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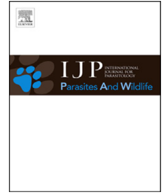

Molecular identification and characterization of partial COX1 gene from caecal worm (*Aulonocephalus pennula*) in Northern bobwhite (*Colinus virginianus*) from the Rolling Plains Ecoregion of Texas

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A B S T R A C T

Aulonocephalus pennula is a nematode living in the caeca of the wild Northern bobwhite quail (*Colinus virginianus*) present throughout the Rolling Plains Ecoregion of Texas. The cytochrome oxidase 1 (COX 1) gene of the mitochondrial genome was used to screen *A. pennula* in wild quail. Through BLAST analysis, similarity of *A. pennula* to other nematode parasites was compared at the nucleotide level. Phylogenetic analysis of *A. pennula* COX1 indicated relationships to Subuluridae, Ascarididae, and Anisakidae. This study on molecular characterization of *A. pennula* provides new insight for the diagnosis of caecal worm infections of quail in the Rolling plains Ecoregion of Texas.
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1. Introduction

The caecum is a valuable part of the avian gastrointestinal system with a variety of functions including cellulose breakdown, weight control, energy conservation, and antibody production (Fenna and Boag, 1974; Clench and Mathias, 1995). The gastrointestinal system, including the caecum, is highly susceptible to parasites (Cyntia, 2011). Avian species infected with parasites of the caecum have reportedly experienced inflammation, hemorrhaging, lesions, hyperplasia, necrosis, and death (Greiner and Ritchie, 1994). Parasites of the caecum have also been documented to result in weight loss and reproductive issues (Lehmann, 1984; Booth et al., 1993).

In recent surveys, Northern bobwhite quail (*Colinus virginianus*, hereafter bobwhite) have appeared with high prevalence and intensity of the caecal worm, *Aulonocephalus pennula*, in the Rolling Plains Ecoregion of Texas (Villarreal et al., 2012; Bruno, 2014; Dunham et al., 2016). The bobwhite is a highly popular gamebird to hunters and is also associated with economic significance in local communities (Johnson et al., 2012). Over the past several decades, the Northern bobwhite quail has been decreasing throughout its native range, with an annual decline of >4% (Sauer et al., 2013). Its decline has been credited to many factors including habitat loss, fragmented populations, agriculture practices, and weather conditions (Bridges et al., 2001; Rollins, 2007; Hernández et al., 2013). However, in the Rolling Plains, known for its general abundance of quail, bobwhite decline has continued despite stable habitat conditions (Rollins, 2007; Bruno, 2014).

A. pennula was first described by Chandler (1935) as *A. lindquisti*. In his analysis, Chandler defines the nematode as a free-living intestinal parasite often found in the caecum as well as the small and large intestine intermittently. Pathological studies performed by Dunham et al. (2017) identified that *A. pennula* does not penetrate the caecal wall, upholding Chandler's free-living parasite speculation. Dunham et al.'s (2017) findings also indicated a low amount of digesta present, suggesting that *A. pennula* may affect nutrient loss due to consumption of digestive material before it has been absorbed by the caecum. *A. pennula* may also disrupt much of the caeca's vital functions due to this ingestion of digestive material resulting in reproductive issues and death by malnutrition, particularly during times of drought (Lehmann, 1984). This is concerning, as the Rolling Plains region experienced a long-term drought between 2010 and 2013 (Dunham et al., 2014).

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Presently, there is no recorded genomic data for *A. pennula*. With a lack of genomic data on *A. pennula* as well as little experimental data, details in regard to *A. pennula*'s direct and indirect effects on bobwhites is relatively unknown. Genomic data may provide a better understanding of these effects based on the phylogenetic relations of *A. pennula*. Comparing phylogeny to pathogenicity based on genomic data is widely researched on species with quick evolutionary adaptations such as bacteria, fungi, and viruses. In Koch et al. (1991), various isolates of *Leptosphaeria maculans* were analyzed using DNA restriction fragment length polymorphisms to determine phylogenetic relationships. In their study, they also observe whether this phylogenetic data corresponded to isolate pathogenicity. In some cases, phylogeny did relate to pathogenicity. In a study by Leclerc et al. (2003), they suggest that pathogenic behavior of *Mycobacterium* is phylogenetically inherited. Findings such as these may correspond with parasite phylogenies. While much of the virulence of a parasite highly depends on the host environment (Leung and Koprivnikar, 2016) general effects may be similar in parasites that inhabit a certain area of the body, such as that of gastrointestinal parasites. However, definite conclusions cannot be made based solely on phylogenetic relationships.

In this study, we aim to sequence the COX1 gene region of *A. pennula* and develop a PCR-based method to detect this parasite. The mitochondrial COX1 gene region is widely used for parasite species identification and distinguishing evolutionary relationships between parasites (Prosser et al., 2013). In previous publications, it has been evaluated as a potential genetic marker in the phylum of Nematoda (Floyd et al., 2002; Elsasser et al., 2009). By creating a PCR method to detect *A. pennula*, this reduces the variability in misidentification of eggs from the traditional fecal floatation methods (Zajac and Conboy, 2012). Thus, objectives of this study include sequence the COX1 gene region of *A. pennula* (i), develop a diagnostic PCR method to detect *A. pennula* presence in bobwhite (ii), create a phylogenetic tree to visualize relatedness between *A. pennula* and other nematode species (iii), determine potential phylogenetic-pathogenic relationships to further understand *A. pennula* pathogenicity in bobwhite (iv).

2. Materials and methods

2.1. Ethics statement

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

2.2. Study area

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

2.3. Sample collection

Wild bobwhites were collected from the same study area, in the same manner, and using the same techniques previously described by Dunham et al. (2014). The quail collection for the present study occurred in April 2017. Adults of *A. pennula* were collected from bobwhite caecums. All nematodes were washed repeatedly with 1XPBS. Samples were preserved in 95% ethanol and stored at −80 °C until DNA extraction.

2.4. Parasite identification

A. pennula adult worms were identified morphologically based on descriptions by Chandler (1935) and Inglis (1958). Its characterized by complex hexagonal mouth opening with grooves partially enclosed by a cuticle envelope. This species is also defined by presence of cephalic allae and poorly developed pharangeal portions as well as the presence of anterior and posterior bulbs. Posterior ends taper to a point. Females are larger in length and diameter than males, with uteri that reaches throughout the body cavity, pushing the oesophageal bulbs towards the head of the worm and extending past the anus. Ovular eggs occupy the entirety of the uteri. In contrast with females, males have a long, tubular spicule present.

2.5. DNA extraction

Genomic DNA extractions were performed on individual males and females of *A. pennula*. Each individual nematode was macerated in 180 μl of the ATL buffer (Qiagen Inc., Germantown, MD). A volume of 300 μl of chloroform was added, mixed vigorously, and centrifuged at 12,000×g for 10 min. The aqueous phase was transferred to a fresh tube, mixed with 0.5 ml of 100% ethanol and 0.2 volume of 3M Sodium acetate, pH 5.2., incubated at −20 °C for 30 min and centrifuged to precipitate DNA. The DNA pellet was washed with 1 ml of 70% ethanol, centrifuged, and the supernatant discarded. The DNA was air-dried and eluted on 100 μl of sterile nuclease free water. The DNA extraction method used followed Sambrook and Russell (2001) with a few modifications including centrifuge speed and time as well as the volumes of liquid used.

DNA extraction of fecal samples was performed on bobwhite feces using a Qiamp Stool Minikit (Qiagen Inc.) following a fecal extraction protocol described in Kistler et al. (2016). However, instead of eluting with 200 μl of AE buffer as described in the protocol, an elution for 50 μl with molecular grade water was used to increase DNA concentration.

2.6. Primer design

Previously, published nematode cytochrome oxidase subunit I (COX I) degenerative primers were used for initial amplification of COX1 gene from male and female adult worms of *A. pennula* (Prosser et al., 2013) (Table 1). PCR was performed using DNA extracted from adult worm of *A. pennula*. The PCR reaction volume

Table 1
List of nematode-specific degenerative primers.

Primer	Sequence	Melting temperature (°C)
Nem F1	5′ CRACWGTWAATCAYAARAATATTGG 3′	52.2 °C
Nem F2	5′ ARAGATCTAATCATAAAGATATYGG 3′	49.6 °C
Nem F3	5′ ARAGTTCTAATCATAARGATATTGG 3′	50.0 °C
Nem R1	5′ AAACTTCWGGRTGACCAAAAAATCA 3′	55.6 °C
Nem R2	5′ AWACYTCWGGRTGMCCAAAAAAYCA 3′	61.6 °C
Nem R3	5′ AAACCTCWGGATGACCAAAAAATCA 3′	55.2 °C

was 25 μl containing 2 × Red dye master mix 10 μM of each primer and 2 μl of extracted DNA template. The PCR program used was: 95 °C for 3 min, (95 °C for 30s, 56 °C for 30 s, 72 °C for 1 min) × 30 cycles, 72 °C for 10 min. The amplified products were visualized in 1.5% agarose gel stained with ethidium bromide. PCR products were purified using PCR purification kit (Sigma, USA) according to manufacturer's protocol. PCR products were sequenced using Sanger sequencing method in a 3100 Automated DNA Sequencer (Applied Biosystems) at Genscript, USA.

2.7. Diagnostic PCR

Caecal worm DNA was extracted from quail fecal samples using faecal DNA extraction kit according to manufacturer's protocol. Primers were designed in order to develop a diagnostic method to identify *A. pennula* in wild quail. Partial 5′ end region of COX1 was amplified by PCR using the forward primer Apen F (5′-GGCTGGTATGGTTGGTACTGGC - 3′) and reverse primer Apen R1 (5′-GCACCCAAAATAGAACTCACCCC - 3′). Diagnostic PCR was performed using DNA extracted from quail fecal sample. The PCR reaction volume was 25 μL containing 2X Red dye master mix 10 μM of each primer and 2 μl of extracted DNA template. The PCR program used was: 95 °C for 3 min, (95 °C for 30s, 60 °C for 30 s, 72 °C for 30 s) × 30 cycles, 72 °C for 5 min. The amplified products were visualized in 2.0% agarose gel stained with ethidium bromide. PCR products purification and sequencing was done like above mentioned.

2.8. Sequence analysis

Amplicon products of COX1 came at 700–750 bp and were sequenced in both directions using *A. pennula* forward and reverse primers. Raw sequences were trimmed using online tools and a contig analysis was performed. This reduced our original amplicon length to 717bp. Sequence identities of other nematode COX1 gene sequences were generated in comparison to *A. pennula* using BLAST analysis. Nucleotide sequence comparisons were done between *A. pennula* and *Heterakis gallinarum* using pairwise alignment (http://www.ebi.ac.uk/Tools/psa/).

2.9. Phylogenetic tree

The sequences used for the phylogenetic tree were obtained from the GenBank database. All the sequences obtained were validated with their corresponding publications. Sequences were aligned using ClustalW. The genetic distances and phylogenetic analyses were conducted using MEGA 7 (Kumar et al., 2016). Intraspecific and interspecies genetic distances were calculated using the Kimura 2-parameter (K2-p) model. Complete deletion treatment and standard error estimated (SE) were determined using a bootstrap procedure (1000 replicates). The phylogenetic tree was constructed using Maximum Livelihood (ML) algorithm with the K2-p model. The bootstrap consensus results inferred from the 1000 replicates were used to represent the evolutionary history of the taxa. Intraspecific and interspecific pairwise distances of *A. pennula* were calculated using complete deletion in the ML algorithm and K2-p model.

3. Results

3.1. Sequence analysis

Female and male *A. pennula* were morphologically identified based on descriptions by Chandler (1935) (Fig. 1). PCR reactions were performed with extracted *A. pennula* DNA and amplified (Fig. 2A). Sequencing of all products were completed and analyzed. BLAST analyzed results showed that the 717 bp COX1 gene of *A. pennula* has 90% identity to *Toxascaris leonina* (KC902750) and *Ascaris lumbricoides* (JN801161) as well as 89% identity to *Ascaris suum* (X54253) and *Baylisascaris procyonis* (JF951366). The pairwise alignment of COX1 sequences of *A. pennula* and *Heterakis gallinarum*, a caecal worm found in chickens and other Galliform species (Lund and Chute, 1973), showed variation in size and nucleotide composition (Fig. 3). These results of *A. pennula* COX1 alignment revealed 84% of identity to *Heterakis gallinarum* (KP308351). A 405bp PCR product was successfully amplified using a set of primers designed from *A. pennula* COX1 gene (Fig. 2B). *A. pennula* COX1 gene sequence was submitted in DDBJ (Accession No: LC228775).

3.2. Phylogenetic tree

Phylogenetic tree was constructed to understand the evolutionary distance of *A. pennula* COX1 region with other species COX1 gene (Fig. 4). The maximum likelihood method was inferred based on COX1 of other parasites such as *Ascaridida* and *Strongylida* groups. All the *A. pennula* collected from our study area were observed in same clade (ML = 100). In this result, *A. pennula* was found closely related to *Ascaridida* than *Strongylida*. Low bootstrap values were observed in *Strongylida*. *Heterakis* family observed with moderate bootstrap value (ML = 83). Finally, the phylogenic results revealed that *Aulonocephalus* sp is closely related to *Ascaridida* (*Toxascaris leonine*) and have conserved the mitochondrial genome. It appears useful to choose COX1 as genetic marker in *Aulonocephalus* sp.

4. Discussion

Morphology and histopathology of *A. pennula* has been previously described in detail over the years (Chandler, 1935; Anderson et al., 2009; Dunham et al., 2017). Additionally, Dunham et al. (2017) suggest that high prevalence of caecal worms indirectly affects the digestive tract of quail, weight, and survivability. Previous studies on caecal worms, such as *Trichostrongylus tenuis*, also suggest that these parasites have the ability to regulate host populations and success rate of breeding in other Galliform species (Potts et al., 1984; Hudson, 1986). Recent reports also confirmed hemorrhagic enteritis in the caecum and heavy production loss of up to 10% in Japanese quail (*Coturnix japonica*) parasitized by the intestinal parasite, *Suburula brumpti* (Nagarajan et al., 2012). Because of a lack of molecular information on other caecal worm species known to inhabit bobwhite quail, such as *Subulara brumpti* (Bruno, 2014), this has potentially limited our understanding of *A. pennula's* pathogenicity.

Molecular phylogeny of *A. pennula* is visualized in a generated phylogenetic tree based on genetic diversity of the mitochondrial COX1 gene region among nematodes. Morphologically, *A. pennula* has been identified in the order Ascaridida, family Subuluridae, and subfamily Allodapinae (Anderson et al., 2009). A BLAST analysis showed *A. pennula*'s 88%–90% genetic identity with Anisakidae and Ascardidae, families also belonging to the order Ascaridida.

The Anisakidae species, in particular, are reportedly pathogenic in many mammals (Anderson, 2000). *Ascaris* spp. is often found in a range of hosts including swine, lagomorphs, cattle, sheep, rodents, and humans. *Ascaris* spp. typically originate in the intestines, where the eggs hatch before the larvae move to the lungs and liver (Anderson, 2000). Many reported symptoms of hosts infected with *Ascaris* spp. include weight loss as well as reduction in fat, vitamin A, and protein absorption (O'Lorcain and Holland, 2000; Blumenthal and Schultz, 1976; Venkatachalam and Patwardhan,

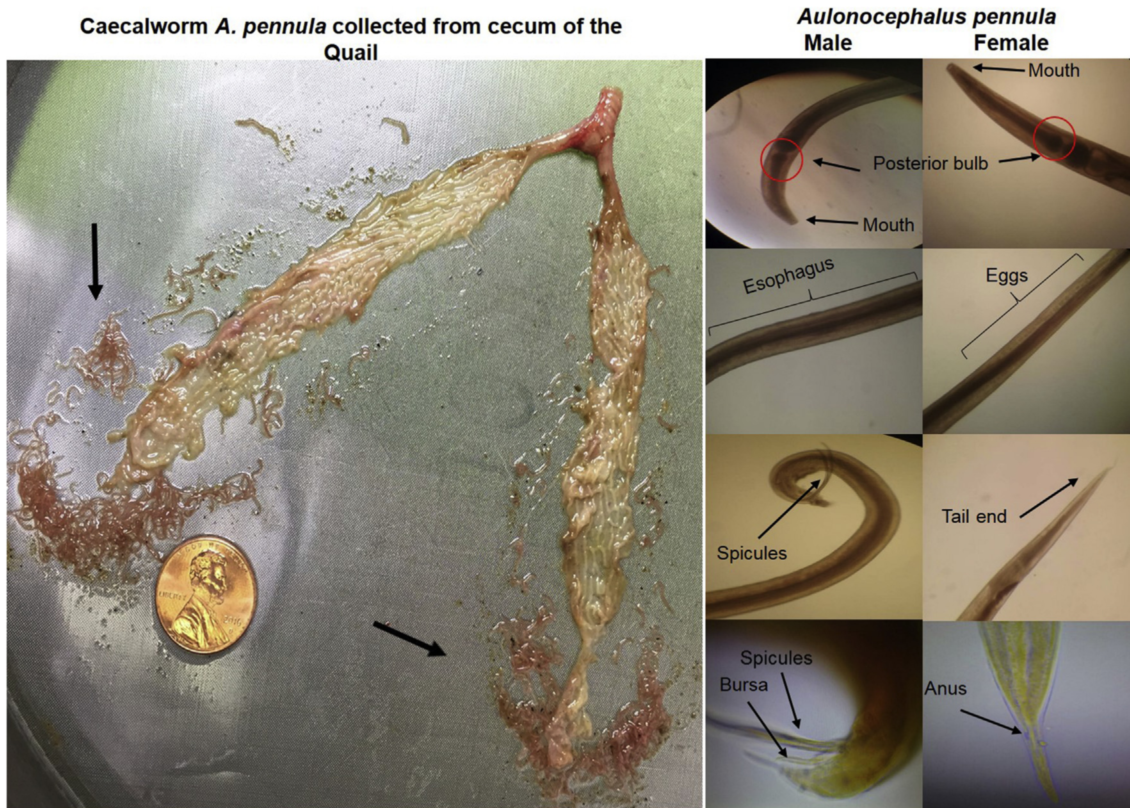

Fig. 1. A. **Caecum of the wild quail** B. **Morphology of male and female caecal worm**. All the parts of male and female caecal worm *Aulonocephalus pennula* are marked in Fig. 1B.

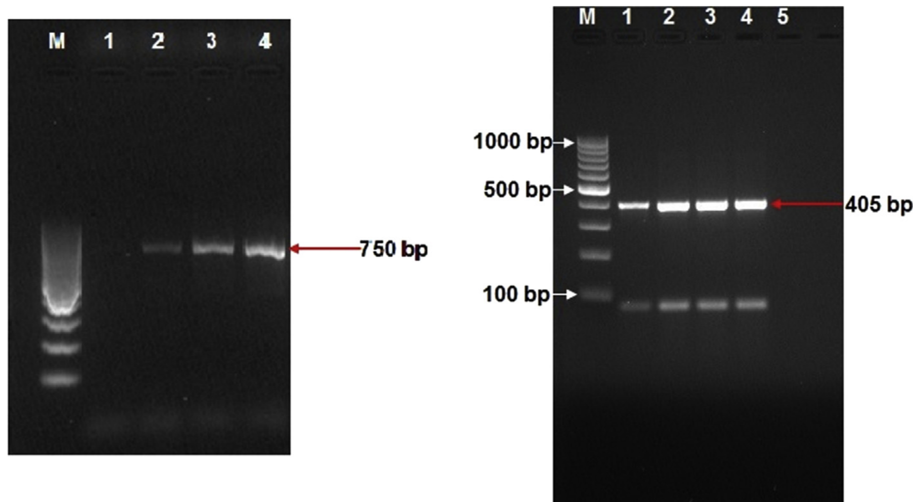

Fig. 2. A. **PCR amplification of COX1 gene using nematode primers**. Lane M: 100 bp DNA ladder (Fermentas); lane 1–4 COX1 gene amplicon (750 bp). B. **PCR amplification of partial COX1 gene using gene specific primers**. Lane M: 100 bp DNA Marker (Fermentas); lane 1–4 partial COX1 amplified products (405bp).

1953). *Ascaris* spp. have also been documented to compete for nutrients in the intestines, decreasing resistance in the host to fight high infections (O'Lorcain and Holland, 2000).

Parascaris spp. is a parasite in the family Anisakidae also closely related to *A. pennula* (88%). It is predominantly found in the small intestine of young horses (Anderson, 2000). The larvae penetrate through the intestinal mucosa before migrating to the liver and lungs in their first stages. During these first stages, the host experiences pneumonia and hepatitis. The larvae then move up the bronchial tree before re-ingestion occurs and life cycles are continued in the small intestine of their host. The final stages of the life cycle induce parascariasis, a disease associated with lethargy, weight loss, and low levels of protein, among many other symptoms. It is also suggested that infections with large numbers of worms can cause abdominal pain due to enteritis and intestinal interference (Cribb et al., 2006; Jabbar et al., 2014).

Additionally, *Toxascaris leonina* (KC902750) has a genetic relatedness of 90%. *T. leonina* is an intestinal parasite commonly infecting canids and felids. Previously thought to be in Anisakidae, genetic investigations performed by Liu et al. (2014) have

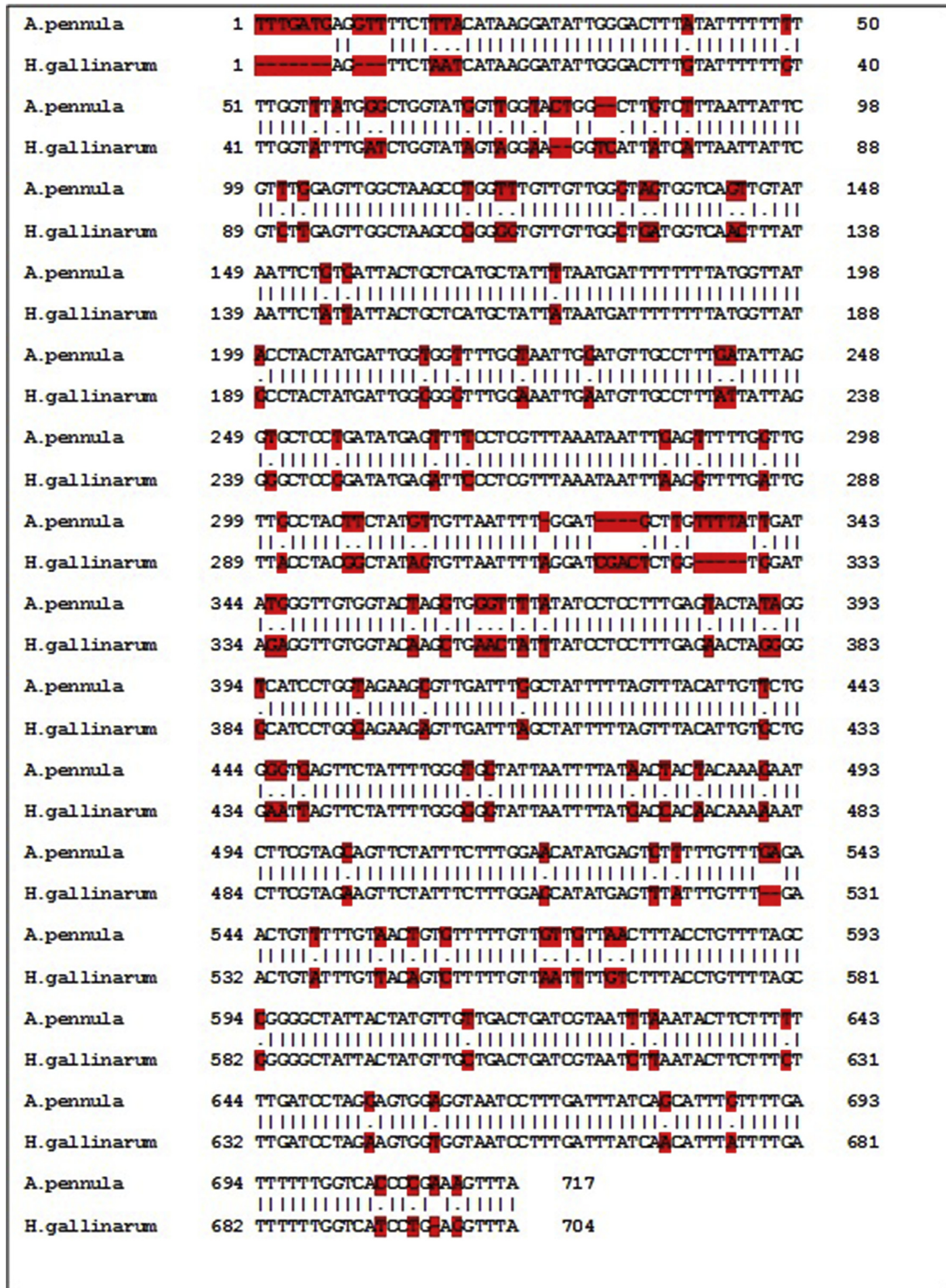

Fig. 3. Pairwise alignment of the sequences of *A. pennula* and *H. gallinarum*. Sequence variations between *A. pennula* and *H. gallinarum* are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reclassified it to Toxocaridae. This species absorbs nutrients from the host's small intestine with recorded symptoms of weight loss, dulling of hair, and a pot-bellied appearance (Liu et al., 2014). They also induce diarrhea and vomiting, with mortalities resulting in younger individuals. The worms often begin in the intestines of their definitive hosts before migrating to the liver, lungs, kidneys, muscles, and heart with a majority situating in the brain (Okulewicz et al., 2012).

Lastly, with an 89% genetic relation to *A. pennula*, *Baylisascaris procyonis* of the Ascarididae family is a highly infectious

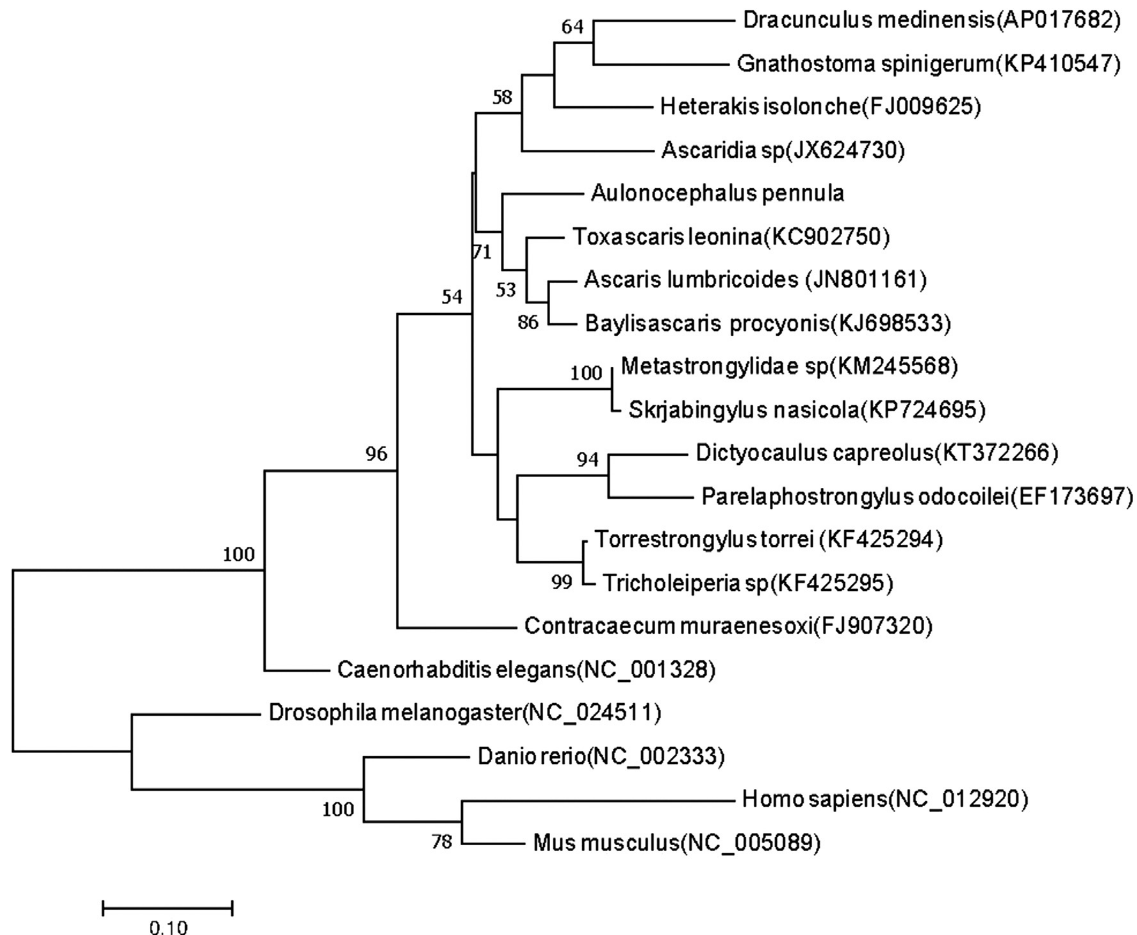

Fig. 4. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred using the ML method based on the General Time Reversible model. The phylogenetic tree illustrates COX1 gene sequences of nematodes related to *A. pennula*. Bootstrap values above 50 are shown in the tree. The tree is drawn to scale, with branch lengths measured in the number of substitutions-per-site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7.

roundworm that uses the North American raccoon (*Procyon lotor*) as its definitive host (Gavin et al., 2005). Contrastingly to other ascarids, this particular parasite exhibits no signs of infection in its definitive host (Roussere et al., 2003) and does not migrate past their gastrointestinal system, remaining in the small intestine and bowel (Sorvillo et al., 2002). However, the roundworm is transmissible to a variety of hosts including commercial rodents, chickens, and bobwhites, as well as natural hosts such as foxes, primates, and humans if any are in contact with raccoon feces. In other hosts, the roundworm can migrate to the eye, organs, and even invades the central nervous system resulting in a severe or fatal neurological disease (Sorvillo et al., 2002; Roussere et al., 2003; Gavin et al., 2005).

As these species only have an 88–90% genetic relatedness to *A. pennula*, these phylogenetic relationships cannot conclusively detail pathogenic effects of *A. pennula* on bobwhites. However, the close relatedness may hint at effects *A. pennula* could potentially have on the bobwhite. Because of *T. leonina*'s 90% genetic relation to *A. pennula*, previous speculations of bobwhite malnutrition due to *A. pennula* (Lehmann, 1984; Dunham et al., 2017) may be correct. Additionally, effects of related species in Anisakidae should not be overlooked, particularly in their role regarding protein deficiency in their hosts as protein in bobwhites is crucial.

Under controlled conditions, protein deficiency has led to reduced growth and development in juvenile bobwhites (Nestler et al., 1942). In many analyses, diets of bobwhite chicks should contain at least 28% of protein for optimal growth (Nestler et al., 1942; Baldini et al., 1950; Andrews et al., 1973). In a study conducted by Lochmiller et al. (1993), chicks of six weeks of age that consumed diets of less than 28% protein experienced significant losses in body mass. Additionally, reduction in protein in this study revealed decreased sizes of bobwhite bursa and spleen. As these two organs play a significant role in antibody development (Giambrone et al., 1977; Toivanen et al., 1987), this may also indicate a reduction in immunoresponses due to protein deficiency (Lochmiller et al., 1993). However, experimental investigation as well as sequencing of nematode species with >90% identity to *A. pennula* is necessary to fully understand *A. pennula*'s effects on bobwhites.

Sequencing of *A. pennula* can also provide valuable molecular methods to understand both the spread of *A. pennula* throughout the Rolling Plains ecoregion but also the potential virulence of this parasite in bobwhite quail. Additionally, sequencing of species belonging to Subuluridae may also provide less genetic variation gaps (>90%) as well as supplementary information to *A. pennula*'s effects on its host. This study concludes that the sequencing of *A. pennula's* COX1 gene region, with further research, can allow complete studies of its lifecycle, epidemiology, and taxonomy that might aid in methods to mitigate parasites in bobwhite quail in the Rolling Plains Ecoregion of Texas.

Conflict of interest statement

The authors have no conflict of interest related to this paper.

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