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Phylogenetic analysis of eyeworm (*Oxyspirura petrowi*) in northern bobwhite (*Colinus virginianus*) based on the nuclear 18S rDNA and mitochondrial cytochrome oxidase 1 gene (COX1)

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Abstract

Oxyspirura petrowi is a heteroxenous nematode found in northern bobwhite (*Colinus virginianus*) of the Rolling Plains ecoregion of Texas. Despite its impact on this popular gamebird, genetic level studies on *O. petrowi* remain relatively unexplored. To accomplish this, we chose the previously studied nuclear rDNA 18S region as well as the mitochondrial COX1 gene region of *O. petrowi* to investigate phylogenetic relations between *O. petrowi* and other nematode species. In this study, we generate primers using multiple alignment and universal nematode primers to obtain a near-complete 18S and partial COX1 sequence of *O. petrowi*, respectively. Phylogenetic trees for *O. petrowi*'s 18S and COX1 gene regions were constructed using the Maximum Likelihood and Maximum Parsimony method. A comparative analysis was done based on the nuclear and mitochondrial region similarities between *O. petrowi* and other nematode species that infect both humans and animals. Results revealed a close relation to the zoonotic eyeworm *Thelazia callipaeda* as well as a close relation with filarial super family (Filarioidea) such as the human eyeworm *Loa loa* and *Dirofilaria repens* eyeworm of dog and carnivores.

Introduction

Oxyspirura petrowi (Spirurida: Thelaziidae) is a heteroxenous nematode found in a variety of avian species in the USA. *O. petrowi* infects the eyes of its hosts, situating on the surface of the eye, underneath the nictitating membrane and eyelids, as well as in the ducts and glands behind the eye (Dunham *et al.* 2014*a*, *b*). First identified in Germany in the family Laniidae (Skrjabin, 1929), *O. petrowi* has since been identified in several other orders of birds including Galliformes and Passeriformes in Michigan (Cram, 1937) as well as various parts of the USA since (Saunders, 1935; McClure, 1949; Pence, 1972; Dunham and Kendall, 2017).

Of the regions that *O. petrowi* has been identified, the Rolling Plains ecoregion of west Texas is one of the most targeted areas of research on this parasite. This is largely because of the decline in northern bobwhite (*Colinus virginianus;* hereafter bobwhite) within this region. A highly popular gamebird in the USA, bobwhites in the rolling plains have experienced an annual decline of >4% over the past several decades (Sauer *et al.* 2013). The decline has been credited to many factors including habitat loss, habitat fragmentation, agricultural practices and weather conditions (Brennan, 1991; Rollins, 2007; Hernandez *et al.* 2013). However, until recently, parasites have remained undervalued in their potential effects on the decline.

Impacts of eyeworm infection in quail was first speculated by Jackson and Galley (1963) in Rolling Plains for *Oxyspirura sigmoides* (=*O. petrowi*). In his findings, Jackson reported potential damage to the eyes of the bobwhite containing more than 15 eyeworms (Jackson and Green, 1964), as well as strange behaviour that was suspected to be a result of vision impairment (Jackson and Galley, 1963). Further analysis by Dunham *et al.* (2016) found lesions and adenitis in the Harderian gland, a gland associated with immune defense (Payne, 1994), and corneal scaring in bobwhites infected with *O. petrowi*. It is likely that the damage caused by these worm burdens can result in reduced foraging efficiency, an inability to effectively escape predators, as well as an inability to avoid stationary objects like a fence or building (Dunham *et al.* 2016).

Despite the increased interest in recent years, *O. petrowi*'s evolutionary relationships with other parasites are still relatively unexplored. Phylogenetic studies of eyeworms in both humans and animals could be useful in understanding epidemiological, ecological and evolutionary influences on their hosts. A previous phylogenetic analysis using the 18S gene region of *O. petrowi* showed filarial nematode families to have a close genetic relation to *O. petrowi* (Xiang *et al.* 2013). However, Xiang *et al.* (2013) suggest that their results are not strongly reliable for the evolutionary affinity of *Oxyspirura* with other parasites due to lack of sequences in

the Thelazioidea super family. This issue can be addressed by constructing multiple phylogenetic trees using different gene regions.

To construct representative and reliable phylogenetic relationships, selecting the appropriate gene regions for analysis is the most important step. Hwang and Kim (1999) suggest an improper selection of a gene region can lead to poor understanding of the evolutionary relationship. For this reason, they also note that highly conserved markers of nuclear DNA (rDNA) and hyper variable regions of mitochondrial DNA (mtDNA) have been identified as useful in investigating phylogenetic relationships of higher categorical levels (deep branches) and lower categorical levels (recently diverged branches) of taxonomy, respectively.

The 18S ribosomal subunit (SSU) of nuclear rDNA is suitable due to its highly conserved region for strong evolutionary links as compared with 28S or LSU, 5.8, and Internal Spacers (ITS1 & ITS2) (Hwang and Kim, 1999). Additionally, 18S has been completely characterized of its V1–V9 variable regions with V4 as the region representing 18S variability in eukaryotes (Nickrent and Sargent, 1991). The V4 region is significant and allows us to distinguish between family and genera and even species in nematode diversity studies. For these reasons, 18S is one of the most popular genetic markers for phylogenetic studies in eukaryotes.

Similarly, mitochondrial DNA has also been used as a popular molecular marker in genetic diversity studies for nearly three decades. In recent years, hundreds of complete parasite mitochondrial genomes have been studied and characterized (Hu and Gasser, 2006). Among the mitochondrial genes, cytochrome oxidase I (COXI) is preferred as a standardized tool for molecular taxonomy and identification of species (Ratnasingham and Hebert, 2007). This gene region is often used as a marker for phylogenetic studies because of its strongly conserved region across species, easiness to amplify in polymerase chain reaction (PCR), lack of introns, lack of recombination, and very small intergenic regions (Galtier *et al.* 2009). It is also an efficient tool used for DNA barcoding and nematode identification on species level (Derycke *et al.* 2010).

In order to provide more detail to the evolutionary relationships of *O. petrowi* to other parasites, as previously done with 18S, we use a near-complete 18S and a partial COX1 gene sequence of *O. petrowi* to generate phylogenetic trees. Presently, there are no phylogenetic studies reported on *O. petrowi*'s mitochondrial COX1 gene. In this study, we analyze the phylogenetic relationships with the Maximum Likelihood (ML) and Maximum Parsimony (MP) method using MEGA 7 software. By combining the analyses of both 18S and COX1, these results could be useful in understanding *O. petrowi*'s relationship to other eyeworms as well as its potential effects on the bobwhite based on these evolutionary relationships.

Materials and methods

Ethics statement

This experiment was approved by Texas Tech University Animal Care and Use Committee under protocol 16071-08. All bobwhites were trapped and handled according to Texas Parks and Wildlife permit SRP-0715-095.

Data availability statement

All data generated or analyzed during this study are included in this paper. Sequencing data obtained from this study has been submitted to DNA Data Bank of Japan (DDBJ) (Acc No. LC316613 and LC333364).

Study area

The experimental study area of the present paper is consistent with the study area described in Dunham *et al.* (2014*b*). The broader range of application (e.g., Rolling Plains) was described by Rollins (2007).

Sample collection

Wild bobwhites were collected in July of 2017 from the same study area, in the same manner and using the same techniques previously described by Dunham *et al.* (2014*b*). *O. petrowi* were collected, aged and sexed as previously described by Dunham *et al.* (2014*b*). Adult eyeworm were washed repeatedly with 1X Phosphate-buffered Saline (PBS). Samples were preserved in 95% ethanol and stored at −80 °C until DNA extraction.

DNA extraction

Genomic DNA of adult *O. petrowi* was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instruction with slight modifications. Modifications included homogenization of eyeworms in 180 μL of ATL buffer with a micro-pestle (Sigma, USA) followed by an addition of 20 μL proteinase K. Additionally, samples were incubated at 56 °C for 20 min and an elution of 100 μL sterile water was performed as the final step. Extracted DNA was stored at −20 °C until further use.

Primer designing

Primers for 18S were designed based on CLUSTAL W2 multiple sequence alignment results. Two forward primers and a reverse primer for 18S were designed and validated using online primer designing tools (Table 1). An internal primer was designed based on the sequence results of 18S to obtain an internal sequence. For COX1, initial amplification was done using degenerative nematode-specific primers (Prosser *et al.* 2013) and primers were designed (Table 1) using sequencing results of primary amplification based on methods described in Kalyanasundaram *et al.* (2017).

Amplification of O. petrowi *18S and COX1*

Both sets of primers were optimized using an annealing temperature gradient from 55 to 60 °C. PCR reactions contained 5 μL of 2X Red Dye Master Mix (Bioline, England), 0.5 μL of 10 μM forward and reverse 18S and COX1 primers, 3.0 μL of molecular grade water, and 1 μL of *O. petrowi* template DNA for a total reaction volume of 10 μL for 18S and COX1, respectively. PCR reactions were run under the following parameters: 95 °C for 3 min, 95 °C for 30 s, 57 and 60 °C for 30 s, for both COX1 and 18S. Elongation temperature was kept at 72 °C for 2 min for 18S reactions and 30 s for

Table 1. Oligonucleotide primers for amplifying and sequencing 18S and COX1 regions of *O. petrowi*

Primer	Oligonucleotide sequences	Melting temp (°C)
Op18SF	5′ CCGATTGATTCTGTCGGCGGTTA 3′	59.3
Op18SR	5′ CACCTACGGAAACCTTGTTACGAC 3′	57.5
Op18SIntF[a]	5′ CTCAACACGGGAAAACTCACCTG 3′	58.1
OxyCOX1F	5′TGAGCTGGTTTAGGTGGTGCTA 3′	58.2
Oxy COX1R	5′GAACCAGCTAACACAGGTACAGC 3′	57.7

[a]Used to obtain missing 18S region by sequencing.

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COX1 reactions with 29 cycles. Final elongation at 7 °C for 5 min was used to check extended chain. Amplification of the 18S and COX1 products were visualized on 1.5% agarose gels.

Sequencing

Purified PCR products of 18S and COX1 reactions were sequenced in both directions using their respective forward and reverse primers. Based on the sequencing results of 18S, an internal primer was designed and used to amplify 18S in PCR again. This PCR product was then sequenced to obtain the near complete 18S rDNA sequence. The partial COX1 sequence was confirmed with similar methods as described by Kalyanasundaram *et al.* (2017) using *Thelazia callipaeda* COX1 sequence (Liu *et al.* 2013) as a comparison. Raw sequences were trimmed using DNA chromatogram explorer (www.dnabaser.com). Final sequences used for analysis totaled at 1811 bp for 18S and 598 bp for COX1. Sequence similarity was performed using BLAST analyses.

Phylogenetic analysis

MEGA 7 software was used to generate phylogenies of 18S and COX1 gene regions. *O. petrowi* 18S and COX1 were separately aligned with selected sequences of other parasites from the GenBank, NCBI. Initially, we did multiple alignments with nearly 150 sequences of 18S retrieved from GenBank using CLUSTAL W program and simple trees were constructed by ML method (Larkin *et al.* 2007). We used taxa from order Spirurida for constructing 18S phylogenetic tree. Based on the alignment results, identical and unfit/short sequences were removed until enough quality congregate sequences were made. All gaps were removed and the total 1634 positions were used in the final dataset. Similarly, we constructed phylogenetic tree for COX1 sequences retrieved from GenBank. We used Filarioidea and Thelazioidea super family as major taxa to generate COX1 phylogeny both ML and MP method. Species specifically analyzed in this study include *Brugia malayi*, *Wuchereia bancrofti*, *Loa loa*, *Dirofilaria repens* from Filarioidea and *O. petrowi*, *T. callipaeda* from Thelazioidea super family. The complete deletion was used to treat gaps as missing information and totalled 405 positions in the final dataset. Phylogenetic tree constructions were performed using character state including ML and MP method. The bootstrap value was set at 1000 in order to represent strong evolutionary relationships between *O. petrowi* and other parasites of the Nematoda phylum.

Results

BLAST analysis results of the 1811 bp sequence of 18S showed a 100% identity to *O. petrowi* isolates (KF110800-KF110799), confirming that our sequence corresponds with previously submitted sequences of *O. petrowi*. Sequence results of *O. petrowi*'s 18S gene region revealed a 95% to 96% similarity to the 18S region of *B. malayi* (AF036588), *W. bancrofti* (LM006781), *L. loa* (XR-002251421) and a 92% similarity to *T. callipaeda* (LK982445). *Oxyspirura petrowi*'s COX1 shows an 86% similarity to *Dirofilaria* spp. (KX265050) and an 85% similarity to *Dirofilaria repens* (KX265049). Lastly, there is also an 84% similarity between *O. petrowi's* and *T. callipaeda*'s COX1 gene region (KY908318-KY908318). Both *O. petrowi*'s 1811 bp 18S sequence (Fig. 1A) and 598 bp COX1 sequence (Fig. 1B) were submitted in DDBJ (Acc No. LC316613 and LC333364).

A phylogenetic tree was constructed for both 18S and COX1 gene regions of *O. petrowi* to determine its evolutionary relationship within the Nematoda phylum (Figs 2 and 3). All *O. petrowi* isolates from different geographical locations were placed in one cluster and received strong support by ML and MP bootstrap analysis (100%). All clades in the 18S tree received moderate to high (50–100%) support by ML bootstrap analyses. Bootstrap values below 50% were removed from the COX1 trees (Fig. 3A and B). In both 18S and COX1 trees, species of the Filarioidea superfamily placed closely to species of the Thelazioidea superfamily (Figs 2 and 3). *T. callipaeda* (KY908318) is located within the same clade of *O. petrowi* in the 18S tree. Similarly, in the COX1 trees, *O. petrowi* shares a branch of the tree with *T. callipaeda*. *Heliconema longissimum* (GQ332423) and *Spirocerca* spp. (KJ605487) in the Spiroroidea superfamily were also placed in the same clade of the COX1 trees (Fig. 3A and B).

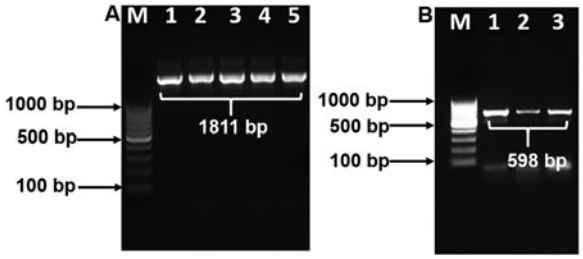

Fig. 1. Polymerase chain reaction (PCR) amplification of 18S and COX1 gene using specific primers. (A) 18S rDNA amplification Lane M: 100 bp DNA ladder (Fermentas); Lane 1–5: 18S rDNA amplicon (1811 bp). (B) partial COX1 gene amplification. Lane M: 100 bp DNA Marker (Fermentas); Lane 1–3: partial COX1 amplified products (598 bp).

Discussion

Over the past several decades, molecular phylogenetic studies have received widespread attention in determining evolutionary relationships between various specimens as proposed by Nadler (1995) in their phylogenetic case study of Ascaridinae nematodes. When morphological features are not similar in parasites, a molecular comparison involving phylogenetic investigation is a useful method to infer the genetic relationship between species (Nadler, 1995). Undoubtedly, morphological evolution can happen strictly on a genetic basis. Comparisons on the genetic level can also decisively confirm or deny relationships between parasites previously examined using morphological characteristics. In this study, we observe the nuclear 18S region and mitochondrial COX1 region of *O. petrowi* to better understand these relationships not readily available by morphology alone.

Our sequences for 18S and COX1 were confirmed using BLAST analyses. Constructed phylogenetic trees following BLAST analyses for both 18S and COX1 sequences show strong support for the monophyly of the genus *Oxyspirura*. We also found our phylogenetic results of 18S in congruence with results of Xiang *et al.* (2013). Based on 18S results from both studies, all the parasite species within the superfamilies of the phylogenetic trees are within the order Spirurida, and the phylogenetic trees reveal the *Oxyspirura* genus as a sister group for the Filarioidea superfamily.

Although the various species of Filarioidea identified in this study all have a 96% similarity with *O. petrowi's* 18S region, *L. loa* has lower nucleotide variation, indicating that *L. loa* is of closer relation to *O. petrowi.* While the filarial nematodes *W. bancrofti* and *B. pahangi* cause lymphatic filariasis in the definitive hosts, *L. loa* causes loaiasis (Chandy *et al.* 2011; Tan *et al.* 2011). Typically found in humans of west and central Africa, *L. loa* is transmitted through a deerfly (*Chrysops* spp.) vector, with infective larvae entering the wound produced by the deerfly and maturing in subcutaneous tissue (CDC, 2015). Loiasis is caused by both adult worms and microfilaria with clinical symptoms of eosinophilia, Calabar swelling, and eyeworm migration in

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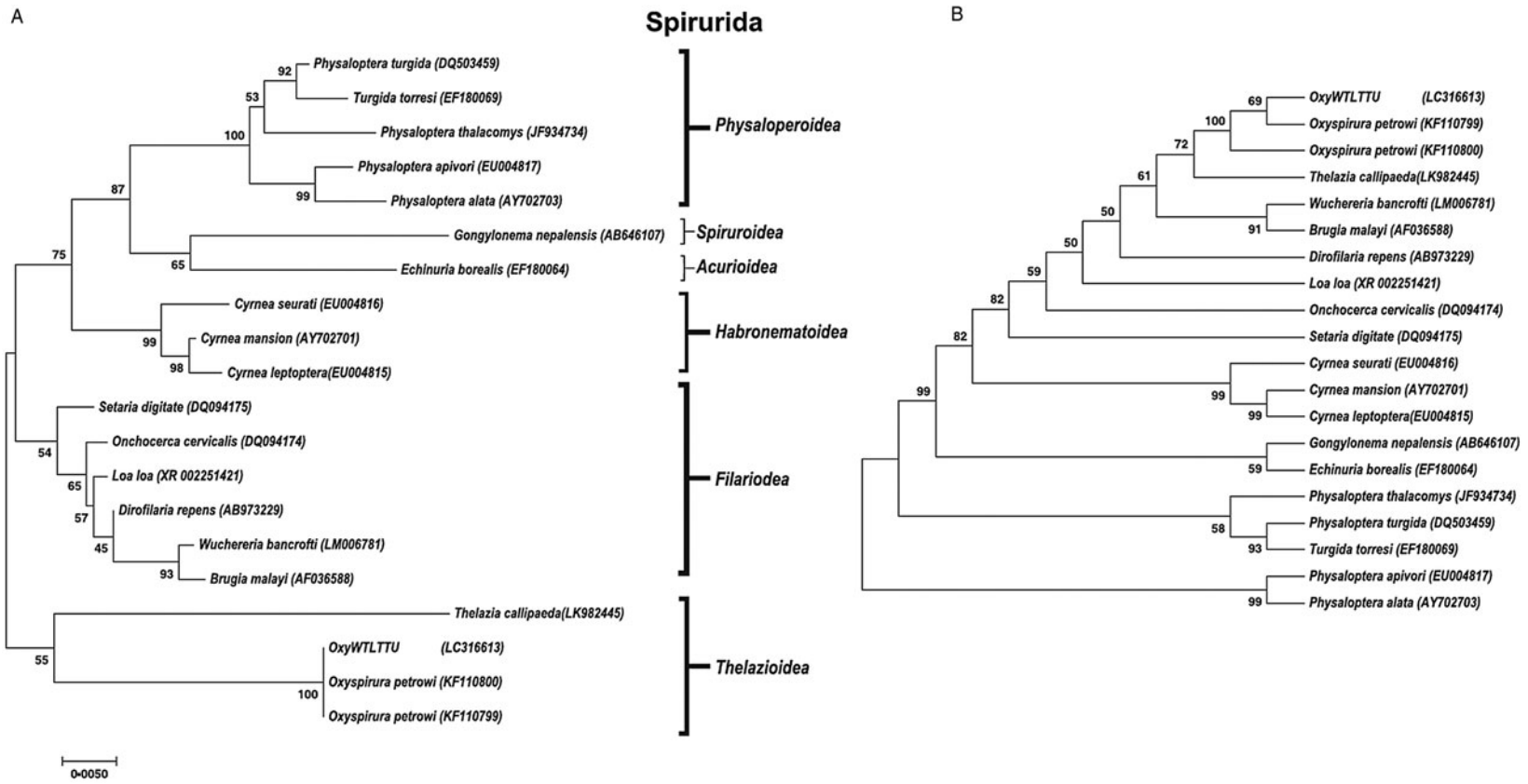

Fig. 2. Phylogenetic analysis of *O. petrowi* based on a near-complete 18S using Maximum Likelihood and Maximum Parsimony methods. (A) Maximum Likelihood: The evolutionary history was inferred using the ML method based on the Tamura-Nei model. The phylogenetic tree illustrates 18S rDNA sequences of nematodes related to *O. petrowi*. Bootstrap values above 50 are shown in the tree. All positions containing gaps and missing data were eliminated. Species name and their nucleotide accession numbers were included in the tree. There were a total of 1634 positions for 18S in the final dataset. Evolutionary analyses were conducted in MEGA7. (B) Maximum Parsimony: The evolutionary history was inferred using the MP method based on Subtree-Pruning-Regrafting (SPR) algorithm. The phylogenetic tree illustrates 18S rDNA sequences of nematodes related to the eyeworm *O. petrowi*.

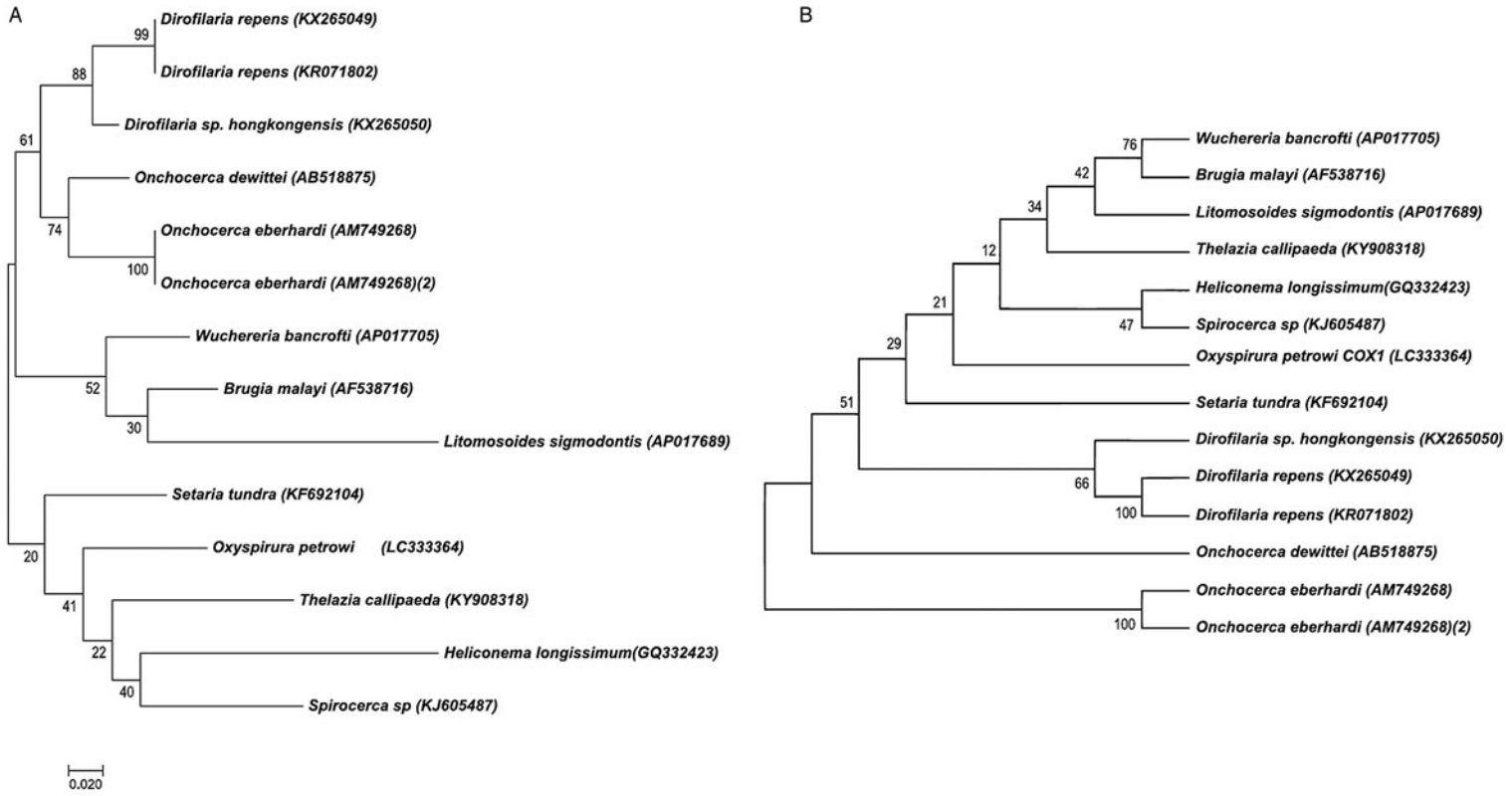

Fig. 3. Phylogenetic analysis of *O. petrowi* based on partial COX1 using Maximum Likelihood and Maximum Parsimony methods. (A) Maximum Likelihood: The evolutionary history was inferred using the ML method based on the Tamura-Nei model. The phylogenetic tree illustrates COX1 sequences of nematodes related to *O. petrowi*. All positions containing gaps and missing data were eliminated. Species names and corresponding nucleotide accession numbers were included in the tree. There was a total of 405 positions for 18S in the final dataset. Evolutionary analyses were conducted in MEGA7. (B) Maximum Parsimony: The evolutionary history was inferred using the MP method based on Subtree-Pruning-Regrafting (SPR) algorithm. The phylogenetic tree illustrates 18S rDNA sequences of nematodes related to *O. petrowi*.

its hosts (Antinori *et al.* 2012). During eye worm migration, the parasite may be seen moving in the vitreous cavity, found in the anterior chamber, or in the cornea, resulting in inflammation and impaired vision (Barua *et al.* 2005; Nayak *et al.* 2016).

Treatment of *L. loa* can be dangerous as it can cause brain inflammation and sometimes complications such as neuropathy and encephalopathy can occur (CDC, 2015). A recent genomic study on *L. loa* found several orthologous kinases that can be targeted by drugs currently approved for use in humans such as imatinib (Desjardins *et al.* 2013), potentially providing a safer treatment. Additionally, Xiang *et al.* (2013) suggest that the treatment strategies used for human eyeworm infection such as *L. loa* would be a model to develop treatment strategies for *O. petrowi* infection in quail.

An additional filarial nematode, *D. repens* of the Filarioidea superfamily, was identified with COX1 sequencing results. Similar to the 18S results, the COX1 phylogenetic tree placed the Thelaziidae family close to the Filarioidea superfamily and revealed less divergence to *D. repens*. These observations signify both are sister groups and likely suggests they evolved from the same ancestor. *Dirofilaria* spp. are responsible for most filarioid eye infections and for nodules on the orbital zone or eyelid (Otranto and Eberhard, 2011; Mateju *et al.* 2016). Found in humans and other domestic and wild animals, *D. repens* is transmitted by mosquitos (Czajka *et al.* 2014). Another sister group to the Thelazioidea superfamily, the Spirocercidae family, causes spirocercosis in their definitive Canidae hosts in tropical and subtropical regions. Clinical symptoms of spirocercosis include vomiting, odynophagia, hyper salivation and lesions, with aortic lesions being the most common and can be deadly (Van der Merwe *et al.* 2008). Van der Merwe *et al.* (2008) also states that species in this family utilize dung beetles as the intermediate host.

Furthermore, our findings in the 18S and COX1 phylogenetic trees demonstrate the relation of *O. petrowi* and *T. callipaeda* in the Thelaziidae family as they were placed in the same clade of our phylogenetic trees. *T. callipaeda* is an eyeworm responsible for the neglected tropical disease known as thelaziasis in humans and carnivores of Europe and the East Asia. Thelaziasis produces clinical signs of epiphora, conjunctivitis, and ulcerative keratitis in their hosts (Otranto *et al.* 2004). Additionally, *T. callipaeda* uses fruit flies, *Phortica* spp., as the intermediate host to transmit infection (Otranto *et al.* 2004; Otranto and Eberhard, 2011).

Based on these results, it is plausible that *O. petrowi* could have similar impacts on the bobwhite as these parasites have on their hosts. Future studies need to be carried out on pathological relations between *O. petrowi* and these parasites to fully understand this comparison and effective treatment strategies. Similarly, all described parasites require an intermediate host to transmit infection. While it is postulated that the plains lubber grasshopper (*Brachystola magna*) is a potential intermediate host (Kistler *et al.* 2016), it has not been determined whether it is capable of transmitting to bobwhite. Future investigations into the intermediate host of *O. petrowi* should prioritize similar species as the intermediate hosts of the related parasites.

This is the first report of examining a partial sequence of the mitochondrial gene region of *O. petrowi* as well as the first report in comparing this region with the almost-complete nuclear 18S gene region of *O. petrowi* with other parasites via phylogenetic analyses. In spite of partial sequences, both 18S and COX1 phylogenetic results strongly concluded the relationship of *O. petrowi* with Thelaziidae family. However, further sequencing of the entire COX1 gene region will help in better understanding inter- and intra-species similarities. Using phylogenetic networking, the COX1 gene region of *O. petrowi* could potentially be used as a biological tag to study the bobwhite population decline. Future genetic analyses could also help in further characterizing *O. petrowi* and how it relates to its contribution to the decline of bobwhites of the Rolling Plains ecoregion of Texas.

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Conflict of interest. None.

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