca* (16,32). Furthermore, this study is the first to use WGS data to validate the qPCR results. LupiQuant represents a critical method that allows researchers to selectively sequence *O. lupi*, conduct population structure studies to understand pathogen spread, develop diagnostics for accurate epidemiological surveillance, and potentially identify novel therapeutics to improve animal outcomes.

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FIGURE LEGENDS

Figure 1. Comparison of *Onchocerca lupi* percentage from three biological canine samples. Samples were screened using LupiQuant which estimated *O. lupi* percentages within each sample. Whole genome sequences of *O. lupi* samples were aligned to *O. lupi* reference genome (BioProject PRJNA802584) to compare LupiQuant ratios. Error bars represent the range of *O. lupi* percentages based on host and parasite technical replicates.

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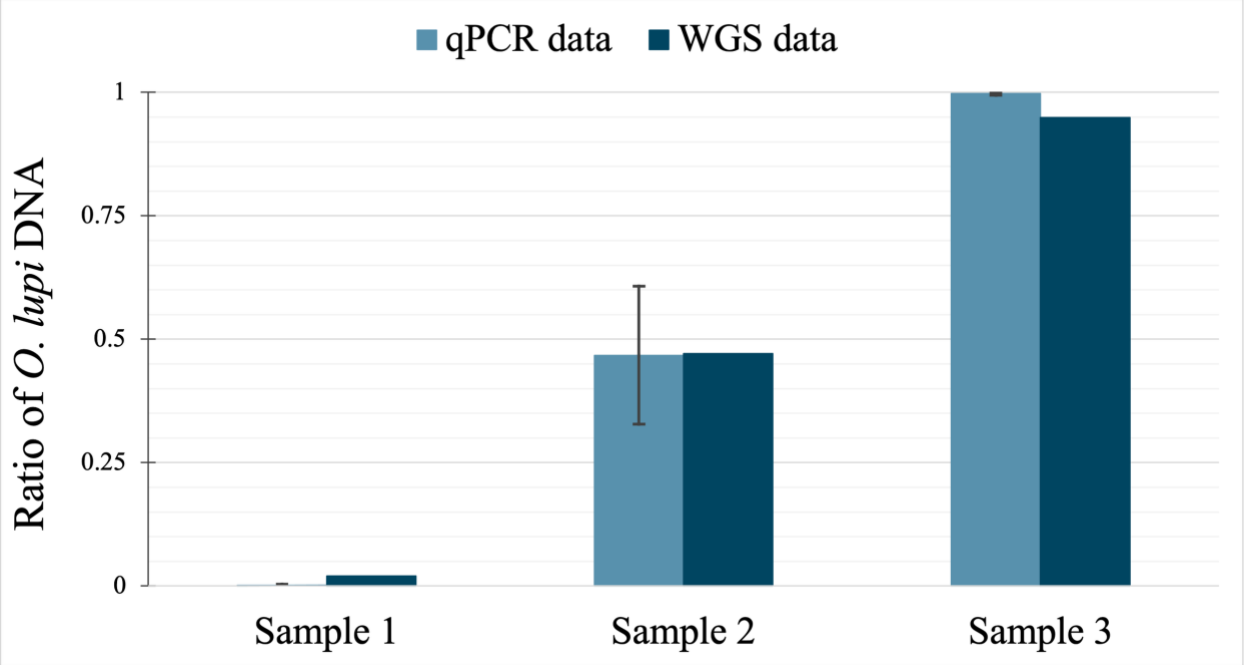

TABLES

Table 1. Primer sequences used to create plasmid standards. Primer sequences for quantitative and conventional PCR for both host and parasite used in this study.

Target	Goal	Sequence 5'-3'	Product Size (bp)
pkd1	plasmid	F: GGCCATAGTCAATTCCAGCG R: CCCAGATCATTGAAGGCACG	951
pkd1	qPCR	F: ACATAGACCGCGGCTTCG R: TGACCTGCAGATGGAAGCG	336
myosin	plasmid	F:GGATATCGCTGGATTCGAGA R:CGGTCATGCTATCATGGAAA	991
myosin	qPCR	F:AACGCGAAGGTATTCAGTGG R:GATCATTCGCTTTAGATTGTTTCA	339

Table 2. qPCR predicted host-to-parasite DNA ratios, WGS read alignment percentages. Table includes data from three adult *O. lupi* nematodes. *Reported ratio is not based on total read numbers.

Sample	Predicted DNA ratio (host to parasite)	WGS alignment ratio (host to parasite)
Isolate 1	99.88 : 0.12	99.48 : 1.98
Isolate 2	53.25 : 46.75	52.85 : 47.15 *
Isolate 3	0.26 : 99.74	3.00 : 94.45

Supplemental Table 1. Accession information for primer design. GenBank accession numbers for polycystin-1 precursor (*pkd1*) gene sequences included for host locus primer design.

Gene Name	Accession Number
Canis familiaris polycystin-1 (*pkd1)*, partial cds	AF483210.1
Canis lupus familiaris breed Labrador retriever chromosome 06b	CP050622.1
Canis lupus familiaris breed Labrador retriever chromosome 06a	CP050586.1
Canis lupus familiaris polycystin 1, transient receptor potential channel interacting (PKD1), mRNA	NM_001006650.1

Supplemental Table 2. *Onchocerca* species used for parasite primer design. Accession information for read data used for parasite locus primer design.

Host	Species	Accession Number
Cattle	*Onchocerca ochengi*	GCA_0009505151
Cattle	*Onchocerca ochengi*	GCA_001077375
Cattle	*Onchocerca ochengi*	GCA_900618345
Deer	*Onchocerca flexuosa*	GCA_002249935
Deer	*Onchocerca flexuosa*	GCA_900618345
Dog	*Onchocerca lupi*	PRJNA802584
Human	*Onchocerca volvulus*	GCA_000280695.1

CHAPTER FIVE

GENETIC DIVERSITY OF THE NUCLEAR, MITOCHONDRIAL, X-CHROMOSE, AND WOLBACHIA GENOMES AMONG ISOLATES OF ONCHOCERCA LUPI IN THE SOUTHWESTERN UNITED STATES

ABSTRACT

Onchocerca lupi is an important zoonotic parasitic filarial nematode that causes canine onchocercosis, a disease that leads to blindness. Since 2010, worldwide incidence in both canines and humans has continued to increase. However, few efforts have been implemented in the US to mitigate the spread of *O. lupi,* with control of the disease dependent entirely on drug administration with limited chemotherapy options. To date, no vaccine or cure exist; the development of both requires an increased and more extensive knowledge of parasite biology. Current research has focused on surveillance and case reports of the disease, but little is known regarding the evolutionary history and genomic landscape of *O. lupi.* To address this knowledge gap, we whole genome sequenced 18 *O. lupi* adult isolates and their endosymbiont *Wolbachia* from Arizona, New Mexico, and Romania collected from 2010-2021. Using LupiQuant to prioritize sample selection, we circumvented the difficulties of whole genome sequencing parasite samples containing host DNA. We predicted 12,344 protein coding genes in the 91Mb nuclear genome and 799 genes in the 0.95Mb *Wolbachia* genome. The gene content between *O. lupi* and the three other *Onchocerca* species with available proteome data was compared. We identified 38 unique orthogroups to *O. lupi.* Gene content regarding ivermectin resistance as well

as known diagnostic serology targets for *O. volvulus* were also identified and characterized. We observed fine-scale parasite population structure with little genetic variation among isolates from the US but observe comparatively high levels of diversity between US and Romanian samples. The mito-nuclear-Xchromosome-*Wolbachia* genome phylogenies agree with each other; there was likely a single introduction of *O. lupi* into the US that rapidly disseminated into the southwest. Furthermore, the genomic repertoire of the nuclear (autosomal + sex chromosomes), mitochondrial, and endosymbiont DNAs produced in this study will support future translational onchocercosis research with particular importance for novel drug, vaccine, and diagnostic targets.

INTRODUCTION

When considering global animal abundance, it is surprising to learn nematodes are the most copious in the world (1). More astonishing, filarial nematodes represents the leading cause of morbidity in the developing world, yet despite over one-third of mankind being infected at any given time, these nematodes remain understudied and poorly understood (2–6). Considering the massive global scale of infection, comparatively little is known regarding the cell biology, ecology, and genomic diversity; knowledge that would allow for the development of surveillance tools and vaccines, novel drug target discovery, and effective control strategies (6). Few studies focusing on the genomic landscape and population structure of both human and wildlife infecting filarial nematodes have been reported, however, when applied, advanced tools were developed that improved elimination strategies (7). Research on human-related filarial parasites has demonstrated the utility of whole genome sequencing regarding improved diagnostics and discovery of novel drug and vaccine targets (7). However, this research has also shown that

multiple draft genomes are necessary to distinguish shared genomic markers that could be exploited for control strategies (7).

Currently, the gold standard of nematode population genetic studies utilizes one or few mitochondrial gene targets that have allowed parasitologists to begin identifying and understand previously unknown nematode population dynamics that includes mating systems, phylogeography, and local scale transmission. The advent of next generation sequencing (NGS) coupled with continually decreasing sequencing costs has allowed the nematode research community the opportunity to conduct genomic studies on organisms with no existing knowledge or resources. These "first generation" nematode genomic studies require a large time and monetary commitment as well as the ability to overcome unique, inherent parasite challenges. The combination of these difficulties has restricted nematode studies to continue to focus on only a few candidate genetic markers, limiting inferences and causing the nematode community to trail behind in the omics era. However, the application of whole genome sequencing (WGS) and parasitology can address fundamental questions regarding nematode biology and evolution. Nematode population genomics is rapidly expanding and WGS data offers fine-scale resolution of parasite populations, biology, and microevolution.

Onchocerca lupi (Rodonaja 1967) is a parasitic nematode of the order Spirurida and the causative agent of canine onchocercosis. *O. lupi*, as well as multiple *Onchocerca* species (e.g. *O. jakutensis (8)* and *O. dewittei* (9)), are capable of causing zoonotic infections. While the definitive host is the dog, *O. lupi* has been reported to infect cats, coyotes, wolves, and rarely, humans (10–17). Disease severity in dogs has a wide spectrum of pathologies and can range from asymptomatic to significant morbidity that includes chronic disability and ocular pathology leading to blindness. In 2011, the first confirmed zoonotic *O. lupi* infection was reported in

Turkey(13). There has since been ~18 confirmed human *O. lupi* cases globally (18–21), eight from the southwestern U.S. starting in 2012 (one case study is unpublished) (14,15). In 2012, a 22-month-old child from northern Arizona was the first reported human case of *O. lupi* in the U.S. Onchocerciasis is described as primarily an ocular and cutaneous disease (22), however, the child did not display typical clinical manifestations of onchocerciasis (14). There were subsequently three more human *O. lupi* cases in Arizona from 2013-2022, all of which were associated with northern Arizona counties (14,15). While there are drugs that are microfilaricidal against *O. lupi* microfilariae (23), the efficacy of ivermectin on adult worms is unknown (15). Furthermore, we have no vaccine to prevent *O. lupi* infection.

To date, researchers have utilized single mitochondrial genes (COI, ND5) to infer low-resolution relationships between global samples (10,24) of *O. lupi*, however, recent research has made available the first *O. lupi* mitochondrial genome (25) as well as the first draft genome (26). To improve our knowledge base of this important but remiss parasite, we implemented a genomics approach for *O. lupi* populations which allows for the discernment of genomic diversity within U.S. *O. lupi* adult worms. This data will allow for discovery of molecular processes that provide the foundation for nematode establishment as well as new insights into the genetic repertoire of their pathogenicity and adaptation to their hosts. Additionally, the predicted *O. lupi* gene content will allow for novel drug target discovery through previously approved FDA drug databases. The goal of this project was to provide this first comparative genomic analysis of *O. lupi* samples from the US. Herein, we describe nuclear (autosomal), sex chromosomes, mitochondrial, and *Wolbachia* genome data for 18 adult *O. lupi* specimens from Arizona, Navajo Nation, New Mexico, and Romania collected from 2010-2021.

METHODS

Parasite collection

18 adult *O. lupi* specimens were collected from infected dogs in Arizona, Navajo Nation, New Mexico (USA), Turkey, and Romania by nodulectomy between 2019-2021. Specimens were stored in 1X DNAShield and shipped to Northern Arizona University for sequencing. To decrease host DNA contamination, adult parasites were liberated from host tissue through a 0.3% collagenase enzymatic digestion followed by four washes with PBS (27). Single, adult worms were prioritized for DNA extraction and sequencing; however, multiple worm fragments were also used in this experiment for samples without intact specimens.

DNA extraction

Genomic DNA was extracted as previously described (26). Briefly, specimens underwent three freeze/thaw cycles consisting of three minutes in liquid nitrogen followed by three minutes at 80ºC. Samples were transferred to 2mL round bottom tubes with a single 5mm stainless steel bead along with 250µL PBS and 100µL lysis buffer. Samples were vortexed on maximum speed for 45 minutes with a special vortex adapter and tubes were rotated every 10 minutes. 30µL 10% SDS was added to each sample along with 2µL of beta-mercaptoethanol and 60µL of proteinase K (20mg/µL). Samples were incubated overnight at 65ºC followed by an RNase A treatment (15µl of 10mg/mL) at 37ºC for 1 hour. The Qiagen DNeasy Blood and Tissue kit (Qiagen) was used with the addition of buffer AL added at a 1:1 ratio with sample volume. DNA was stored at -20ºC until further use.

Informed WGS sample selection and library preparation

To assess the host-to-parasite DNA ratio within each sample and make informed sample selection, the LupiQuant qPCR assay was utilized (26). This SYBR assay targets the conserved polycystin-1 precursor (*pkd1*) gene for the canine host and a conserved myosin gene for *O. lupi*.

This qPCR was carried out in 10µl reactions containing 1X PowerUp SYBR Green Master Mix (Applied Biosystems), 0.3µM of each primer, and 2µl of template DNA or plasmid standards. Thermal cycling protocol was as follows: an initial hot start of 10 min at 95ºC, followed by 40 cycles of 15 sec at 95ºC and 1 min at 60ºC was performed on Quant Studio 12 (Thermo Fisher). The following equation was used to calculate gene copy number (a) followed by (b) host-to-parasite DNA ratios:

a)

$$GCN = \frac{6.02\ X\ 10^{23}\ \frac{copy}{mol}\ X\ DNA\ amount\ (g)}{DNA\ length\ (bp) X\ 660(\frac{g}{mol/bp})}$$

b)

$$Ratio = [Parasite\ DNA/Total\ DNA\ (Host + Parasite)]\ x\ 100$$

Samples were selected for sequencing based on high parasite to low host DNA ratios (Supplemental Table 1).

The genomes of 18 *O. lupi* samples were sequenced using Illumina MiSeq, Nextseq, and Novaseq platforms. DNA was prepared for paired-end sequencing using a Kapa Biosystems Hyper library preparation kit with an 8bp index modification as previously described (25). DNA was sheared to an average size of 650bp and indexed with an 8bp barcode. Prepared libraries were pooled and sequenced to a read length of 150bp on an Illumina NextSeq or NovaSeq systems or to 300bp on an Illumina MiSeq system.

Host DNA Read Removal

Raw reads were trimmed for adapter sequences using Trimmomatic v0.36 (28). To remove any residual host DNA, trimmed reads were aligned to the dog reference genome GCA_008641055.1 using bowtie2 and the --local parameter. Unmapped reads were extracted

from the alignment files and converted into fastq files using samtools v1.2 (29) and bam2fastq within BEDtools v2.20.0-3-g18eb978a (30). Reads moving forward were considered host-free and cleaned and will be referenced as cleaned reads.

Nuclear, mitochondrial, and *Wolbachia* genome assemblies

Cleaned Illumina short-read data were assembled using both SPAdes v3.15.3 (31) and MEGAHIT v1.2.9 (32). Test assemblies using other assemblers and a range of parameters within SPAdes and MEGAHIT were tested. No one assembly method was optimal for both the nuclear, *Wolbachia,* and mitochondrial genomes; the nuclear were assembled using SPAdes and the *Wolbachia* reference genome was generated using MEGAHIT. The *Wolbachia* reference genome assembly was assessed for circularity using circlator v1.5.5 (33). *Wolbachia* reference genome gene content was predicted using BAKTA v1.6.1 (34). The draft reference genome GCA_028564675.1 (Rommel_NM) was used as it had the best overall contig number, total size, and N50. Mitochondrial genome contigs were identified from the assemblies by binning contigs that mapped to the *O. lupi* reference mitochondrial genome NC_056960.1 (25). Both mitochondrial and *Wolbachia* contigs were removed from the assembly to create the *O. lupi* nuclear assembly. Repetitive elements were identified and masked in the nuclear *O. lupi* draft genome using RepeatMasker v4.0.7 (35). Summary statistics on identified repeat sequence families classified as DNA transposons and retroelements were generated using parseRM.pl (36).

Additionally, the completeness of the entire reference genome Rommel_NM was assessed using the BUSCO pipeline v5.4.4 (37) with the "nematoda_odb10" reference gene set.

Genome annotations and comparative analysis

Unsupervised training of two gene model prediction tools (Augustus (38), PASA (39)) was carried out within the funannotate pipeline v1.8.9 (40). Ideally, RNASeq data is available for

evidence-based gene prediction in newly sequenced genomes, however, there is currently no such data for *O. lupi.* To circumvent this, the near neighbor *Onchocerca ochengi* genome (accession number GCA_900537205.1) and RNASeq triplicate data (PRJEB1686) was used for unsupervised training of two gene prediction tools (Augustus, PASA) within the funannotate (40) pipeline. This generated gene prediction models that were applied to our repeat masked *O. lupi* reference genome Rommel_NM (26). The annotation of the *O. lupi* genome was carried out within the funannotate pipeline that included *ab initio* gene prediction, and functional annotation of the predicted protein-coding genes.

The proteomes of *Onchocerca volvulus, Onchocerca ochengi,* and *Onchocerca flexuosa* were downloaded from WormBase ParaSite (41). OrthoMCL v2.0.9 (42) was used to identify orthologs of protein-coding genes across these *Onchocerca* species in addition to *O. lupi*. Ortholog cluster data was visualized using OrthoVenn2 (43). GO annotations were performed within OrthoVenn2.

Currently, the gold standard in commercial diagnostics for onchocerciasis in humans utilizes OV16 serology by ELISA or Rapid Detection Test (RDT). To identify the OV16 ortholog within *O. lupi,* the software OrthoMCL v 2.0.9 was applied and the protein-coding gene that clustered with OV16 was used as a reference gene to screen 15 *O. lupi* genomes (two genomes were excluded from downstream analysis because of low coverage) for presence and SNP content. β-tubulin, an additional gene of interest that has been shown to confer resistance to the *Onchocerca* treatment ivermectin, was identified within the *O. lupi* predicted protein-coding genes using OrthoMCL v2.0.9. As heterozygosity within this gene allows for the ivermectin resistance phenotype, cleaned *O. lupi* reads were aligned against the β-tubulin *O. lupi* ortholog using BWA-mem v 0.7.17-r1188 (44) and GATK toolkit v3.4-46-gbc02625 (45) with the ploidy option set at

2. Heterozygous positions within this region were examined, tallied, and compared across 15 *O. lupi* genomes.

To identify highly repetitive genomic regions for improved surveillance of *O. lupi*, a Kmer-based analysis was conducted independently on each clean read dataset. Briefly, cleaned reads were fragmented into 54 and 99 base pair Kmers using bbmap (46). Kmer counts were recorded and the highest 1,000 kmers were investigated as potential surveillance targets. Blast (47) was used locally to identify the contigs the Kmers of interest were located on.

Sex-linked Chromosome Detection

Predicted *O. lupi* gene content was compared to known sex-linked protein-coding regions from the *Onchocerca volvulus* genome (48) using OrthoMCL v 2.0.9 (42). Furthermore, the X-chromosome was identified in the nuclear assembly using the *Onchocerca volvulus* X-chromosome OM2 contig (48). Gene content homology of the reference X-chromosome was screened for in the *O. lupi* nuclear genome using OrthoMCL. Protein-coding regions identified as single-copy orthologs for sex-linked genes were binned according to X-chromosome, XY-linked pseudoautosomal region (PAR), and the remaining nuclear (autosomal) contigs.

SNP phylogenetics

To understand relationships between US and Romanian samples, phylogenetic analyses were carried out for the nuclear (autosomal), X-chromosome, XY-lined PAR region, mitochondrial, and the *O. lupi* endosymbiont *Wolbachia* separately. Cleaned reads were aligned to the five different *O. lupi* references using bowtie2 (49) as a global aligner. Mismatching intervals were removed from each data set with the RealignerTargetCreator and IndelRealigner tools within the GATK toolkit v3.4-46-gbc02625 (45). SNPs were called using the UnifiedGenotyper with the “het” parameter set to 0.01. Resulting .vcf files were filtered using

the following parameters: QD = 2.0 || FS_filter = 60.0 || MQ_filter = 30.0 || MQ_Rank_Sum_filter = -12.5 || Read_Pos_Rank_Sum_filter = -8.0. Filtered .vcf files for each dataset were passed into NASP v1.2.0 (50) which further filtered SNPs with less than 3X coverage or with less than 66% variant allele calls. Duplicated regions in the reference genome were removed from the final data set using NUCmer v3.0 (51). To investigate the genealogical relationships among *O. lupi* adult worms, we built maximum likelihood (ML) phylogenetic trees using four different SNP datasets (mitogenome, *Wolbachia*, X-chromosome, and nuclear), each drawn with and without the Romanian sample within IQ-TREE (52).

Mitochondrial genome amplification and sequencing

To include *O. lupi* mitochondrial genomes within our analyses without whole genome sequencing, we developed a long-range PCR targeting Onchocercidae mitochondrial genomes from complex sample types (e.g. skin snip samples, insects). Primers were designed using 3 species of *Onchocerca* (*O. lupi, O. volvulus, O. gutterosa*) and *Dirofilaria immitis* (canine heartworm) (Supplemental Table 2) mitochondrial genomes. All mitogenomes were aligned to the *O. lupi* mitochondrial reference genome using NUCmer v3.0 (51) within the SNP pipeline NASP (50). The master alignment matrix was used to reverse and extend previously published cytochrome c oxidase (COI) primers (53) to create suitable primers for long range PCR amplification. Primer sequences were evaluated in Primer3 and a single primer pair were selected (54) (Table 1). Long range PCR targeting mitochondrial genomes were amplified from 38 *O. lupi* PCR-positive canine skin biopsy samples using 50μL reactions with GoTaq Long PCR Master Mix (Promega), a final primer concentration of 0.3μM, 4μL molecular grade water, and 18μL DNA with the following thermocycler conditions:

94ºC for 2 min, followed by 45 cycles of 94ºC for 15 sec, 65ºC for 20 sec, 65ºC for 14 min, and a final extension of 72ºC for 10 min.

Long range PCR products were visualized on a 0.6% agarose gel and prepared for short read sequencing. Briefly, a 1X AMPure XP (Beckman Coulter) bead cleanup was applied to all PCR products prior to library prep. Sequencing libraries were prepared as previously described for whole genome sequencing using a dual-indexing approach (55). Final sequence libraries were pooled and sequenced on either a MiSeq using 600-cycle v3 kit or a NextSeq 500 v2 chemistry (Illumina).

Mitochondrial genome analyses

Raw reads from 8 skin snip samples that were subjected to mitochondrial genome long-range PCR and sequencing were trimmed for adapter sequences using trimmomatic v0.36 (28). These and the 18 cleaned WGS reads from adult worms were aligned to *O. lupi* mitochondrial reference genome MW266120.1 (25) using bowtie2 v2.2.5 (44) and SNPs were identified with GATK v3.4-46-gbc02625 (45) as part of the NASP pipeline v1.2.0 (50). The final SNP matrix excluded sites with lower than 5X coverage or lower than 0.8 variant allele frequency for any one sample. A maximum likelihood (ML) tree was produced from the final SNP matrix in IQ-TREE (52).

RESULTS

We sequenced the genomes of 18 adult *O. lupi* samples collected from infected dogs in Arizona, New Mexico, Navajo Nation, Turkey, and Romania. Three of the genomes, Lola_NM, Puggles_rep1_NM, and Turkey, had too low of coverage for most of the genomic analyses. They were included in the mitochondrial genome analysis but were removed from downstream datasets. The assembly for sample Rommel_NM was chosen as the reference genome because it

had the lowest number of total contigs (2,320), the highest overall N50 value (96,536 bases), and assembled into a total of 92,492,139 total bases. Funannotate predicted 12,344 putative coding regions for the entire metagenomic assembly, and the estimated GC content was 29.2%. Contigs that mapped to the XY-linked pseudoautosomal region (PAR) and X-chromosome within the *O. volvulus* reference genome were removed from the Rommel_NM *O. lupi* draft genome and binned into subsequent files creating references for *O. lupi* X-chromosome and PAR region. The X-chromosome draft *O. lupi* reference consisted of 376 contigs totaling 23,339,636 bases and had a GC content of 30.5 %. Within the draft X-chromosome, a total of 3,460 putative coding sequences were predicted. The PAR region made up 102 contigs that totaled 7,042,042 bases, had a GC content of 29.3%. It consisted of 936 predicted coding sequences.

The assembly for sample Montigue_NM had the *Wolbachia* genome assembly. It assembled into a single circularized contig that was 954,902 bases in length and had a GC content of 32.1%. Prodigal was used to predict protein coding regions and identified 799 putative genes within the *O. lupi Wolbachia* genome. The *Wolbachia* genome was mapped against the metagenomic Rommel_NM draft reference genome and mapped contigs were removed from Rommel_NM assembly. Finally, the mitochondrial reference genome, NC_056960.1, for *O. lupi* was queried against the Rommel_NM metagenomic assembly and mapped contigs were removed. This left a nuclear (autosomal) draft reference genome for *O. lupi* that was 1,792 contigs, 61,066,388 bases in length with a GC content of 28.6%. There were a predicted 7,504 putative coding regions within this autosomal assembly. A repetitive element analysis was conducted on each of the above assembled genomes using RepeatMasker and 16 repeat element Nematode families in ancestor taxa as well as 180 lineage-specific repeat families. Within the

61Mb nuclear genome assembly, RepeatMasker identified and masked 4.41% repetitive elements and the X-chromosome had 3.19% of its genome masked due to repeat elements.

Onchocerca orthology analysis

Gene prediction models generated in this study were used for *ab initio* gene prediction in *O. lupi.* We predicted 12,344 protein-coding genes across the entire *O. lupi* genome. To identify the core genome in the four *Onchocerca* species that have been published to date and discover unique protein-coding regions within *O. lupi,* we conducted an OrthoMCL analysis of all four proteomes and identified 6,888 orthogroups shared across all four species (Figure 1). This analysis identified 38 orthogroups specific to *Onchocerca lupi.* Most of these *O. lupi* specific orthogroups were categorized as biological processes (84.2%), followed by metabolic processes (10.5%) and cellular components (2.6%) (Figure 2). GO annotations are listed in Supplemental Tables 3, 4, and 5.

Genes of interest to this study included the orthologs for OV16 and β-tubulin due to their utility as a serology diagnostic and association with ivermectin resistance respectively. Using OrthoMCL, *O. lupi* specific orthologs were identified for both gene targets. The Ol-OV16 ortholog is 816 bp total in length. The presence of Ol-OV16 in the 15 newly sequenced *O. lupi* genomes was investigated by alignment and conservation across the US strains was verified. A total of 5 SNP loci were called within these samples total. The Romanian sample aligned to only 88% of the reference length. More testing is necessary to confirm whether this is an artifact of the low DNA input for sequencing (Romania aligns to the reference genomes at 40% of the total reference length). The Ol-OV16 gene has potential for use in creating a serological diagnostic for *O. lupi*.

We investigated the presence of the *O. volvulus* β-tubulin gene as it is an important marker for ivermectin resistance. Using OrthoMCL, the *O. lupi* β-tubulin ortholog was identified and heterozygosity investigated among the 15 *O. lupi* genomes. No homozygous SNP positions were identified; however, heterozygous positions were called among all isolates. The number of heterozygous positions for each sample ranged between 2 to 15 loci. Interestingly, these heterozygous loci overlap in 14 isolates except for 4 heterozygous singleton positions. The Romanian sample aligned to just 7% of this gene and was removed from the analysis.

Kmer analysis using 54mers and 99mers of clean read data was run on 15 *O. lupi* genomes to identify highly repetitive regions of DNA. Kmer analysis did not allow for mismatches so candidate kmers were used as a reference gene to generate read coverage per *O. lupi* sample. The top kmer candidates were consistently located within either the 5S rRNA subunit or the mitogenome. Read coverage of these top targets revealed conflicting results, with 3 genomes showing the highest read coverage of the 5S rRNA target and 12 genomes showing the highest read coverage of the mitochondrial target. PCR testing is required to determine the most highly repetitive region of the *O. lupi* genome for the development of improved surveillance tools.

Mitochondrial genome phylogeny

Our long-range mitogenome PCR produced the correct band size above 13kb for 38 canine skin samples from Northern Arizona that were prepared and sequenced using the whole genome library preparation methods listed above. 8 of these samples covered the entire *O. lupi* mitogenome reference, with the remaining 30 skin samples covering at least 13,000 bases of the 13.766Kb reference. These 30 samples were excluded from the analyses to include all possible SNP positions within our samples. We examined the SNP content among these 8 mitogenomes

from canine skin snips in combination with our 18 *O. lupi* mitogenomes from the US, Romania, and Turkey generated from whole genome sequencing (Figure 3). In total, 13 SNPs were identified across the mitogenomes of these 26 taxa. There were 8 shared SNPs that separated both Romania and Turkey taxa from the US clade that were parsimony informative. Additionally, autapomorphic SNPs were identified within the Romanian taxa (2 SNPs) and the Turkey taxa (3 SNPs). We did not identify SNPs within the 24 US taxa while examining 13,675 bases of the 13,766 base mitogenome reference. The mitochondrial genome maximum likelihood phylogenetic analysis suggests a single *O. lupi* introduction into the US followed by dissemination across the southwest.

Wolbachia genome SNP phylogeny

A total of 8 whole genome samples had >90% coverage of the *Wolbachia O. lupi* endosymbiont reference genome. The high-quality core genome for these 8 samples covered 846,356 bases (88.63%) of the 955kb reference genome and identified 618 high-quality, core SNP loci (Figure 4). 460 SNPs separated the Romanian sample from the US strains. The US clade included 97.02% (926,424 bases) of the reference genome and had a total of 192 SNPs identified, of which 54 were parsimony informative. The overall low total SNP count for the US only clade suggests these *Wolbachia* strains are highly related and are likely the result of a recent, single introduction, and agrees with the mitogenome phylogeny.

X-chromosome SNP phylogenies

The impact of mutations differs significantly between sex chromosomes and autosomes, as such, the X-chromosome was removed from the nuclear reference genome and analyzed separately. Additionally, due to the shared nature of the XY-linked pseudoautosomal region (PAR) between both the X and Y chromosomes, the PAR was identified within the X-

chromosome and masked. This analysis included 15 *O. lupi* samples including the Romanian sample (Figure 5). The Romanian sample only aligned to 45.96% of the *O. lupi* X-chromosome. The Romanian taxa accounts for 11,754 SNPs of the 12,366 total SNPs. The consistency index (CI) indicated a high level of uncertainty within the phylogenetic tree, with a value of 0.41. To observe a higher level of detail and include more loci in the analysis, we removed the Romanian sample. This US phylogeny included 14 genomes that aligned to 22,203,171 bases of the reference X-chromosome and included 2,303 total SNP loci with 804 parsimony informative SNPs (Figure 6). The bootstrap values further support this uncertainty as only two branches have a bootstrap score above 90.

Nuclear (autosomal) results

WGS data from 15 *O. lupi* adult worms were aligned to the nuclear *O. lupi* genome. Of the 61Mb that makeup the *O. lupi* nuclear (autosomal) reference, the high-quality, core nuclear genome alignment that included the Romanian sample aligned 42.50% of the reference genome which drastically reduced the overall bases included in this analysis to 24,665,611 bases. This dataset identified 47,288 SNP loci shared by all strains (Figure 7) with 4,991 parsimony informative SNPs and 42,251 singleton SNPs. The phylogenetic tree shows two populations, the US strains, and the strain from Romania, which is separated by 39,486 SNPs. The CI value was low, 0.42, again suggesting phylogenetic uncertainty within this sample set. To maximize the loci included in this analysis, we removed the Romanian sample. This US-only nuclear SNP matrix covered 87% of the nuclear reference genome (Figure 8). The phylogenetic tree had slightly elevated CI value (0.50) in comparison to the rest of the genome, however, these values still indicate high amounts of homoplasy.

DISCUSSION

Here we present results of whole-genome sequence analyses of *Onchocerca lupi* strains from the endemic southwestern US. Using SNP phylogenetic analyses of the mitochondrial genomes, *Wolbachia* genomes, X-chromosome, XY-linked PAR region, and the nuclear (autosomal) genomes, we provide evidence of the genetic subdivision of the US strains from a single, partial Romanian strain and demonstrate the lack of diversity and population structure within the US. This study represents the first genomic population survey of *O. lupi* in the US and an initial step towards a comprehensive understanding of the parasite evolution worldwide. Low genomic diversity observed within the US clade necessitates the sequencing of additional isolates from multiple endemic regions in Europe, the Middle East, and Northern Africa for a robust and complete understanding of population structure and parasite dispersal worldwide. Regardless, the data produced in this study has provided a framework for future onchocercosis research including novel surveillance tools, diagnostics, vaccine targets, and novel drug-target discovery.

Until now, the evolutionary relatedness of *O. lupi* has been characterized primarily using single mitochondrial gene COI (10,13,56,57). Single mitochondrial gene phylogenies often lack the fine-scale resolution necessary for population structure inferences. Other nematode studies have demonstrated the utility of whole mitochondrial genome analyses, which allow for robust phylogenetic studies differentiating closely related taxa. For metazoans, the mitogenome has higher mutation rates than their nuclear genomes (58,59). As this maternally inherited DNA is not subject to genetic recombination from sexual re-assortment (60,61), mitochondrial gene rearrangements can be conserved across distant-related taxa. The application of whole mitogenome analyses for the investigation of both mutations and gene synteny among closely related or highly diverse taxa has produced well-supported results in the phylum Nematoda (62–65). As mitogenome characterization has been used as an informative and powerful molecular

418 tool for nematode mitogenome evolution, we aimed to characterize the mitogenome diversity of
419 *O. lupi*. The resulting phylogenetic tree covering all 13,766 bases of the reference mitogenome
420 showed the US samples as a monophyletic clade with zero SNPs across the 26 taxa suggesting a
421 single and recent introduction into the southwestern US. While past research has described
422 nematode mitogenomic evolution from closely related taxa, the *O. lupi* population within the US
423 appears to have disseminated too recently for any accumulation of mutations to have occurred.
424 However, applying this tool across disparate, endemic regions such as Europe, the Middle East,
425 and Northern Africa may prove useful for understanding the life history and population
426 dynamics of *O. lupi.* A larger geographic area, or perhaps regions where the parasite has been
427 endemic for a longer period, will aid in the understanding of both the historical and
428 contemporary *O. lupi* population demographics. Applying the long-range mitogenome PCR tool
429 we developed here to a large global sample set will facilitate epidemiological studies to infer
430 geographic origins that can directly inform *O. lupi* elimination efforts worldwide.
431 *Wolbachia* is an alpha-proteobacterial symbiont that is widely distributed among
432 arthropods but are also present in two different families of nematodes (66). With the ability to
433 effect diverse phenotypes in their hosts, from reproductive parasites to obligate mutualism,
434 defining the 'species' of *Wolbachia* has been challenging (66). Co-evolutionary signals between
435 arthropods and *Wolbachia* are generally considered weak. Within Onchocercidae, however, a
436 strict co-evolutionary signature has been observed between filariae and their symbiont (67). The
437 genomic diversity within the *Wolbachia pipientis* species defined 17 monophyletic clades
438 (supergroups labeled alphabetically A-F,H-Q, and S) that are characterized by different host
439 ranges and lifestyles (68). Research has focused on the evolutionary relationships of *Wolbachia*
440 across filariae species, but little research has investigated *Wolbachia* diversity with a single

441 species of filariae. Here we present the first complete *Wolbachia* genome of *O. lupi* (*w*Ol) as
442 well as characterize the genomic diversity within US *O. lupi* populations. Interestingly, like the
443 mitogenome, *Wolbachia* is also maternally inherited. From 9 whole genome datasets, we were
444 able to cover 88.63% of the *wOl* genome. In total, we identified 844 SNPs within the 9 samples,
445 however, considering only the shared loci across all samples that had more than 5X coverage and
446 a proportion filter set to 0.8 (4/5 positions), the high-quality, core genome had 342 SNPs. Only
447 74 of those SNPs were placed within the US monophyletic clade which appears as a highly
448 related dataset. However, a pairwise comparison between each sample and the reference revealed
449 we observed between 1 to 476 (Romania) SNPs. Upon closer inspection, 40% of SNP positions
450 have been excluded from the phylogeny due to regions of heterozygosity across each *Wolbachia*
451 genome.
452 Regions of heterozygosity were identified across all samples in the autosomes, X-
453 chromosome, and *Wolbachia* genomes. Heterozygosity was not identified in the mitochondrial
454 genomes. We hypothesize this heterozygosity is the result of sequencing adult, gravid females
455 that contained ovaries and a uterus. Published data on the cattle parasite *Onchocerca ochengi*
456 reported the whole genome sequencing of 3 adult male nematodes, however, they mention
457 "heterozygous positions" in a single figure description (69). This footnote of heterozygous
458 positions in male samples suggests two possibilities regarding the heterozygosity of our *O. lupi*
459 data. We hypothesize the heterozygous positions in our samples most likely reflect repeat regions
460 within the reference genome that the assembler was unable to resolve and therefore collapsed
461 into a single region. This would cause reads from both regions to align to the single locus in the
462 reference genome, appearing as heterozygosity.

Anthelmintics have been heavily relied upon for nematode parasite control in human, veterinary, and livestock which has led to a five-decade-long effort against anthelmintic resistance in these parasites (70). Pan-resistance of anthelmintics has been reported particularly in veterinary parasites; for example, sheep and goat farms in the southern hemisphere have identified pan-resistant nematodes as a common occurrence (71). This finding garnered a global interest resulting in extensive research focused on both markers of selection and mechanisms contributing to anthelmintic resistance (72,73). The identification of individual drug-resistant candidate genes has generally been unsuccessful due to limited assumptions, however, as genomic data for these nematodes became available, genome-wide approaches allowed for the identification of genomic regions that are highly correlated with resistance (74). The new availability of *O. lupi* genomic data has provided an initial framework for anthelmintic resistance surveillance to identify drug-resistant genotypes in *O. lupi* populations.

Within the field of parasitology, the generation of genomic data will appreciably advance parasite diagnostics through sensitivity and specificity. Currently, 'gold standard' parasite diagnostics require visual identification of parasite life stages in various bodily fluids and tissues or serological detection (75–78). These current diagnostic methods are highly subjective and limited in relative sensitivity (2). *O. lupi* predicted gene content generated in this study has the potential for identification of novel biomarkers for diagnosis of *O. lupi* infections. Here we have an unprecedented opportunity to translate *O. lupi* genomics into a sensitive and specific early detection molecular diagnostic tool.

Disentangling phylogenetic relationships from complex parasite sample types that recently/or rapidly diverged is extremely challenging. Hurdles encountered include minimal differentiation as well as regions of heterozygosity (70). Herein we applied a high-resolution,

whole-genome sequencing approach to gain insight of the complex parasite *O. lupi.* Relatively little is known regarding genomic variability of the nuclear genomes of filarial nematodes (71,72). In 2012, researchers investigated the relatedness of heartworm, *Dirofilaria immitis*, of two isolates from Europe and the US and reported very little variation within the mitogenome and nuclear genome (73). Other nematode species studies have used mitochondrial genomes to determine relatedness within a species (71). To date, past studies have investigated the genetic variation within isolates of *O. lupi* using the mitochondrial COI gene (10,23,56). Our study provides the most comprehensive *O. lupi* genomic variation analysis to date examining nuclear, mitochondrial, X-chromosome, and *Wolbachia* genomes from 15 *O. lupi* adult worms. Gene content analysis will allow for data mining of known FDA-approved drug databases to create a prioritized list of potential treatments of *O. lupi*. We have provided a critical genomic resource for future endeavors of novel drug target discovery, development of improved surveillance tools and commercial diagnostics, and vaccine therapies. These data will inform control strategies through improved surveillance and epidemiological inferences for forward momentum needed toward *O. lupi* elimination.

FIGURE LEGENDS

Figure 1. Venn diagram showing shared and unique orthologs among the four *Onchocerca* species with available genomic data. A) shows the overlapping and unique orthologous clusters across *O. volvulus, O. lupi, O. ochengi,* and *O. flexuosa*. B) shows the number of starting orthogroups for each *Onchocerca* species. C) represents the number of shared orthogroups between all four species, three species, two species, and unique orthogroups.

Figure 2. 32 *O. lupi* unique orthogroups according to biological function.

Figure 3. Maximum likelihood phylogenetic tree spanning 13,675 bases of the 13,766 base mitogenome reference (Wyatt_AZ). This tree includes 13 SNPs and is rooted using those 13 SNP positions only in *O. volvulus*.

Figure 4. Maximum likelihood phylogenetic tree of 8 *Wolbachia* endosymbionts of *O. lupi.* This analysis spanned 97.02% (926,424 bases) of the reference genome and included 192 SNP loci.

Figure 5. Maximum likelihood phylogenetic analysis of the *O. lupi* X-chromosome from the US and Romania. Our high-quality core genome included 10,287,808 bases (44.08%) and identified 12,366 SNPs. Within those SNP positions, only 599 were parsimony-informative, 11,766 SNPs are singletons.

Figure 6. Maximum likelihood phylogenetic analysis of 14 *O. lupi* X-chromosomes rooted with *O. volvulus.* This ML analysis covered 95.13% of the reference chromosome and included 2,303 total SNP loci. The CI value was 0.40, indicating uncertainty.

Figure 7. Maximum likelihood phylogenetic analysis of *O. lupi's* nuclear genome. This analysis spanned 24,665,611bases of the 61Mb reference genome.

Figure 8. Maximum likelihood phylogenetic analysis of the high-quality, core nuclear genome of the US-only samples. This analysis had a breadth of coverage of 52,929,440 bases across the 61Mb reference genome with a total of 10,213 SNPs with 3,105 parsimony informative SNPs.

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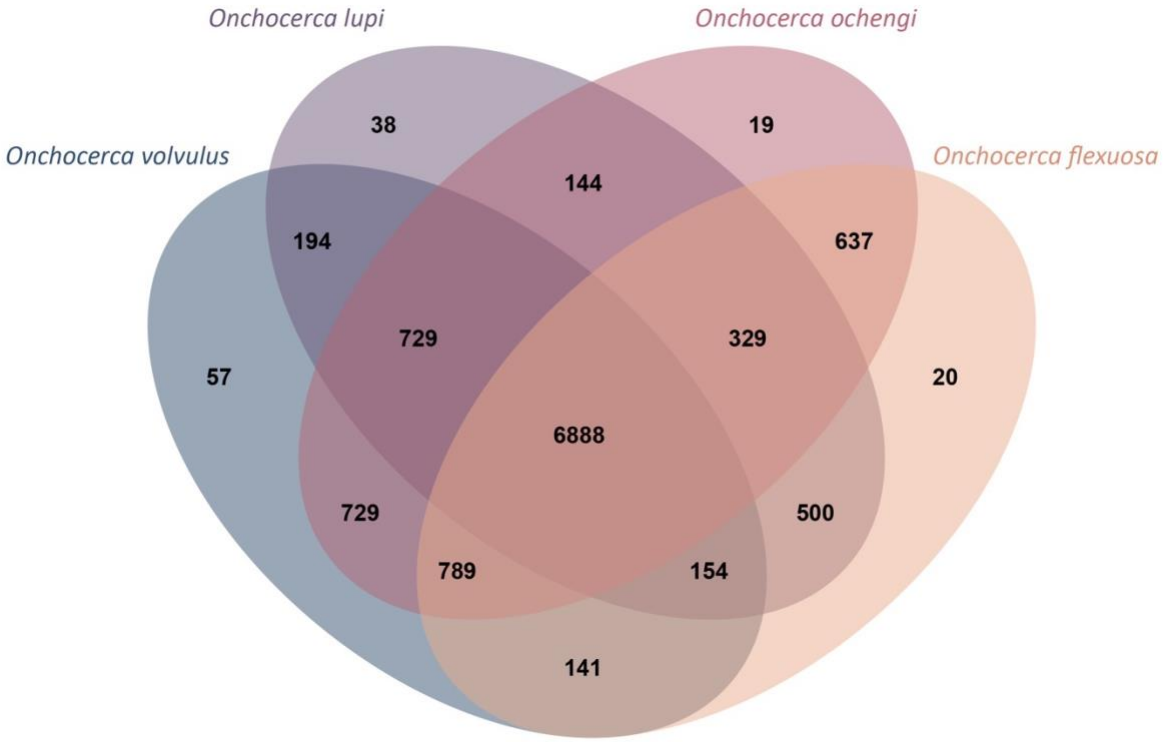

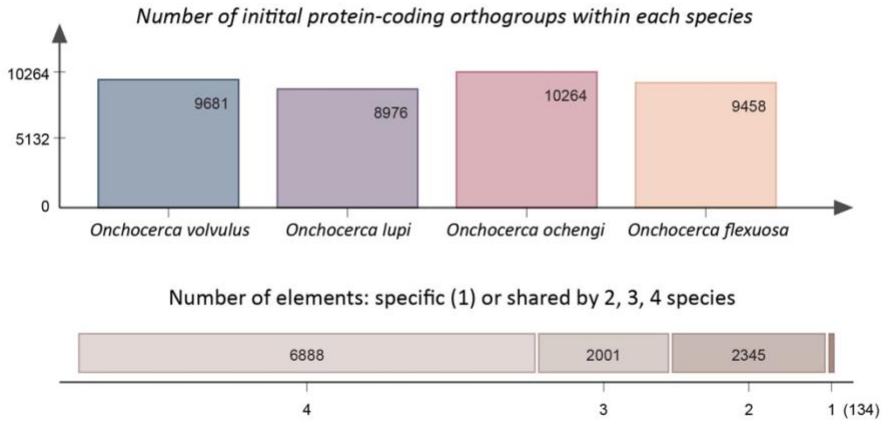

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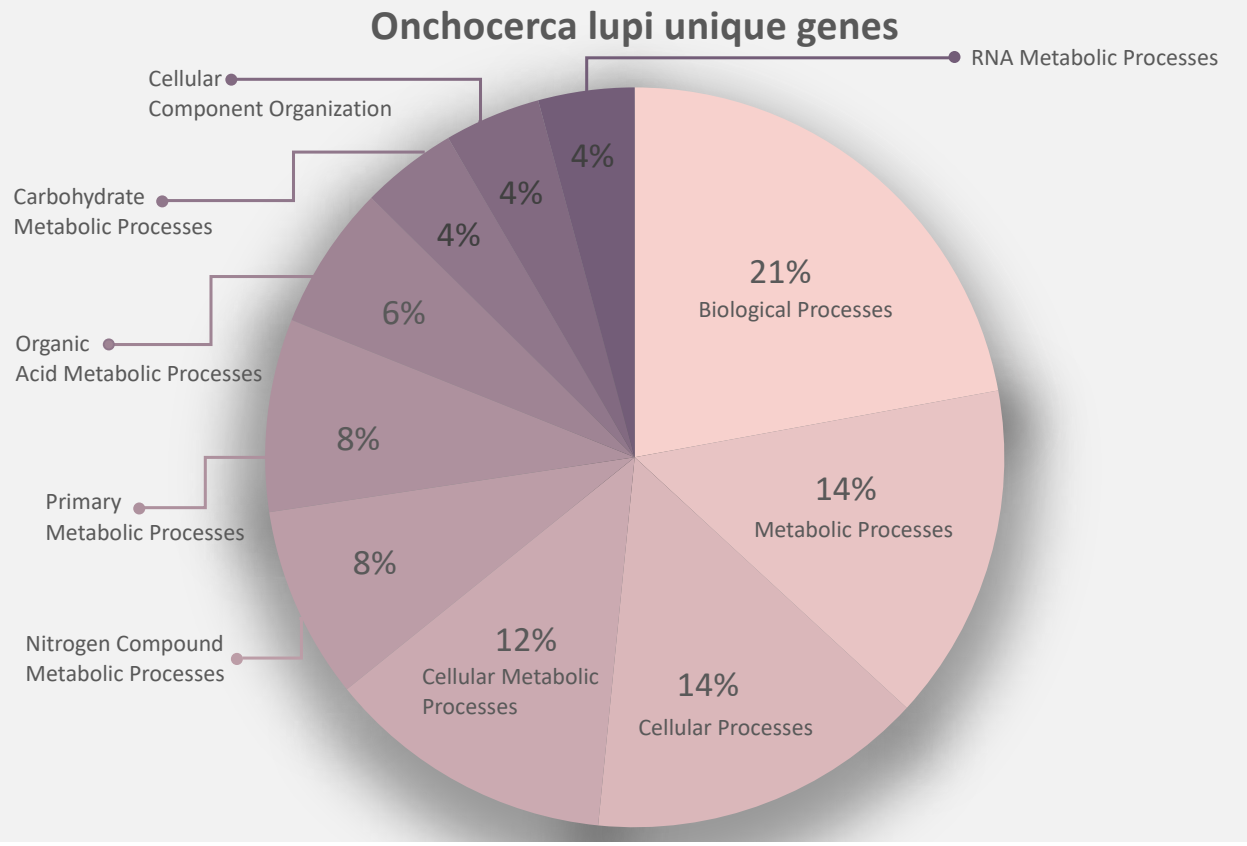

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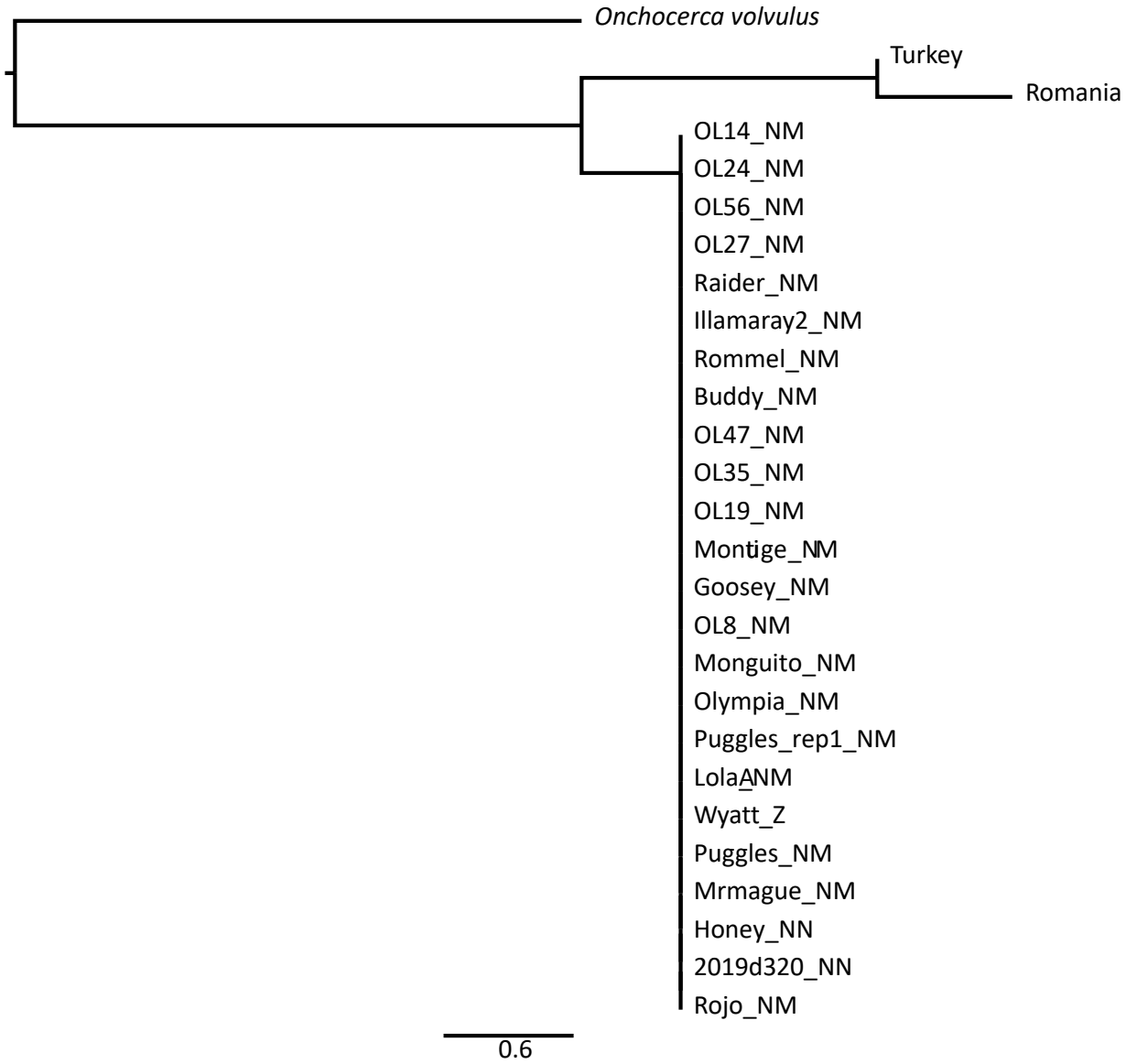
551



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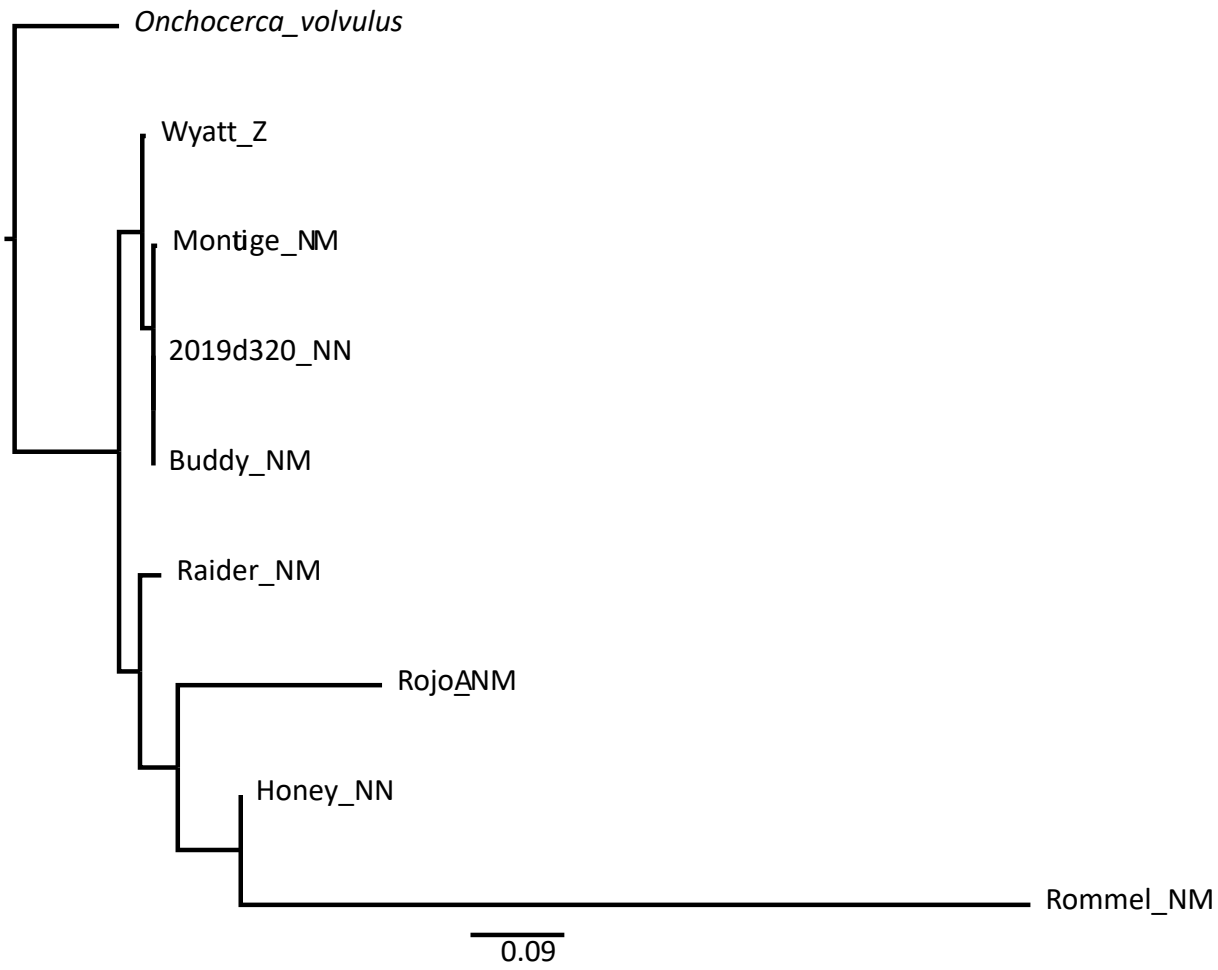

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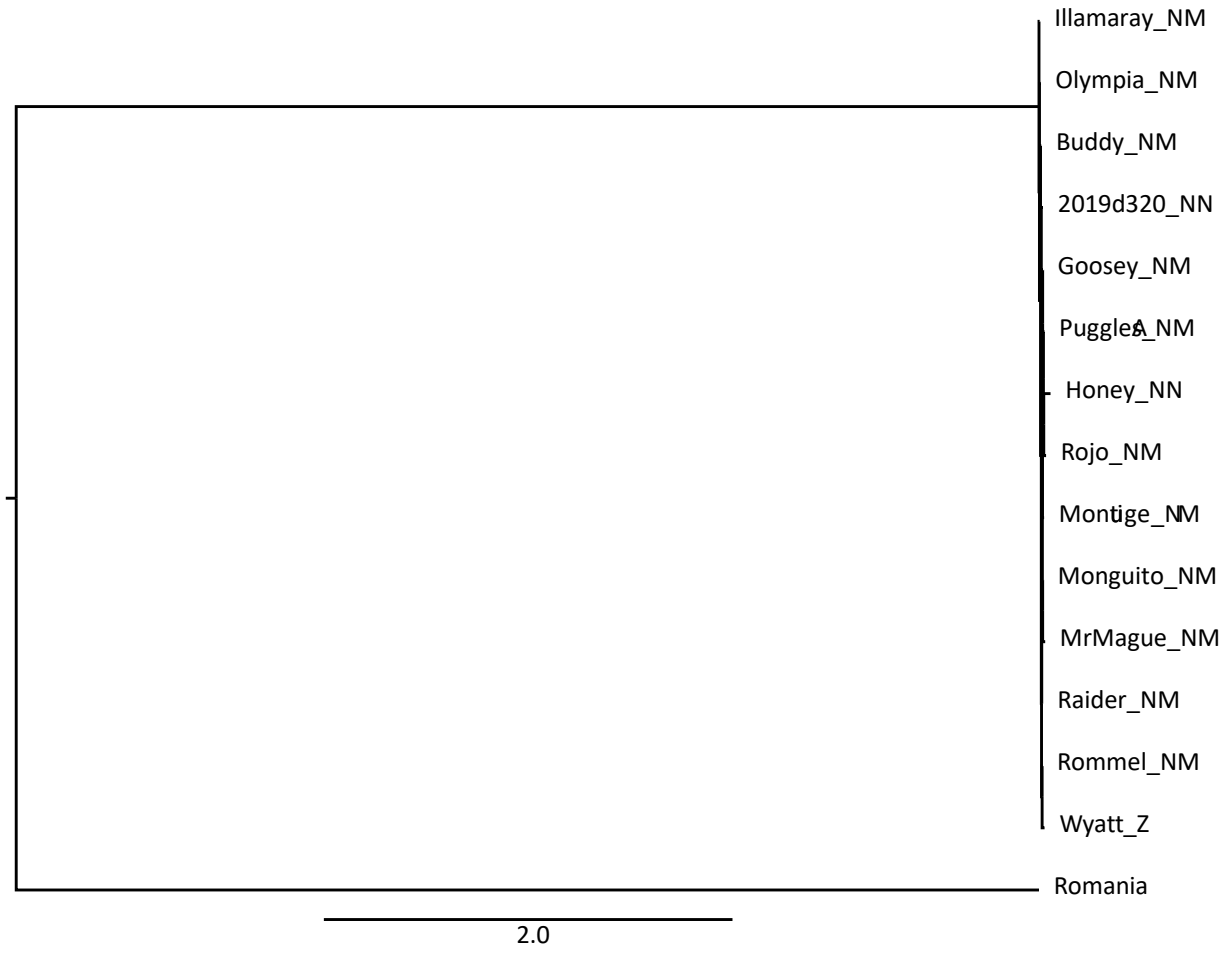

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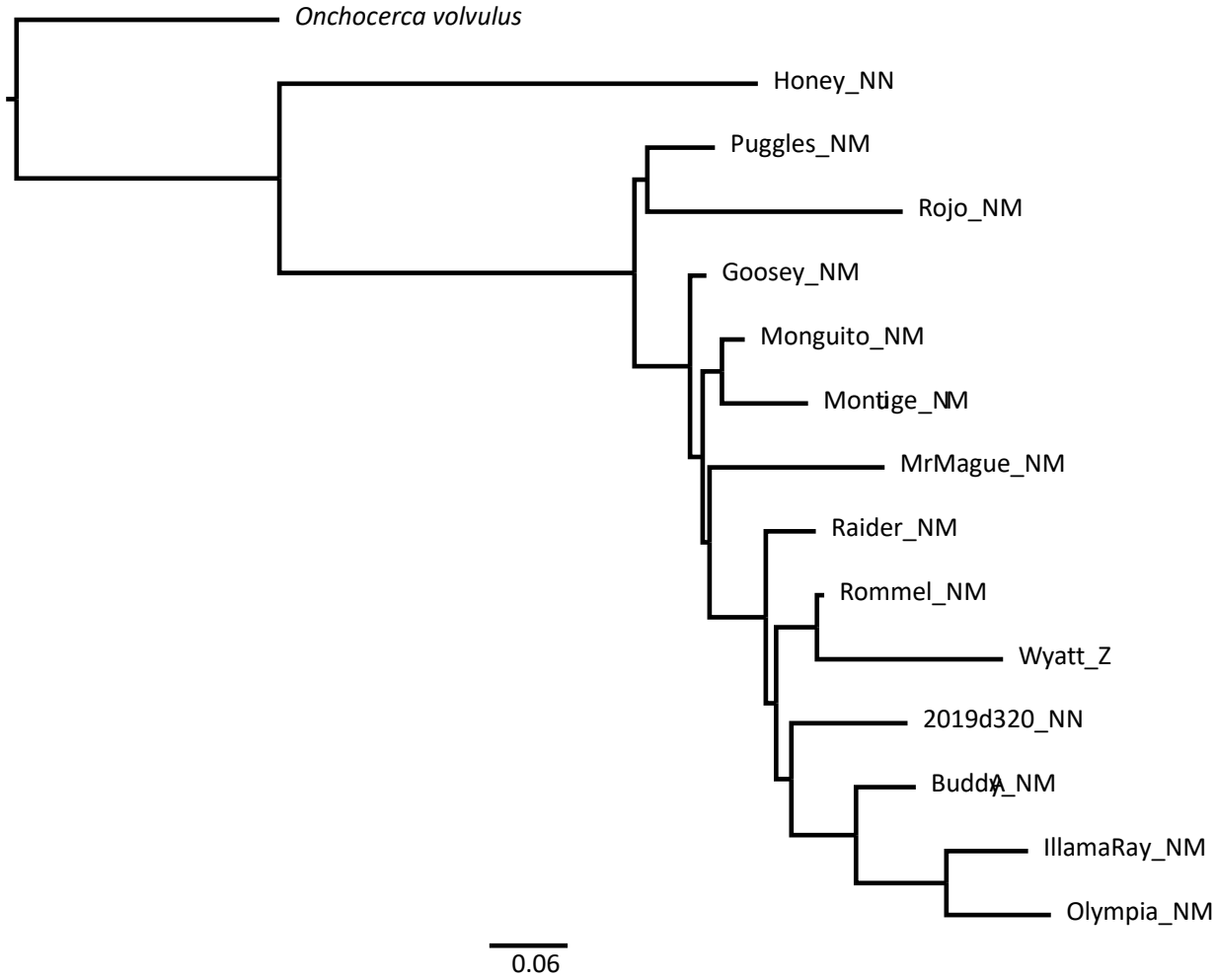

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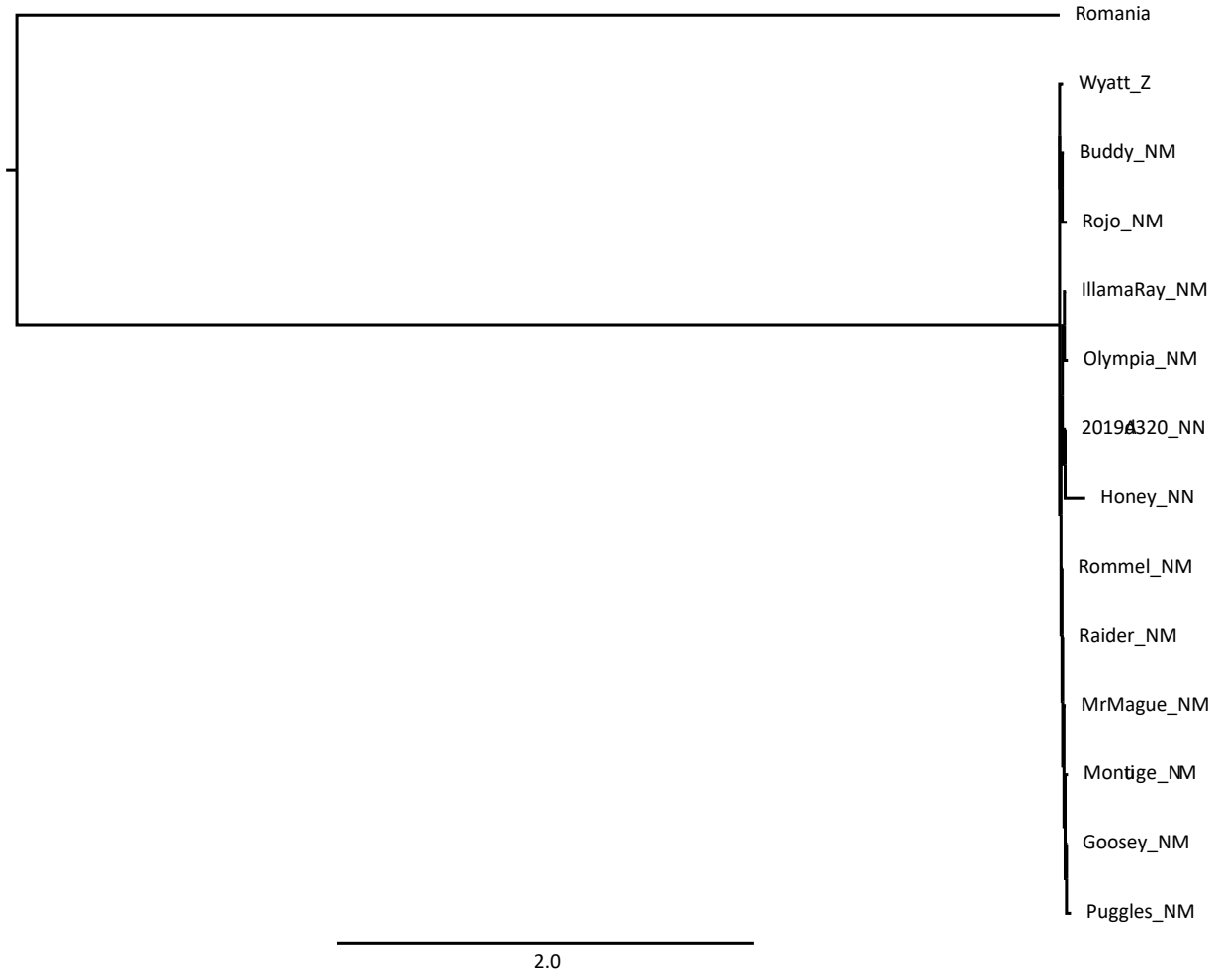

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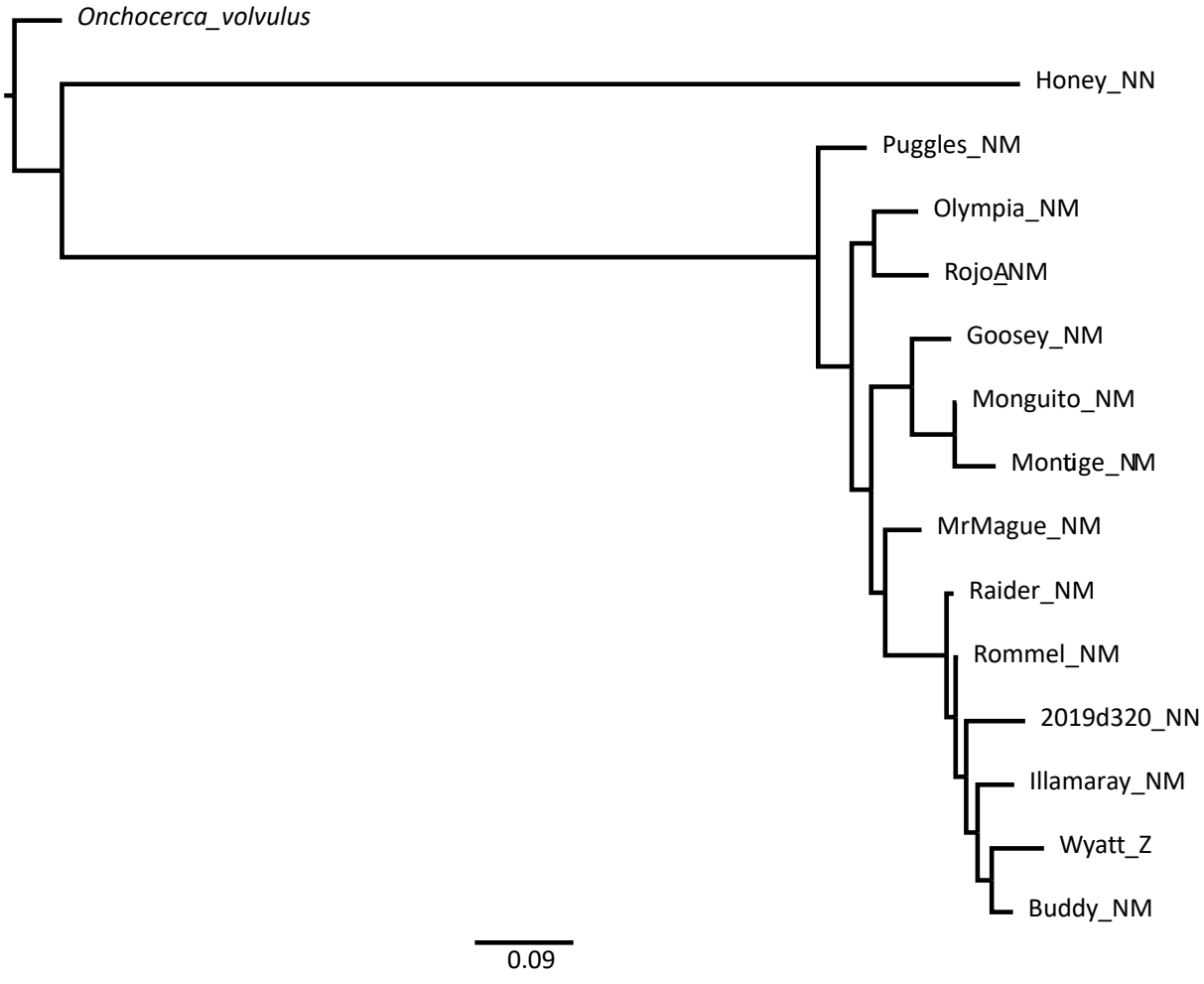

584 TABLES
585
586 Table 1. Primer sequences for long range mitochondrial genome PCR used in this study.
587 Locations are based on *O. lupi* mitochondrial reference genome MW266120.1.

	Location	Primer Sequence 5'-3'	Product Size (bp)
Forward	3189	GATATTGATACTCGTACTTATTTTAGTGCT	13,766
Reverse	2521	CATCCAATTACCAAAACCACCAATCA	

588

589 Supplemental Table 1. Predicted host-to-parasite DNA ratios using LupiQuant.

Sample Name	Predicted *O. lupi* DNA %	Predicted canine DNA %
2019d-320_NM	99.95	0.05
Buddy_NM	97.48	2.52
Goosey_NM	84.21	15.79
IlamaRay_NM	98.69	1.31
Lola_NM	100.00	0.00
Monguito_NM	100.00	0.00
Montigue_NM	94.18	5.82
MrMague_NM	100.00	0.00
Olympia_NM	91.41	8.59
Puggles_rep1_NM	100.00	0.00
Puggles_rep2_NM	64.12	35.88
Raider_NM	99.74	0.26
Honey_NN	91.53	8.47
Rojo_NM	100.00	0.00
Romania	53.51	46.49
Rommel_NM	100	0.00
Turkey	100	0.00
Wyatt_AZ_2010	46.75	53.25

590

591 Supplemental Table 2. Accession information for mitochondrial genome and whole genome
592 sequences included for long range mitochondrial genome primer design.

Species	Accession Number
Onchocerca lupi	MW266120.1
Onchocerca volvulus	NC_001861.1
Onchocerca flexuosa	HQ214004.1
Onchocerca ochengi	KX181289.1
Onchocerca ochengi	KX181209.2
Onchocerca gutturosa	-
Dirofilaria immitis	NC_005305.1

593

Supplemental Table 3. Unique orthogroups categorized as biological processes that were identified in *O. lupi* in comparison to the proteomes of O. *volvulus, O. ochengi,* and *O. flexusosa*. Table includes GO match, description, and number of proteins per orthogroup.

GO ID	Name	Count of proteins within orthogroup
GO:0008150	biological_process	10
GO:0008152	metabolic process	7
GO:0009987	cellular process	7
GO:0044237	cellular metabolic process	6
GO:0006807	nitrogen compound metabolic process	4
GO:0044238	primary metabolic process	4
GO:0006082	organic acid metabolic process	3
GO:0005975	carbohydrate metabolic process	2
GO:0016043	cellular component organization	2
GO:0016070	RNA metabolic process	2
GO:0043170	macromolecule metabolic process	2
GO:0050896	response to stimulus	2
GO:0065007	biological regulation	2
GO:0006066	alcohol metabolic process	1
GO:0006091	generation of precursor metabolites and energy	1
GO:0006119	oxidative phosphorylation	1
GO:0006139	nucleobase-containing compound metabolic process	1
GO:0006412	translation	1
GO:0006518	peptide metabolic process	1
GO:0006725	cellular aromatic compound metabolic process	1
GO:0006810	transport	1
GO:0007154	cell communication	1
GO:0015031	protein transport	1
GO:0019538	protein metabolic process	1
GO:0032502	developmental process	1
GO:0032989	cellular component morphogenesis	1
GO:0043603	cellular amide metabolic process	1
GO:0045333	cellular respiration	1
GO:0046483	heterocycle metabolic process	1
GO:0051179	localization	1
GO:0051234	establishment of localization	1
GO:0051641	cellular localization	1

Supplemental Table 4. 4 unique orthogroups categorized as molecular function that were identified in *O. lupi* in comparison to the proteomes of O. *volvulus, O. ochengi,* and *O. flexusosa.* Table includes GO match, description, and number of proteins per orthogroup.

GO ID	Name	Count of proteins within orthogroup
GO:0003674	molecular_function	2
GO:0005215	transporter activity	1
GO:0008233	peptidase activity	1
GO:0030234	enzyme regulator activity	1

Supplemental Table 5. 1 unique orthogroups categorized as a cellular component that was identified in *O. lupi* in comparison to the proteomes of O. *volvulus, O. ochengi,* and *O. flexusosa.* Table includes GO match, description, and number of proteins per orthogroup.

GO ID	Name	Count of proteins within orthogroup
GO:0031143	Pseudopodium	1

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CHAPTER SIX

CONCLUSIONS

Currently, several barriers exist for *O. lupi* control within the US that include anthropogenic movement of animals, low compliance with recommended monthly ivermectin as a preventative, unknown true endemic ranges, the lack of a commercial diagnostic, limited treatment options, and a general lack of knowledge regarding vector species and transmission cycle. The goal of this dissertation was to gain forward momentum necessary to stop the spread of this parasite in the southwestern US. Identifying the putative vector species in Northern Arizona and New Mexico has bridged a critical gap of knowledge regarding the transmission cycle in the southwestern US.

In Chapter three, we sequenced and assembled the first *O. lupi* mitochondrial genome. Generating this mitogenome allowed for the design of long-range PCR primers that covered the entire mitogenome from complex canine skin samples. mtDNA has been shown as a highly lucrative source of markers for population genetic studies of multiple species of nematodes (1). This mitochondrial genome has provided the basis for fundamental global population studies of *O. lupi.* Additionally, our long-range PCR has successfully amplified whole mitogenomes from five filarial nematode species that affect a range of host species (monkey, bison, moose, cattle, white-tailed deer). Population studies within these species are currently underway using these mitogenomes.

Sequencing an *O. lupi* whole genome sample using the minION technology provided valuable insight necessary for whole genome sequencing: the host DNA can swamp out the signal of *O. lupi* during sequencing. This sample had been first freed from a nodule enzymatically and further washed to remove all contaminants. The sequence data was identified as 99% canine. Realizing the need for informed sample selection, I designed and produced the LupiQuant assay. This allowed for the generation of the first *O. lupi* draft genome as well as whole genome sequencing of 15 adult worms in Chapter five. The data produced in Chapter five has bridged a major gap of knowledge; improved surveillance tools are being tested, data mining for novel drug targets using pre-approved FDA drug databases has been conducted, and the predicted *O. lupi* proteome will be used to identify the most immunoreactive protein for a diagnostic. Control strategies are needed to stop the spread of this pathogen, the data provided within this dissertation is a step toward *O. lupi* control with the eventual goal of elimination.

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