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Molecular Evidence for Vector Implication of Onchocerca lupi in Los Angeles County, CA

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Molecular Evidence for Vector Implication of *Onchocerca lupi* in Los Angeles County, CA

by

Shanna J. Bolcen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
Department of Global Health
College of Public Health
University of South Florida

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Azliyati Azizan, Ph.D.

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March 27, 2014

Keywords: Canine, Ocular lesions, PCR Design, *Simulium* species

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DEDICATION

I would like to dedicate this thesis to my husband, James Webster, and my parents, JuneAnn and Lee Williams. Without their love and support, my work on this project would have been significantly more difficult.
ACKNOWLEDGMENTS

I would like to acknowledge my major professor, Dr. Thomas Unnasch, for allowing me the opportunity to participate in a public health research investigation as a principle investigator. I would also like to acknowledge the assistance of the Hassan K. Hassan in understanding the laboratory techniques used in this project. Lastly, I would like to acknowledge Dr. Emily Beeler and the Los Angeles County Department of Public Health for bringing this project to the attention of the Unnasch Laboratory.
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Abstract

*Onchocerca* is a genus of roundworm most commonly associated with the human infection onchocerciasis, or river blindness. While typically a zoonotic infection of ungulate populations, canine cases (*Onchocerca lupi*) have been identified in the United States and Greece. In 2012, Los Angeles County, Veterinary Public Health Program identified 3 cases of *Onchocerca* spp. infections in domestic canines. Samples from the ensuing blackfly collections were sent to the Global Health Infectious Disease Research Unnasch Laboratory for parasite isolation and vector species identification. Species-specific primers were designed and optimized for *O. lupi* using a non-specific cytochrome oxidase (COI) gene target (689bp) previously utilized in *Onchocerca* identification as a base. A secondary, nested PCR primer set (115bp) was additionally designed to aid in the screening process. Extracted DNA samples from the collected blackflies were screened for the presence of the parasite and positive samples were further tested against the larger fragment for confirmation. The primers designed for the study were determined to be specific to *O. lupi* and not cross-reactive with other *Onchocerca* species or *Dirofilaria immitis*. Of the 213 blackflies screened, 6 samples tested positive for parasite presence. The blackfly species was identified as *Simulium* spp. The presence of the *O. lupi* parasite in the blackfly population implicates that this is the likely vector in the LA County cases. While the blackfly identification was unable to be determined to the species level, the identification of *Simulium* spp. confirms the typically suspected vector in *Onchocerca* infections. Concerns of cross-species transmission make *O. lupi* an important parasite for public health study.
Chapter One:

Introduction

*Onchocerca* is a genus of filarial parasites with worldwide distribution and is typically associated with ungulates, including horses and cattle. The causative agent of human “river blindness” also resides within the same genus (Zarfoss, Dubielzig, Eberhard, & Schmidt, 2005). Historically, canids were not thought to be natural hosts for any *Onchocerca* parasite (Eberhard et al., 2000) until 1967, when *Onchocerca lupi* was described in the periocular tissues of a Caucasian wolf (*Canis lupus*) from what is now the country of Georgia (Rodonaja, 1967).

As with most of the zoonotic *Onchocerca* infection, very little is understood about the life cycle of *O. lupi* (Otranto et al., 2012a); however some extrapolation may be made as larval and adult stages based on other *Onchocerca* species (Sréter & Széll, 2008). In all *Onchocerca* spp., the life cycle is indirect with blackflies (*Simulium* spp.) or biting midges (*Culicoides* spp.) acting as the intermediate host. Previously described *Onchocerca* spp. (Table 1) exhibit narrow host ranges so it is suspected the *O. lupi* most likely only infects canids including wolves, foxes, jackals, and coyotes (Sréter & Széll, 2008). However, since the initial identification in a feral wolf, all canine *O. lupi* have only been identified in domestic dogs (Labelle et al., 2013).

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. cervicalis</em></td>
<td>Horse</td>
<td><em>Culicoides variipennis</em></td>
</tr>
<tr>
<td><em>O. reticulata</em></td>
<td>Horse</td>
<td><em>Culicoides variipennis</em></td>
</tr>
<tr>
<td><em>O. gutturosa</em></td>
<td>Cattle</td>
<td><em>Culicoides</em> spp., <em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. lienalis</em></td>
<td>Cattle</td>
<td><em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. gibsoni</em></td>
<td>Cattle</td>
<td><em>Culicoides</em> spp.</td>
</tr>
</tbody>
</table>
Table 1 continued. Host range of select *Onchocerca* spp. in wild and domestic animals

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. ochengi</em></td>
<td>Cattle</td>
<td><em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. jakutensis</em></td>
<td>Red deer</td>
<td><em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. cervipes</em></td>
<td>White-tailed deer, Black-tailed deer</td>
<td><em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. tarsicola</em></td>
<td>Red deer, reindeer</td>
<td><em>Odagmia ornata</em></td>
</tr>
<tr>
<td><em>O. alcis</em></td>
<td>Elk</td>
<td><em>Simulium</em> spp.</td>
</tr>
<tr>
<td><em>O. volvulus</em></td>
<td>Humans</td>
<td><em>Simulium</em> spp.</td>
</tr>
</tbody>
</table>

Table modified from Sréter & Széll, 2008

Morphologically, there are distinct characteristics for the male and female worms. Male worms are smaller and slender, typically measuring 43-50mm in length and 120-200µm in diameter. The anterior end is rounded with a nerve ring located 320-380µm proximally. The cuticle is in 4-5µm thick and has faint transverse striations. For internal organs, the esophagus is 550-650µm in length and increases slightly in diameter towards the posterior end, the intestines are approximately 12-15µm in diameter and the testes measure 50-70µm in diameter and occupy 80% of the pseudocoel. The left spicule is curved, tubular, slightly tapered, and 172-191µm in length while the right spicule is curved, tubular, and approximately 87-94µm in length. Additionally, the right spicule has a broad, heavy cuticle at the proximal end that tapers to a knobbed end (Egyed et al., 2001).

Adult female worms are difficult to remove intact from the nodule so the total length is unknown; however, the longest sections recorded range from 100-165mm (Sréter & Széll, 2008). The average width of the parasite range from 275-420µm in diameter. Internally, the esophagus is 1100-1200µm long, the intestines are approximately 34-40µm in diameter, and the uterine tubes are parallel, straight, about 118-143µm in length, and fill about 70% of the body cavity. The nerve ring and vulva resides 350-390µm and 800-1000µm respectively from the anterior end. The cuticle for the female worm is composed of two distinct layers with the outer layer...
exhibiting ring-like ridges that are small and close together anteriorly and become taller and farther apart posteriorly (Egyed et al., 2001).

Additionally, the microfilariae for the species have distinct characteristics including smaller length of 100-111µm and a width of 4-6µm with a body ratio of 15-28:1. The anterior end is bluntly rounded and contains two to three nuclei in the head. The remaining nuclei are found in the body with three to four nuclei per row until the posterior end where the nuclear column is reduced to a single row of three. From the anterior end, the nerve ring is found 23-27µm, the excretory pore 30-37µm, and the anal pore 80-91µm (Egyed et al., 2001).

Initially, canine-presenting cases were postulated to be an aberrant infection by *O. lienalis* (Orihel et al, 1991; Gardiner et al., 1993; Eberhard et al., 2000); however, recent molecular work and morphological comparisons have determined *O. lupi* to be a valid, separate species. Common genes to molecularly characterize the parasite include the 5S ribosomal RNA (5S) gene spacer sequences, cytochrome oxidase I (COI) gene, and the NADH dehydrogenase subunit 5 (ND5) (Egyed et al., 2001; Egyed et al., 2002; Sréter-Lancz, Széll, & Sréter, 2007; Sréter & Széll, 2008). The 16S ribosomal RNA (16S) gene has been used to characterize the intracellular *Wolbachia* bacteria that are present in most filarial parasites. Typically, the phylogeny of the endosymbiont matches that of the parasite and can therefore be used in parasite identification (Egyed et al., 2002).

Clinically the canine cases of *O. lupi* present as either acute or chronic cases. In acute cases, periorbital swelling, exophthalmos, photophobia, lacrimation, discharge, conjunctival congestion, protrusion of the nictitating membrane, and corneal edema are common observations. Chronic cases often present, with the worm incorporated in subconjunctival granulomatous nodules or cyst-like formations that penetrate the retrobulbar space, the orbital
fascia, eyelid, nictitating membrane, and sclera (Komnenou, Egyed, Sréter, & Eberhard, 2003). The nodules are primarily composed of eosinophils, histiocytes, fibroblasts, and newly formed blood vessels (Zarfoss, Dubielzig, Eberhard, & Schmidt, 2005). This is typically associated with exophthalmos and possible third-eyelid protrusion (Komnenou, Egyed, Sréter, & Eberhard, 2003). In almost all cases of canine onchocerciasis, enucleation is required.

In the past 20 years approximately 70 cases of subjunctival onchocercosis from O. lupi have been reported in domesticated canines (Sréter-Lancz, Széll, & Sréter, 2007; Otranto et al. 2013). Confirmed O. lupi canine onchocerciasis cases have occurred in the United States, Greece, and Portugal (Komnenou, Eberhard, Kaldrymidou, Tsalie, & Dessiris, 2002; Komnenou, Egyed, Sréter, & Eberhard, 2003; Zarfoss, Dubielzig, Eberhard, & Schmidt, 2005; Faísca et al., 2010; Labelle et al., 2013; Otranto et al., 2013). There are also possible, but unconfirmed, cases in Germany, Hungry, Switzerland, & Canada (Széll et al., 2001; Faísca et al., 2010; Hermosilla et al., 2011). In 2011, two cases of onchocerciasis attributed to O. lupi were documented in cats from the western regions of the United States. Both cats had been previously diagnosed with Feline Leukemia Virus (FeLV) but without prior ocular infections. These cases mark the first feline infection of O. lupi (Labelle, Daniels, Dix, & Labelle, 2011).

Between 1965 and 2002, ten cases of Onchocerca infection were recorded in humans worldwide. In eight cases, a single immature worm was isolated from various regions of the body including the wrist, the knee, and the sole of the foot and all were determined to be a variant of a previously described enzootic parasite. The other two cases were never definitively established beyond the genus level; however, both occurred in the subconjunctiva. A re-examination of the case data in a 2002 study has led the authors to suspect O. lupi in the latter two cases based on similarity of the clinical presentations, the histopathology, and the cuticle
morphology, particularly the ridge height, shape, and distance. The first of these infections pre-
dates the earliest description of *O. lupi* by two years but was described in a young female living
in the Crimean region of the former U.S.S.R. less than 800km from the site of the Russian wolf
infection. The other case was identified in an Albanian woman which is a similar distance to
Hungary, a country known to have endemic *O. lupi* canine infections (Sréter, Széll, Egyed, &
Varga, 2002).

The first case of *O. lupi* infection in a human patient documented at the time of infection
occurred in 2011. At Trakya University’s Department of Ophthalmology in Turkey, an 18-year-
old-female presented painless redness of the left eye and a history of a fly bite to the left, upper
eyelid approximately 30 days prior. At that time, a subconjunctival mass was noted in the
superonasal quadrant of the bulbar conjunctiva. Twenty-eight days after the initial visit, the
patient experienced pain in the left eye and a foreign body was observed in the subconjunctival
mass. A nematode was removed from the eye and sent for identification. Both morphological and
molecular identification using COI and 12S ribosomal RNA matched to *O. lupi* (Otranto et al.
2011).

Two more human onchocerciasis cases were retroactively attributed to *O. lupi* were
described in 2012. A 26-year-old male patient from Turkey underwent explorative surgery for a
suspected foreign body in the eye. Tiny, filamentous nematodes were extracted from beneath the
subconjunctiva and were tentatively identified as *Dirofilaria repens*. In the second case, an 8-
year-old patient from Tunisia had a subconjunctival mass surgically excised in which parasite
presence was noted. At the time, the parasite was identified as *D. immitis*. In both cases, the
histological specimens were re-examined for morphology and molecular characterization and
retroactively identified as *O. lupi* (Otranto et al., 2012a).
Most recently, the first case of human ocular onchocerciasis infection from *O. lupi* was reported in the United States. A 22-month-old female from northern Arizona presented with gradually progressive neck pain and stiffness. A magnetic resonance imaging (MRI) scan revealed an extradural mass in the cervical spine region with moderate to severe compression on the spinal cord. An excisional biopsy was performed during which the mass was determined to be of a rubbery consistency not conducive to removal except for a small biopsy. Histological examination revealed the presence of a parasitic worm which was morphologically identical to *O. lupi*. Sufficient parasite DNA was not available for molecular identification (Eberhard et al., 2013).

In 2012, the Los Angeles County, Veterinary Public Health Program (LACVPH) received a report of an *Onchocerca* infection in a domestic Boxer dog (Figure 1.) with corneal ulcers. A veterinary ophthalmologist provided additional information on two more such cases identified in 2004 and 2006 and outreach investigations of the neighboring communities identified more cases of similar infection, including one from San Diego County. *Onchocerca* spp were identified by histology of the conjunctival mass by Dr. Richard Dubielzig of the University of Wisconsin College of Veterinary Medicine.

LACVPH contacted the Unnasch laboratory to assist in the molecular identification of the parasite as well as the isolation and identification of the parasite from the suspected vector: a *Simulium* spp. Paraffin-embedded tissue samples from the ocular lesions containing the parasite were sent by Dr. Dubielzig from the histopathology work. The San Gabriel Valley Mosquito and Vector Control District (SGVMVCD) had kept incidentally collected blackflies from the San Gabriel Valley and these were sent to the laboratory for parasite analysis and fly identification. To evaluate the samples, an assay was developed for molecular characterization.
Figure 1. Infected right eye of an LA County dog. Lesions included generalized conjunctival inflammation, corneal degeneration, and elevated intraocular pressure of 31 mmHg. The condition was refractory to antibiotics, steroids, and other treatments. Eventually enucleation was performed, and histopathologic examination of tissue confirmed infection with *Onchocerca lupi*. (Photo: Dr. Bruce Silverman, 2004)
Chapter Two:

Material and Methods

Paraffin-fixed Tissue Extraction

Sections of paraffin-fixed microfilaria tissue from the three canine *Onchocerca* cases were received for test development. Individual slices of tissue were separated and divided into three sections: two ends and the center portion containing the microfilaria. The ends and the center portion were placed in separate 1.5ml microcentrifuge tubes. DNA was extracted from the samples using Qiagen’s QIAamp®DNA FFPE Tissue Kit’s Isolation of Genomic DNA from FFPE Tissue Sections protocol. The procedure was performed as described in the handbook with a longer lysing period of overnight (at least 12 hours).

Briefly, after the samples were trimmed and placed into separate tubes, 1ml of xylene was added to each sample and the sample vortexed for 10 seconds. A supernatant was obtained by full speed centrifugation for 2 minutes and then removed by pipette. Any residual xylene was removed from the remaining pellet by adding 1 ml of 100% ethanol and vortexing. A second pellet was obtained by full speed centrifugation for 2 minutes and the supernatant then removed. Residual ethanol was evaporated at room temperature for 20 minutes. The pellet was then resuspended in 180µl of Buffer ATL. 20µl of proteinase K was added, mixed by vortexing, and then the sample was incubated overnight at 56°C. The next morning, a second incubation at 90°C for 1 hour was performed and the tubes centrifuged to remove lid condensation. To each sample, 200µl of Buffer AL was added and mixed by vortexing followed by 200µl of ethanol. After
centrifugation to remove any drops from the lid, the samples were transferred to a QIAamp MinElute column provided in the kit and centrifuged at 6000 x g. The filter was transferred to a clean tube and underwent two rounds of washing with Buffer AW1 and AW2. Each time, the filter was moved to a clean tube and centrifuged at 6000 x g. The columns were dried by centrifugation at full speed for 3 minutes and the filter moved to a final clean tube. Elutions were performed at 50µL and stored at -80°C.

**PCR Target Selection and Optimization**

Four mitochondrial gene targets were selected for testing: 12S rRNA (533bp), 16S rRNA (493bp), ND5 (471bp), and cytochrome oxidase subunit I or COI (689bp) (Casiraghi et al., 2001). All primers were tested against a well performing stock 10⁻¹ dilution of *O. volvulus* DNA using polymerase chain reaction (PCR). All amplifications were conducted in a solution containing 20mM Tris·HCl (pH 8.0), 50mM KCl (10X Invitrogen PCR Buffer I), 200µM each of dATP, dCTP, dGTP, and dTTP, 0.5µM of primer, 2.5 units of Taq DNA polymerase (Invitrogen), and 2µL of DNA template. Each primer was tested against a gradient annealing temperature program running from 45°C to 52°C. Reactions began at an initializing temperature of 94°C for 3 minutes, followed by 35 cycles of denaturing, annealing, and extension at 94°C for 45 seconds, 45°C to 52°C for 30 seconds, and 72°C for 90 seconds respectively. Final elongation occurred at 72°C for 10 minutes. As amplification was not found to be affected by the annealing temperature gradient, 52°C was selected for further testing. The four primer sets were then tested against the DNA extracted from the tissue samples under the PCR conditions described above with the selected 52°C annealing temperature to determine the most suitable gene target.
Cytochrome oxidase Cloning

After gene target selection, the gene was cloned into a plasmid vector using Invitrogen’s TA Cloning® Kit as per the manufacturer’s protocol. Once the necessary amount of amplicon was generated, the product was ligated into the pCR®2.1 vector using a reaction of 3μl PCR product, 3μl of 10X ligation buffer, 6μl of pCR®2.1 vector, 3μl T4 DNA ligase, and 15μl of PCR grade water. The reaction was incubated overnight at 14°C. After ligation, the construct was transformed into competent E. coli One Shot TOP10 cells provided in the kit.

Prior to cell transformation, agar plates containing Luria-Bertani (LB) media were prepared using a composition of 1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, and deionized water and brought to a pH of 7.0 with NaOH. A total volume of 1 liter was prepared. The media was autoclaved and allowed to cool to room temperature before 100μg/ml of ampicillin was added. Agar plates were stored at 4°C after hardening.

All reagents for the transformation were kept on ice unless otherwise noted in the protocol. Each transformation required 50μl of TOP10 cells to which 2μl of the ligation reaction were added. The mixture was incubated in the ice bath for 30 minutes, heat shocked for 30 seconds at 42°C, and immediately returned to the ice bath. Each vial received 250μl of room temperature S.O.C. medium and was transferred to a shaking incubator where they were shook for 1 hour at 225 rpms in 37°C. Two different volumes (30μl and 300μl) of the transformation mixture were plated on room temperature LB agar plates to ensure that adequately spaced colonies were obtained. Plates were allowed to incubate overnight at 37°C. Selected colonies were grown overnight in LB broth, tested by PCR to confirm the presence of the plasmid, and sent for sequencing to confirm the sequence of the cloned amplicon. Clones with confirmed gene
inserts were regrown in LB broth overnight and the plasmid DNA was isolated by a Promega Pure Yield Plasmid Miniprep System (catalog no. A1222).

**Primer Design and PCR Conditions**

Species-specific primers were designed from the COI clone using the National Center for Biotechnology Information’s (NCBI) Primer-BLAST and MacVector primer analysis software. A multiple alignment consisting of the COI target region for *Dirofilaria immitis* (Genbank accession number EU159111), *Onchocerca gibsoni* (Genbank accession number AJ271616), *Wuchereria bancrofti* (Genbank accession number JF775522), and the *O. lupi* clone was used to determine regions of least similarity among the isolates for more specified gene regions. One set of primers was designed to capture a larger (475bp) fragment and a second, nested set was positioned within the larger fragment for screening purposes (Table 2.)

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>5’- 3’ Sequence (Forward)</th>
<th>5’- 3’ Sequence (Reverse)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF1R1</td>
<td>TGTTGCCTTTGATGGGGGG</td>
<td>GGATGACCGAAAAACCAAAACAAG</td>
<td>475</td>
</tr>
<tr>
<td>NF2R2</td>
<td>TCAAAATATGCCTACTGCTGTG</td>
<td>CAAAGACCCAGCTAAAACAGGAAC</td>
<td>115</td>
</tr>
</tbody>
</table>

All PCR reactions for the COI gene were designed to run in reaction mixtures containing 300mM Tris-HCL (pH 9.0), 75mM (NH$_4$)$_2$SO$_4$, 10mM MgCl$_2$ (10X Invitrogen PCR Buffer F), 200µM each of dATP, dCTP, dGTP, and dTTP, 0.5µM of primer, 2.5 units of Taq DNA polymerase (Invitrogen), and 2µL of DNA template. PCR amplification for the larger, non-nested fragment primers were conducted at an initialization step of 94°C for 3 minutes followed by 35 cycles of denaturing, annealing, and extension at 94°C for 45 seconds, 52°C for 30 seconds, and 72°C for 90 seconds and a final extension of 72°C for 10 minutes. The PCR
conditions for the nested fragment differed slightly with an initialization step of 94°C for 4 minutes followed by 40 cycles of 94°C for 45 seconds, 50°C for 45 seconds, and 72°C for 90 seconds and a final extension of 72°C for 10 minutes. For the nested PCR, 1µl of the initial, larger fragment amplicon was added to the reaction.

**Blackfly Processing**

Blackflies were collected incidentally by the San Gabriel Valley Mosquito and Vector Control District (SGVMVCD) in mosquito collection traps set throughout the San Gabriel Valley. The flies were collected 21 separate times in 21 fly pools from 15 locations. In total, 253 blackflies were submitted for testing with a median pool size of 7 flies and an average pool size of 13. Upon receipt, the flies were separated into individual 1.5ml microcentrifuge tubes containing 0.5ml of 70% ethanol and assigned a USF identification number. All USF ID numbers retained original collection location information.

For DNA extraction, flies were selected in groups of eleven flies using a random number generator. In total, 215 flies underwent DNA extraction with the remaining flies kept for possible further study in 70% ethanol. A Qiagen DNeasy® Blood and Tissue Kit was used following homogenization as per the tissue protocol using Kimble Chase 1.5mL RNase-Free Disposable Pellet Pestles (Article No. 749521-1590). All samples were removed from their original tubes with forceps, placed in new tubes, and any transferred ethanol allowed to evaporate. Individual flies were homogenized in 200µl Buffer ATL, followed by the addition of 20µl of proteinase K and 200µl of Buffer AL. The samples were then incubated for 30 minutes at 56°C followed by centrifugation for 5 minutes at 6000 x g, the supernatant transferred via pipette to a new 1.5ml tube, and 200µl of 100% ethanol were added to each tube. The samples were vortexed and left
for 5 minutes. All samples were then transferred to a clean spin column provided in the kit and centrifuged for 1 min at 6000 x g. Each sample then received two cycles of washing (Buffers AW1 and AW2) and centrifugations (1 minutes and 3 minutes respectively) at 6000 x g. After a 3 minute centrifugation at 6000 x g to dry the samples, the filters were transferred to a new 1.5ml tube, 60µl of elution buffer added, and the samples incubated at room temperature for 1 minute. After a final centrifugation for 1 minute at 6000 x g, the filters were discarded, and the sample stored at -80°C. All extractions were performed manually through the 30 minute incubation at 56°C and subsequent transfer of the supernatant.

Of the extracted samples, 182 were completed manually and 33 were completed using a Qiagen QIAcube machine protocol for the Qiagen DNeasy® Blood and Tissue Kit, Tissue and Rodent Tail program. The extractions were performed according to the kit’s instructions in both methods. The first elution from the manual extractions and all samples processed by machine were transferred to a 96-well Cell Culture Cluster Round Bottom Plate with Lid from Corning Incorporated (Cat No. 0720095) and stored at -80°C.

**Parasite Screening**

All extracted samples were screened for parasite presence using the 115bp nested (NF2R2) COI primer set. Samples were considered to be positive in the initial screening if an appropriate band was present on a 1.5% agarose gel after amplification. Positive samples from the nested PCR were rescreened using the same protocol. Amplicons from samples positive in both reactions were purified and sent for sequencing. All sample sequencing was processed by Eurofins MWG Operon. All PCR amplifications were conducted as described in the primer design section.
While the HF1R1 primer set yielded more consistent results with the screening assay on 1.5% agarose gel, upon sequencing it was discovered that the DNA was too degraded to produce usable sequences possibly as the result of too many freeze-thaw cycles. In order to amplify the fragmented DNA, smaller sections were amplified using overlapping fragments and combining them together into a consensus contig.

**Simulium spp. Identification**

To molecularly determine *Simulium* spp. identification, a multi-locus (COI, 16S and ND4) approach was utilized. Gene targets for comparison were selected based upon their previously successful use in laboratory identification of *O. volvulus* vectors. Cytochrome oxidase reactions were performed as described for the parasite screening; however, PCR conditions did vary with an initialization step of 96°C for 1 minute followed by 35 cycles of denaturing, annealing, and extension at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 90 seconds and a final extension of 72°C for 10 minutes. Reactions for 16S gene amplification were performed in solutions containing 20mM Tris-HCL (pH 8.0), 50mM KCL (10X Invitrogen PCR Buffer I), 200µM each of dATP, dCTP, dGTP, and dTTP, 0.5µM of primer, 2.5 units of Taq DNA polymerase (Invitrogen), and 2µL of DNA template. Reaction mixtures for ND4 amplification utilized the basic formula as the COI gene with addition 2.5 mM MgCl₂ added. Both the 16S and the ND4 PCR condition utilized an initialization step of 95°C for 3.5 minute followed by 40 cycles of 95°C for 30 seconds, 45°C for 50 seconds, and 72°C for 50 seconds and a final extension of 72°C for 7 minutes. Primer sequences for the *Simulium* spp. identification can be found in Table 2. All positive samples on a 1.5% agarose gel were purified and sent for sequencing.
Chapter Three:

Results

Extraction, Cloning, and Selection

Three tubes of paraffin-embed tissue samples tubes were obtained from LA County with several histological sections per tube. Extraction was attempted on two sections from sample 12RD0544, one from 12RD1242, and one from 12RD0301 before successful isolation of DNA occurred. Extractions from the 12RD0301 sample were used in the gene target selection and cloning process. From this sample, two extractions were successfully used in the COI cloning and were labeled as Clone 3-1 through 3-4 and Clone 4-1 thorough 4-4. Prior to cloning, two gene targets (12S and ND5) were rejected for further study based on poor and inconsistent results against the *O. volvulus* template. The 16S and the COI gene targets both performed equally well (Figure 2); however, the COI gene was chosen for further study based on its use as the main target for the Barcode of Life project. The other gene targets, 12S and ND5, were not found to yield consecutively consistent results against the *O. lupi* DNA template.

Primer Design

Two sets of COI primers were designed for determining parasite presence in this study. The smaller nested NF2R2 (115bp) was designed for initial parasite screening and a larger fragment HF1R1 (475bp) set was designed for parasite identification. Figure 3 shows the locations of the two primer sets relative to each other.
Figure 2. Comparison of the COI and 16S rRNA genes via PCR amplification.

Figure 3. Location of primer sets designed for this study in the Genbank COI gene (accession number JX080028.1)

**Gene Sensitivity and Specificity**

After random selection, Clone 3-4 was used in the serial dilution process. Figure 4A shows the same information with the cloned DNA tested against primer set HF1R1 with the detection level determined to be down to $8 \times 10^4$ copies of DNA sequence. Serial dilutions were
also performed on the nested primer set (NF2R2) with detection level set at 8 copies of DNA sequence (Figure 4B).

Figure 4. Detection levels for the HF1R1 primer set (A) for sample A = 8x10^9 copies of DNA, B = 8x10^8, C = 8x10^7, D = 8x10^6, E = 8x10^5, and F = 8x10^4 copies. Detection levels for the NF2R2 primers (B) for the following concentrations: A = 8x10^9, C = 8x10^7, E = 8x10^6, G = 8x10^4, I = 8x10, and J = 8 copies of DNA template. Expected amplicon size is denoted by the arrow.

Designed O. lupi primers were tested against undiluted and diluted concentrations of O. lupi, Caenorhabditis elegans, Brugia malayi, O. volvulus microfilaria, O. volvulus, O. ochengi genomic DNA, and Dirofilaria immitis (Figure 5). The nested set of primers proved to be less
discriminative than the larger fragment with only *C. elegans* and *B. malayi* not detectable at the 115bp fragment. The HF1R1 primers were more selective in their amplification.

![Image](image.png)

**Figure 5.** Specificity test with HF1R1 primers (A) and the NF2R2 primers (B). Arrow indicates expected band placement.

**Parasite Screening**

Of the 213 blackflies screened, 15 samples appeared to have appropriately-sized amplicons for the 115bp, nested fragment. A second, independent PCR amplification was performed to generate enough template for sequencing and verify the results. From this, 7 samples (LAC 3-2, LAC 5-1, LAC7-45, LAC 12-26, LAC 17-5, LAC17-18, and LAC 21-1) exhibited adequate template presence on 1.5% agarose gel to send for genomic sequencing. Of
these samples, 6 samples (all except LAC 21-1) were found to be a 100% identity match to the *O. lupi* Genbank sequence (accession number JX183106). The six samples were matched at a 60bp fragment length, the difference between the original 115bp and the resulting 60bp is due to the trimming of the primer and unusable portions.

Initially, these samples were tested solely against the HF1R1 primer set but resulting sequences did not allow for adequate identification due to degradation of the fragment. Testing of the samples against the combination primer pairs (HF1NR2 and NF2HR1) created overlapping regions that allowed for better coverage when amplifying DNA templates that may potentially be degraded from freeze-thaw cycles (Figure 6). Use of these primer sets produced adequate amplification for sequencing in samples LAC 7-45, LAC 12-26, LAC 17-5, and LAC 17-18.

All four samples matched to the Genbank sequence for 399bp with two sequence polymorphisms noted relative to the Genbank sequence. Samples LAC 17-5 and LAC 17-18 were noted to have a single base-pair change of A to G near the 5’ end at the position 78 which resulted in a silent mutation. In sample LAC 12-26, a G to C substitution was identified near the 3’ end at position 393 which caused a glutamine to histidine (gln -> his) amino acid change (Figure 7.).

**Geographic Location**

During fly collection, GPS coordinates of all trap locations were recorded (Table 3) and, from this information, a map depicting the distribution of the samples was generated (Figure 8.). Of the 15 locations sampled, 6 of those had positive samples and, of these 6 locations, 4 of them fall within a 10 mile radius of each other. All fly collections occurred between April 19, 2012

**Simulium Identification**

A multi-locus suite of mitochondrial genes (COI, 16S, and ND4) were used to try and determine *Simulium* identification down to the species level. While all of the gene targets matched the genus to *Simulium*, none were able to determine genus beyond a 98% identity match when sequencing results were blasted against the Genbank database. Thus, fly identity for this project has been established as *Simulium* spp.
Figure 7. Multiple alignment of the 4 positive contig sequences as compared to the Genbank sequence.

<table>
<thead>
<tr>
<th>Date</th>
<th>Name of Location</th>
<th>Latitude</th>
<th>Longitude</th>
<th>USF Ref #</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/22/2013</td>
<td>San Gabriel River</td>
<td>34° 8'38.41&quot;N</td>
<td>117°56'12.58&quot;W</td>
<td>LAC 2-4</td>
</tr>
<tr>
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<td>LAC 3-2</td>
</tr>
<tr>
<td>4/24/2013</td>
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<td>-117.7125</td>
<td>LAC 5-1</td>
</tr>
<tr>
<td>4/29/2013</td>
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<td>LAC7-45</td>
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<tr>
<td>5/7/2013</td>
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<td>-117.935686</td>
<td>LAC 12-26</td>
</tr>
<tr>
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<td>LAC 17-5</td>
</tr>
<tr>
<td>6/4/2013</td>
<td>Santa Fe Dam</td>
<td>34.116667</td>
<td>-117.95</td>
<td>LAC 17-18</td>
</tr>
</tbody>
</table>
Figure 8. Map of location of positive (red balloons) and negative (yellow balloons) collection sites.
Chapter Four:

Discussion

The ability to positively identify the samples by PCR and DNA sequencing of the amplicons supports the *O. lupi* identification in the fly samples as this provides evidence of repeatability. Matching at the 400bp fragment also provides a larger genomic target than the screening fragment and thus better identification matches. The detection of the two polymorphisms within the sample sequences strengthens the identification for this group of samples as it demonstrates that the results are not likely to be due to positive control contamination. Neither the positive control nor the Genbank sequence contained the polymorphisms. One of the polymorphisms resulted in a silent mutation; however, the base change near the 3’ end resulted in an amino acid change. The gln->his conversion is a relatively conservative amino acid change and is therefore not likely to result in a change in functionality.

In terms of detection with the designed primers, 8 copies of DNA templates are detectable under perfect conditions using the nested, NF2R2 primers. As an individual larva contains approximately 800 cells that each contain about approximately 100 mitochondria, the nested assay is able to detect the presence of one larva within the flies. Both the nested and the HF1R1 primers were tested beyond the limits depicted above, but neither was confirmed beyond the limits shown.

The geographical locations of the positive samples provide some insights as to the ecological factors that may be driving the parasite presence and distribution. The Rainbow
Canyon Ranch, San Gabriel River, and Santa Fe Dam sites all sit adjacent to the San Gabriel River and its watershed basin. In the past 20 years, southern California has made an effort to restore natural watershed and wetland habitats, including those in the San Gabriel Valley area (Stein et al., 2007). The Santa Fe Dam itself houses an 836-acre recreational area with areas for fishing, a chlorinated swim beach, and a nature center. Blackflies rely upon rivers and other bodies of water with aquatic vegetation for egg laying and larval development, all of which would be provided by the San Gabriel Valley watershed and Santa Fe Dam areas.

Directly upstream of the positive sample sites lays the San Gabriel Mountains which provide Los Angeles with more than a third of its drinking water. The mountains are comprised of alpine forests, chaparral hills, elevations reaching 10,064 feet above sea level, and over 600,000 acres of recreational use land with known populations of larger mammals such as deer, black bear, coyotes, and mountain lions (San Gabriel Mountains, 2014). Additionally, encroachment of the coyote population into urban areas is considered to be endemic to the area. A combination of easy access to food, water, and shelter with loss of predator control practices have ensured the coyote’s residential co-habitation and increases contact likelihood (Timm, Coolahan, Baker, & Beckerman, 2007).

It is highly likely that the San Gabriel River, its watershed, and its recreational areas are providing a wildlife corridor that allows for an easily accessible transmission interface. Traditionally, species in the Onchocerca genus utilize a secondary host for replication. In the described cases, domestic dogs serve in this capacity but it is unknown where the parasite resides when not in the companion animals. It is suspected that the parasite utilizes non-domestic canids for this role. While no zoonotic samples from wild population were collected, the ubiquitous presence of the coyote population and other non-domestic canids may provide a convenient
natural reservoir for the parasite and provides potential biologic plausibility for the presence of the parasite.

While there is strong evidence that the collected flies are a Simulium spp., positive identification down to the species level was not possible with molecular techniques alone. Traditionally, fly identification relies on cytotaxonomic analysis of larvae, which was not possible for this study, given that it analyzed adult flies. This provides an area for future study with the collection of larval samples. Ideally, the larva would be bisected with the anterior end analyzed by cytotaxonomy and the posterior end processed for molecular analysis using the same gene targets as described in this study. Identification from the cytotaxonomic work could then be matched to a molecular sequence that would correlate to the sequence generated from the adult flies. Historically, there is an association between Onchocerca and Simulium spp.; however, the presence of the parasite within the fly does not necessarily implicate the vector as the sole source of infections for this species but may be attributed to this series of infections.

Further study of the flies’ life cycle and feeding habits is critical to understanding transmission of this pathogen. Thus, targeted collection of blackflies would allow for a more accurate ecological picture by providing population distribution, broader molecular characterizations, and potential blood meal analyses. From larval collections and subsequent cytotaxonomy of the specimens, accurate identification of the larvae could be determined and then be molecularly matched to the adult flies. This would give a more accurate identification down to the species level of flies suspected of transmission.

The presence of easily accessible non-domestic canines makes for a convenient silent host for O. lupi; however no evidence has been collected to substantiate this hypothesis. Surveys of the wildlife population, specifically wild canids such as coyotes and foxes that are present in
the ecosystem, may expose cases of ocular *O. lupi* infections. Even if the parasite is not immediately apparent, skin snips could show microfilarial presence. Such a find would be the first documented case of *O. lupi* in a non-domesticated animal or human since the initial identification. Although not likely in view of the typical *Onchocerca* host range, examination of local ungulates would rule out the possibility of aberrant infection by another *Onchocerca* species or document further cross-species transmission.

In addition, further case searches may reveal a broader scope of domestic canine and human infection. After the initial case in LA County, two more cases were discovered for a total of three cases in six years in the Los Angeles and San Diego areas. While this only accounts for infections that sought treatment, additional cases may still be non-differentiated from other ocular infections. The scope of the case searches performed for this project was conducted by the LACDPh and the criteria for selection is unknown including the geographical range in which cases were sought. A more detailed description of the infection with a broader scope including non-parasitically classified ocular infections may uncover more cases than previously thought. Similarly, additional human cases may be identified with further retroactive studies of previously described cases of suspected parasitic infections. In both cases, a heightened awareness could provide a more timely diagnosis in both canines and humans.

Ideally, further investigation of *Onchocerca lupi* would lead to preventative measures against the disease. It is important to understand the life cycle and transmission factors before vector control can be initiated. Targets for possible vaccination or anti-parasitic prophylaxis may also come from a better understanding of the host population. Ultimately, prevention of the disease in companion animals is likely to reduce the risk of human infection.
LIST OF REFERENCES


