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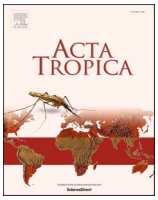

Mosquito-borne parasites in the Great Plains: searching for vectors of nematodes and avian malaria parasites

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ABSTRACT

Vector-borne diseases in the United States have recently increased as a result of the changing nature of vectors, hosts, reservoirs, parasite/pathogens, and the ecological and environmental conditions. While most focus has been on mosquito-borne pathogens affecting humans, little is known regarding parasites of companion animal, livestock and wildlife and their potential mosquito hosts in the United States. This study assessed the prevalence of mature infections of *Dirofilaria immitis* and avian malaria parasites (Haemosporida) within urban mosquito (Diptera, Culicidae) communities in Oklahoma. 2,620 pools consisting of 12,686 mosquitoes from 13 species collected over two summers were tested for the presence of filarioid and haemosporidian DNA. *Dirofilaria immitis*-infected mosquitoes were detected only in *Aedes albopictus* (MIR=0.18-0.22) and *Culex pipiens* complex (MIR=0.12) collected in cities in central and southern Oklahoma. Two other filarioid nematode species with 91-92% similarity with *Onchocerca* spp. and *Mansonella* spp. were also detected. Haemosporidian DNA was detected in 13 mosquito pools (0.9% of pools tested) from seven mosquito species out of 13 species tested. *Plasmodium* DNA in four species (*Cx. coronator, Cx. pipiens* complex, *Cx. tarsalis*, and *Psorophora columbiae*) had high homology with published sequences of avian *Plasmodium* species while DNA in four other species (*Cx. nigripalpus, Ps. columbiae, Anopheles quadrimaculatus*, and *An. punctipennis*) were closely related to *Plasmodium* species from deer. One pool of *Cx. tarsalis* was positive with a 100% sequence identity of *Haemoproteus sacharovi*. This study provides a baseline concerning the diversity of parasites in different mosquito species present in the southern Great Plains. These studies provide important information for understanding the factors of transmission involving the mosquito community, potential hosts, and different mosquito-borne parasites in this important region involved in livestock management and wildlife conservation.

1. Introduction

Mosquitoes transmit a wide variety of pathogens/parasites worldwide. Outbreaks of dengue, chikungunya and Zika virus transmitted by *Aedes* mosquitoes have impacted millions of people in recent years while endemic West Nile Virus (WNV) and Eastern Equine Encephalitis virus occur with occasional regional outbreaks in the United States (Johnson et al., 2015; Rosenberg et al., 2018; Kramer et al., 2019; Lindsey et al., 2019). In addition to affecting humans, mosquitoes also transmit a variety of pathogens/parasites that affect companion animals, livestock, and wildlife (Mullen and Durden, 2018). While most attention has been given to mosquito-borne pathogens/parasites that affect humans, there are gaps in our understanding regarding these zoonotic parasites and their potential mosquito hosts in the United States.

One understudied region in regards to mosquito communities and the parasites they transmit is the southern Great Plains. Currently, there are 65 mosquito species recorded in Oklahoma (Noden et al., 2015; Bradt et al., 2017, 2018). While most species are host-specific and cause no problems for humans or their animals, WNV is mainly transmitted by *Cx. pipiens* complex and *Cx. tarsalis* with *Aedes albopictus* primarily involved in the transmission of canine heartworm (Paras et al., 2014; Noden et al., 2015). Recent studies discovered *Aedes aegypti* through much of the southern urban areas in the state (Bradt et al., 2017; Sanders, 2019) while *Aedes japonicus* and *Culex coronator* are present in other regions (Bradt et al., 2018). Mosquito-borne parasites that cause disease in humans and companion animals may be the most important in impacting health in a given region, but mosquitoes also feed on birds and mammals often infected with other parasites. Knowledge regarding vector competence and transmission dynamics from these relationships can lead to new understandings for how hosts, parasites, and mosquito

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vectors interact within a given environment ('nidus of infection'; Reisen, 2010).

Filarioids are vector-borne parasitic nematodes which dwell in the tissues of different animals worldwide. The most studied mosquito-borne filarioid nematodes are *Brugia* spp. and *Wuchereria* spp. which cause human morbidity in different parts of the world with *Dirofilaria immitis* causing heartworm in canines (Mullen and Durden 2018). While various filarioid nematodes are found in different animals (Rabinowitz et al., 1985; Pung et al., 1996; Netherlands et al., 2020), *Setaria* spp. are mainly found in bovids and cervids and can be transmitted by different species of mosquitoes (Cancrini et al., 1995, 1997; Ubleis et al., 2018). Recently identified in European mosquitoes (Czajka et al., 2012; Kemenesi et al., 2015; Ubleis et al., 2018; Martínez-de la Puente et al., 2019), little is known regarding filarioid nematodes circulating in mosquitoes in the United States. For example, the only information regarding mosquito vectors of filarioid nematodes in Oklahoma is from several heartworm studies in one county (Afolabi et al., 1989; Paras et al., 2014).

Haemosporida are also important protozoan parasites transmitted by blood-feeding arthropods. Studies from other regions of the US have identified avian haemosporida in *Culex, Anopheles, Aedes*, and *Psorophora* mosquitoes (Fryxell et al., 2014; Carlson et al., 2015) while only one study identified cervid haemosporida in *An. punctipennis* (Martinsen et al., 2016). The human malaria parasite, *Plasmodium falciparum*, was finally eradicated from southeastern Oklahoma in the early 1940s (Griffith, 1946), but the main vector, *Anopheles quadrimaculatus*, still occurs throughout the state (Noden et al., 2015; Bradt et al., 2019). *Plasmodium* species have been identified in birds in Oklahoma (Janovy, 1964, 1966), yet, in the south central region, the mosquitoes involved in malaria parasite transmission have not been identified. The aim of the study, then, was to assess the potential presence of filarioid nematodes and haemosporida in mosquitoes collected in urban areas across Oklahoma.

2. Materials and methods

2.1. Study Locations

Adult mosquitoes were collected as part of two surveillance studies in six Oklahoma urban/exurban locations in 2016 (Enid, Midwest City, Ardmore, Idabel, Lawton, and Altus) (May until September (Bradt et al., 2019)) and 2017 (Davis, Ardmore, Marietta, Altus, Mangum, Elk City) (May until August (Sanders, 2019). This study focuses on parasite detection in mosquitoes collected as the mosquito-related components including trapping protocols and species identification were published (Bradt et al., 2019) or submitted for consideration for publication (Sanders, 2019). Collections in 2016 consisted of three trap types (CDC light trap with dry ice, CDC gravid trap with grass-infused water, and BG sentinel traps) in 6 locations/night/city every two weeks (Bradt et al., 2019) while the 2017 collections consisted of two trap types (BioGents Aedes Gravid Traps and BioGents sentinel traps [BioQuip, Rancho Dominguez, CA]) in each city every two weeks (Sanders, 2019). Sites were selected by proximity to urban centers, areas of reported mosquito activity, potential mosquito habitat such as vegetation and container availability, safety for research personnel and limited chance of trap disturbance. Oklahoma State University County Extension agents, city officials and local police aided in site selection and community messaging about the projects. The rationale and procedures behind the mosquito surveys were personally explained to owners at each resident or industry trap sites and verbal authorization was given for mosquito trap placement in the front area of their property. As all sites are located in a hybrid zone, all references to *Culex pipiens* complex denote the *Culex pipiens/quinquefasciatus* complex.

2.2. Parasite testing

Because of the large number of mosquitoes collected in the studies, only species with published record of being vectors of *D. immitis* were processed and tested for the presence of canine heartworm and avian malaria parasites (Ledesma and Harrington, 2011; Fryxell et al., 2014; Paras et al., 2014). *Culex coronator*, while not a known vector for either canine heartworm or avian malaria parasites in the United States, was included due to the relatively large number of individuals collected in 2016. The abdomens of each mosquito were removed prior to testing to maximize detection of L3 *Dirofilaria* and salivary gland *Plasmodium* infections. Pools were created with $\leq$10 specimens by species, collection site and date, and trap type. DNA contamination was minimized by completing the DNA extractions, parasite amplification, and parasite detection via gel electrophoresis in different laboratories using room-dedicated reagents and equipment. Pools were homogenized in a Mini-Beadbeater 16 (Biospec, Bartlesville, OK) for two minutes in sterile 2 ml polypropylene Sarstedt microvials (Biospec, Bartlesville, OK) containing 100 µl of DNAzol (Molecular Research Center, Inc., Cincinnati, OH) and sterilized zirconia/silica beads (Biospec, Bartlesville, OK) (Bradt, 2017; Sanders, 2019). After centrifugation, supernatants were removed and placed into 1.7 ml tubes and frozen at -20°C until analysis.

2.2.1. Filarioid nematode DNA

Mosquito pools were initially screened for *Dirofilaria* DNA using published primers (DIDR-F1/DIDR-R1) which amplify a region of the internal transcribed spacer (ITS) of the ribosomal DNA (Rishniw et al., 2006). Due to low numbers of *D. immitis* detected, a second round of screening of all pools used primers (COIintF/COIintR) that amplify a portion of the filarioid mitochondrial DNA cytochrome oxidase subunit I (COI) gene (Casiraghi et al., 2001). Positive controls consisted of *D. immitis* gDNA generously supplied by Dr. Rebecca Trout-Fryxell (U of Tennessee) and Dr. Michael Reiskind (North Carolina State U) and negative controls were non-template controls (NTC).

2.2.2. Plasmodium DNA

Pools of mosquitoes were initially screened for *Plasmodium* DNA using published primers (343F/496R) which amplify a 154-nucleotide segment of ribosomal RNA coding sequence within the mitochondrial DNA of *Plasmodium* and *Haemoproteus* (Fallon et al., 2003; Fecchio et al., 2013). Due to the low numbers, a nested PCR assay (HaemNF, HaemNR2, HaemF and HaemR2) was used which amplifies a 478-nucleotide segment of mitochondrial cytochrome oxidase subunit-b gene (Waldenström et al., 2004). Positive controls consisted of avian *Plasmodium* gDNA generously supplied by Dr. Ravinder Sehgal (San Francisco State University) and negative controls were non-template controls (NTC).

2.2.3. Sample sequencing

All PCR products were visualized on an ethidium bromide-stained 2% agarose gel in 1x TBE buffer under ultraviolet light. All positive amplicons were bidirectionally sequenced using an Applied Biosystems 3730 DNA analyzer at the Oklahoma State University Core Facility to identify parasite species. We verified each resulting sequence using BioEdit (Ibis Therapeutics, https://bioedit.software.informer.com/7.2/) and aligned bidirectional sequences to create consensus sequences using Clustal Omega (EMBL-EBI, https://www.ebi.ac.uk/Tools/msa/clustalo/). We compared resulting consensus sequences with GenBank submissions using default conditions on NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) where the highest percent sequence identity was used to determine positivity for *D. immitis* or percent identity with closely-related filarioid nematode species and *Plasmodium/Haemoproteus* species and genetic comparisons. Phylogenetic trees were constructed using maximum likelihood method, complete deletion, and discrete gamma distribution with invariant sites for two filarioid and the ITS1 haemosporida gene using Mega 10.1.7 (Tamura et al., 2011).

Outgroups were included for the filarioid nematode and haemosporida analysis. The filarioid nematode spp., *Plasmodium* spp., and *Haemoproteus* spp. DNA sequences were aligned with ITS, COI, and cytochrome b (*cytB*) sequences, respectively, from similar species found in GenBank. Further sequence comparisons with the filarioid COI sequence and the avian malaria parasite *cytoB* sequences were carried out on the Barcode of Life website (BOLD; https://ibol.org/) and the MalAvi website (http://130.235.244.92/Malavi/), respectively. Due to the short sequences (<150 bp) generated by the DIDR primers, sequence homology was reported (Table 1) but sequences were not uploaded to NCBI.

2.3. Statistical analysis

Minimum infection rates (MIR) (number of positive pools/total number of mosquitoes tested) x 100) (Schoener et al., 2017), which are used to estimate the lower limit of infection in pools of mosquitoes due to uneven pool numbers (Fryxell et al., 2014), were calculated for mosquito species in which *D. immitis* and/or avian and cervid malaria were detected by city of collection and pool-positive mosquito species. The results of the two years were analyzed and presented separately due to the differences in study focus and trap types.

3. Results

Of the mosquitoes collected in 2016, 1,790 pools were tested for parasite presence from 9,617 mosquitoes, consisting of *Ae. albopictus* (298 pools; n=1,343), *Ae. canadensis* (3 pools; n=3), *Ae. triseriatus* (121 pools; n=828), *Ae. sollicitans* (137 pools; n=877), *Ae. vexans* (26 pools; n=44), *Anopheles quadrimaculatus* (103 pools; n=247), *An. punctipennis* (27 pools; n=46), *Cx. coronator* (31 pools, n=84), *Cx. nigripalpus* (205 pools; n=1,521), *Cx. pipiens* complex (548 pools, n=3,334), *Cx. salinarius* (12 pools; n=27), *Cx. tarsalis* (113 pools; n=539) and *Psorophora columbiae* (166 pools; n=724) (Tables 1 and 2). Of the mosquitoes collected in 2017, 830 pools were tested for parasite DNA from 4,069 mosquitoes, consisting of *Ae. albopictus* (669 pools; n=3,297) and *Cx. pipiens* complex (161 pools, n=772).

3.1. Filarioid nematode PCR assays

3.1.1. 2016 mosquito pools

Dirofilaria immitis DNA was detected by the COI assay in seven mosquito pools out of 13 species tested, consisting of two mosquito species (*Ae. albopictus* (3/298 pools tested (1.01%)), and *Cx. pipiens* complex (4/548 pools tested (0.73%)) (Table 1). The positive samples were from diverse sites in three cities across southern Oklahoma (Ardmore, Idabel, and Lawton) (Table 1). The MIR for Idabel was the highest (0.18) followed by Ardmore (0.10) and Lawton (0.05). Positive sequences were confirmed using NCBI BLAST with a 99-100% sequence identity with known sequences of *D. immitis* (AJ537512) (Supplemental Table 1). *D. immitis*-positive mosquito pools were collected in diverse settings in gravid (n=4) and CDC light (n=3) traps in June (n=2), July (n=3) and August (n=2) 2016.

The 2016 COI mosquito pool assays also detected DNA from other filarioid nematode species in six pools (6/328 pools tested (1.8%) of *Ae. albopictus* from two cities (Midwest City and Ardmore) (Supplemental Table 1). Positive amplicons were confirmed using NCBI Blast with 91-92% sequence identity with known sequences of 2 species of filarioid nematodes not present in the US (AM749270, AF228559) (Figs. 1 and 2, Supplemental Table 1). Both of the filarioid nematode species were identified in *Ae. albopictus* pools from one central park in Ardmore using all three trap types across a three-month period. Five of the filarioid nematode samples had 92% homology with the ITS gene and aligned with *Mansonia ozzardi* (Supplemental table 1) in the phylogenetic tree (Figure 1). The one sample with 91% homology with the COI gene of *Ochcocerca skrjabini* (Supplemental table 1) aligned with sequences of *Foleyella furcata*, a filarioid nematode species of chameleons (Figure 2). All six sequences were deposited in GenBank (accession numbers MW020300 - MW020304 (ITS1) and MW021557 (COI).

3.1.2. 2017 mosquito pools

Dirofilaria immitis DNA was detected by the COI assay in six *Ae. albopictus* pools (6/669 pools tested; (0.90%)) from three cities in Central Oklahoma (Table 1). No *D. immitis* DNA was identified in *Cx. pipiens*

Table 1
Canine heartworm percentage of positive pools and minimum infection rate (MIR) for mosquitoes collected in 10 Oklahoma cities between May and September, 2016/17.

Year	City / Species	Total no. mosquitoes tested	Pool size (range)	No. pools screened	Canine heartworm			
					No. positive pools	% positive pools	MIR (Lower/ Upper)	County 5-year canine prevalence*
2016	Enid	581	1-10	133	0	0.00	0.00	0.88-3.52%
	Midwest City	893	1-10	200	0	0.00	0.00	1.69-2.12%
	Ardmore	1962	1-10	330	2	0.60	0.10 (0.0-0.24)	No data
	Idabel	1636	1-10	298	4	1.28	0.18 (0.0-0.39)	No data
	Lawton	1929	1-10	342	1	0.29	0.05 (0.0-0.15)	0.91-2.15%
	Altus	837	1-10	188	0	0.00	0.00	2.16-3.78%
	Total^	**7838**		**1491**	**7**	**0.47**		
	Cx. pipiens	3334	1-10	548	4	0.73	0.12 (0.0-0.24)	
	Ae. albopictus	1343	1-10	298	3	1.00	0.22 (0.0-0.48)	
	Total	**4677**		**846**	**7**	**0.83**		
2017	Davis	755	1-10	119	4	3.36	0.53 (0.01-1.05)	4.94-6.31%
	Ardmore	535	1-10	103	1	0.97	0.19 (0.0-0.55)	No data
	Marietta	347	1-10	95	1	1.05	0.29 (0.0-0.85)	No data
	Elk City	385	1-10	92	0	0.00	0.00	No data
	Mangum	844	1-10	158	0	0.00	0.00	No data
	Altus	431	1-10	102	0	0.00	0.00	2.16-3.78%
	Total#	**3297**		**669**	**6**	**0.90**		
	Ae. albopictus	3297	1-10	669	6	0.90	0.18 (0.04-0.33)	

*CAPCVET reports 2015-2019, prevalence rates in years when over 100 dogs tested.
^Does not include pool-negative *Ae. canadensis. Ae. sollicitans, Ae. triseriatus, Ae. vexans, An. quadrimaculatus, An. punctipennis, Cx. coronator, Cx. nigripalpus, Cx. salinarius, Cx. tarsalis*, and *Ps. columbiae*
Does not include pools-negative *Cx. pipiens*.

Table 2
Haemosporida percentage of positive pools and minimum infection rate (MIR) for mosquitoes collected in six Oklahoma cities between May and August 2016. Mosquitoes collected in 2017 were not included due to lack of positive pools.

City	Total no. mosquitoes	Pool size (range)	No. pools screened	No. positive pools	% positive pools	MIR (Lower/Upper)
Enid	581	1-10	133	4	3.01	0.69 (0.02-1.36)
Midwest City	893	1-10	200	1	0.50	0.11 (0.0-0.33)
Ardmore	1962	1-10	330	2	0.61	0.10 (0.0-0.24)
Idabel	1636	1-10	298	2	1.01	0.18 (0.0-0.39)
Lawton	1929	1-10	342	4	1.17	0.21 (0.0-0.41)
Altus	837	1-10	188	0	0.00	0.00 (0.0)
Total*	**7838**		**1491**	**13**	**0.87**	
Species						
Cx. pipiens	3334	1-10	548	3	0.55	0.09 (0.0-0.24)
Cx. tarsalis	539	1-10	113	3	2.65	0.56 (0.0-1.18)
Cx. coronator	84	1-10	31	1	3.22	1.19 (0.0-3.50)
Cx. nigripalpus	1521	1-10	205	2	0.98	0.13 (0.0-0.31)
An. quadrimaculatus	247	1-10	103	1	0.97	0.40 (0.0-1.20)
An. punctipennis	46	1-10	27	1	3.70	2.17 (0.0-6.39)
Ps. columbiae	724	1-10	166	2	1.20	0.28 (0.0-0.66)
Ae. albopictus	1343	1-10	298	0	0.00	0.00 (0.0)
Total	**7838**		**1491**	**13**	**0.87**	

* Does not include pool-negative *Ae. triseriatus, Ae. sollicitans, Cx. salinarius, Ae. vexans*, and *Ae. canadensis*.

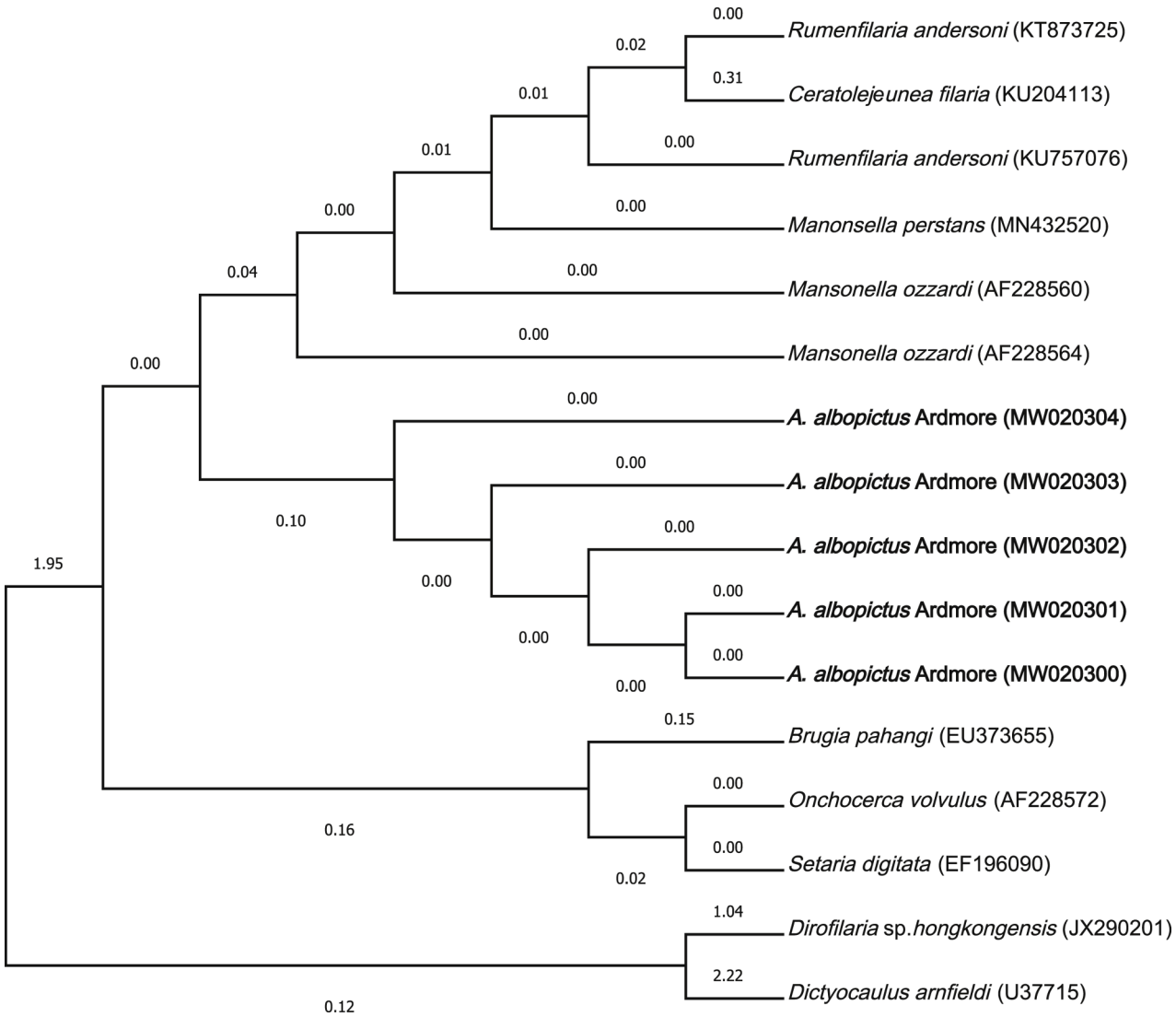

Fig. 1. Phylogenetic relationships between the unknown filaroid nematode parasite in *Aedes albopictus* and other nematodes based on sequence variation of the internal transcribed spacer (ITS) of the ribosomal DNA. The tree was constructed by aligning 310 bp using Maximum likelihood method. Numbers near branches indicate branch length values. Additional NCBI GenBank sequences were used for comparison and *Dictyocaulus arnfieldi* was used as an outgroup.

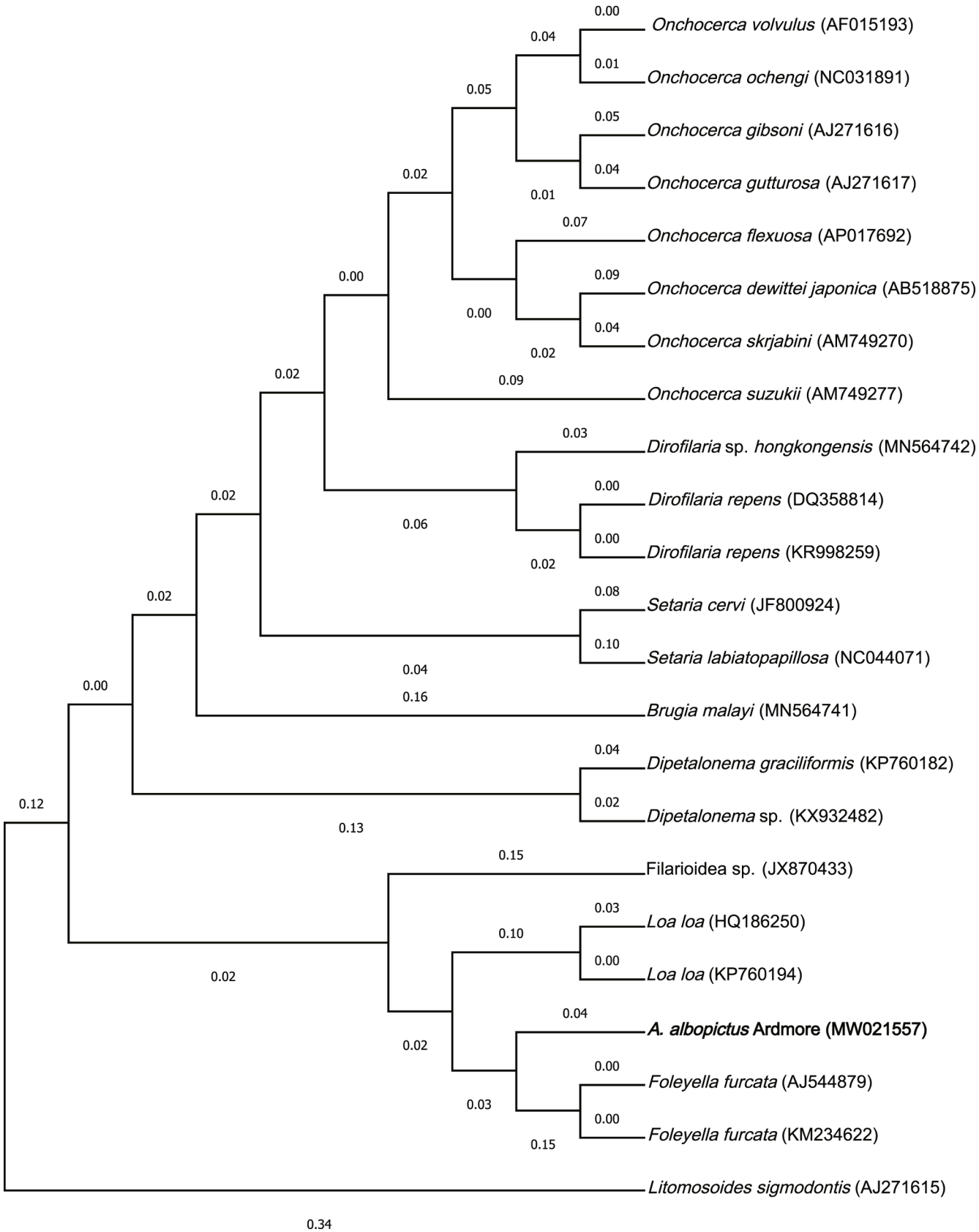

Fig. 2. Phylogenetic relationships between the unknown filaroid nematode parasite in *Aedes albopictus* and other nematodes based on sequence variation of the mitochondrial cytochrome c oxidase subunit I gene. The tree was constructed by aligning 586 bp using Maximum likelihood method. Numbers near branches indicate branch length values. Additional NCBI GenBank sequences were used for comparison and *Litomosoides sigmodontis* was used as an outgroup.

complex pools (0/165 pools tested; (0%)). The MIR for Davis was the highest (0.53) followed by Marietta (0.29) and Ardmore (0.19). No *D. immitis* infected mosquitoes (n=1,660) were collected in western Oklahoma (Table 1). Positive sequences were confirmed using NCBI BLAST with a 99-100% sequence identity with known sequences of *D. immitis* (AJ537512) (Supplemental Table 1). The majority (n=4) of the *D. immitis*-infected *Ae. albopictus* were collected in July in urban residential communities in areas with low or medium clutter and half of the sites had visible dogs present (Supplemental Table 1).

3.2. Haemosporida assays

Haemosporidian DNA was detected in 13 mosquito pools (0.90% of pools tested) from seven mosquito species out of 13 species tested (*Cx. pipiens* complex, *Cx. tarsalis, Cx. coronator, Cx. nigripalpus, An. quadrimaculatus, An. punctipennis*, and *Ps. columbiae*) across 5 of the 6 cities sampled in 2016 (Table 2). None of the *Ae. albopictus* or *Cx. pipiens* complex pools collected in 2017 were positive for haemosporidian DNA. The highest MIR was in mosquitoes tested from Enid (0.69) followed by Lawton (0.21) and Idabel (0.18). Positive amplicons were confirmed using NCBI Blast with a 95-100% sequence identity with known sequences of *Plasmodium* spp. (Supplemental Table 2). *Plasmodium* DNA in four species (*Cx. coronator, Cx. pipiens* complex, *Cx. tarsalis*, and *Ps. columbiae*) had high homology with published sequences of avian *Plasmodium* species while four species (*Cx. nigripalpus, Ps. columbiae, An. quadrimaculatus*, and *An. punctipennis*) were closely related to *Plasmodium* species from deer (Figure 3, Supplemental Table 2). One pool of *Cx. tarsalis* from a Gravid trap from a park in Enid, OK was positive with a 100% sequence identity with a known sequence of *Haemoproteus sacharovi* (KY653811.1) (Figure 3, Supplemental Table 2). All nine haemosporidian cytB sequences were deposited in GenBank (accession numbers MW019448 - MW019456).

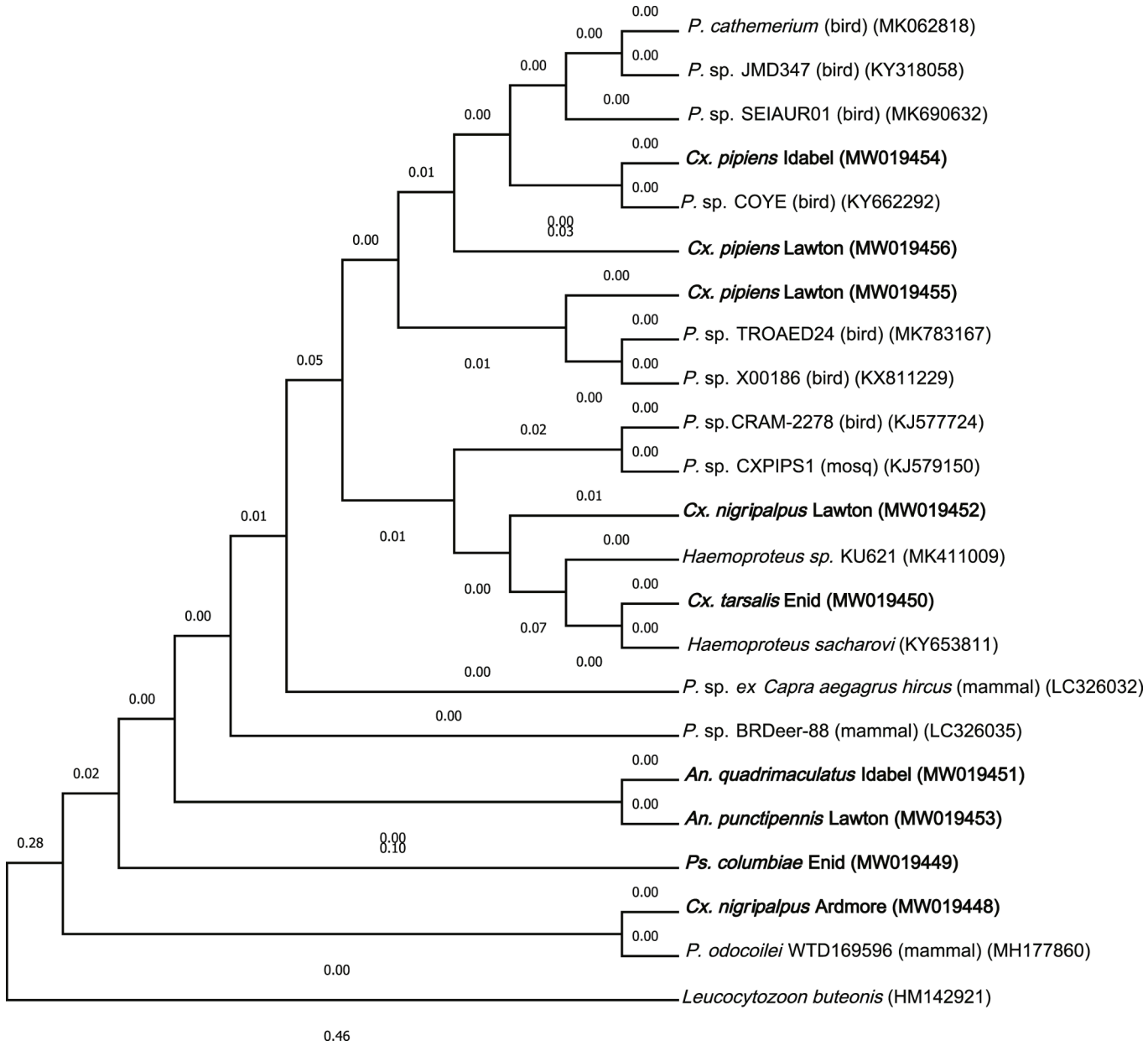

Fig. 3. Phylogenetic relationships between the unknown *Plasmodium* spp. and *Haemoproteus* spp. and other haemosporida species based on sequence variation of the mitochondrial cytochrome oxidase subunit-b gene. The tree was constructed by aligning 478 bp using Maximum likelihood method. Numbers near branches indicate branch length values. Additional NCBI GenBank sequences were used for comparison (in addition to host animal, if recorded in the sequence description) and *Leucocytozoon buteonis* was used as an outgroup.

4. Discussion

The data from this study indicates that different filarioid nematode and haemosporida species are circulating in diverse mosquito species in urban areas across the southern Great Plains. *Dirofilaria immitis*, the causative agent of canine heartworm, was detected in two mosquito species in 50% of urban areas sampled while two unidentified filarioid nematodes were detected in *Aedes albopictus*. Several species of *Plasmodium* spp. and a *Haemoproteus* spp. were detected in 7 different mosquito species in 50% of the urban areas sampled. Most samples were related to bird malaria species but several aligned with known cervid malaria species.

4.1. Heartworm

Dirofilaria immitis is a concern for companion canines and felines throughout the southern United States, reaching into the Great Plains region (Capcvet, 2020). Yet, within the southern Great Plains, little is known regarding the mosquito vectors outside of a few studies conducted in one town in central Oklahoma (Afolabi et al., 1989; Paras et al., 2014). While we know that there are high prevalence rates of heartworm in central and eastern Oklahoma (Capcvet, 2020), no study has surveyed mosquito species throughout the state for the presence of *D. immitis* DNA. The current study detected *D. immitis* in mosquitoes in half of the urban areas sampled with the majority of *D. immitis*-infected mosquito pools (61%) collected in three central Oklahoma cities with the highest MIRs (Davis, Marietta, and Ardmore). These MIRs correlate with annual county canine prevalence rates where the cities reside (between 4.94-7.41%) (Capcvet, 2020), which may have enhanced the likelihood of detection. While mosquito infections may have corresponded with local prevalence rates, only two of the 13 mosquito species tested were positive for *D. immitis* – *Aedes albopictus* and *Culex pipiens* complex. *Aedes albopictus* has been identified as the principle vector species for canine heartworm in Oklahoma (Paras et al. 2014) and other regions of the United States (Ledesma and Harrington, 2011). However, the detection of *D. immitis* DNA in *Culex pipiens* was not anticipated. It is important to note that Oklahoma is in the *Culex pipiens/quinquefaciatus* hybrid zone in the United States (Rochlin et al., 2019) and the two species were pooled for testing as it was not possible to differentiate between them at the time of identification. *Culex pipiens* prefers to feed on birds while *Cx. quinquefasciatus* prefers to feed on mammals, including dogs and cats (Hamer et al., 2008; Farajollahi et al., 2011; Molaei et al., 2012). This difference in feeding preference may have played a role in the results (Savage and Kothera, 2012). While preliminary, these results demonstrate the continued need for attention to this important complex in regards to vector-parasite relationships (Bartholomay et al., 2010; Huang et al., 2013; Fryxell et al., 2014).

The low diversity of mosquito species implicated in *D. immitis* transmission was surprising as *D. immitis* DNA was detected in 15 species of mosquitoes in an earlier Oklahoma-based study (Paras et al., 2014). There are potential reasons for these differences. First, the earlier study (Paras et al., 2014) occurred in diverse habitats in one city while most trapping in the current study occurred in urbanized habitats in 5 cities where 11 (6 residential and 5 industrial) of the 13 *D. immitis*-infected pools were collected. The potential urban-related decrease in mosquito diversity in the current study (Bradt et al., 2019), also reported elsewhere (Spence Beaulieu et al., 2020), may have contributed to the lower prevalence of *D. immitis*. Secondly, we extracted DNA from thorax and head instead of the whole body (Spence Beaulieu et al., 2020) which may have limited detection for only mature infections instead of developing infections. The low diversity of *D. immitis* infected mosquito species collected in only half of the cities sampled indicates that more work is needed in the region to understand the ecology of this parasite of companion animals.

4.2. Other filarioid nematodes

This was the first study to detect other filarioid nematodes in mosquitoes collected in the southern Great Plains of the United States. Two different filarioid nematode species were detected in two of the ten urban areas surveyed (Midwest City and Ardmore). Five (83%) of the samples were 92% similar to *Mansonella ozzardi* which is a blackfly-transmitted filarioid nematode species in Central and South America (Mullens and Durden, 2018). While *M. ozzardi* is not a parasite found in the United States, potential species include filarioid nematode species commonly found in raccoons in the United States (*Dirofilaria tenuis* and *Brugia beaveri*). The most probable closely related species to *M. ozzardi*, however, is *Mansonella llewellyni* for which comparative sequences were not available in GenBank or Barcode of Life (Dr. Jefferson Vaughan (U North Dakota) and Dr. Matthew Bolek (Oklahoma State Un.), personal communication) (Ash and Little, 1964; Herman and Price, 1965; Rabinowitz et al., 1985; Isaza and Courtney, 1988; Pung et al., 1996). To date, no mosquito species tested, including *Cx. pipiens* complex, are known to be competent for *M. llewillyni*, but *A. albopictus* has never been tested (Herman and Price, 1965). The influence of such filarioid nematodes in human and animal health in the United States are unknown; however, they occasionally infect humans as occurred in an Oklahoma-based immunodeficient infant infected with mosquito-borne *Brugia beaveri* (Simmons et al., 1984).

The other filarioid nematode species detected was 91% similar to *Onchocerca skrjabini*, a European cervid filarioid nematode, but more closely related to *Folyella furcata*, a filarioid nematode found in chameleons, when phylogenetically compared with known sequences. Similar unidentified filarioid nematode have also been reported from *Culex* mosquitoes in Europe in which the consensus sequences did not cluster with any particular genera of known filarioid nematode species in the tree analysis but the NCBI Blast search revealed an 86% homology with another *Onchocerca* spp. (Czajka et al., 2012; Kemensei et al., 2015). In North America, *Onchocerca cervipedis* infects North American cervids in addition to another uncharacterized *Onchocerca* species that infects white-tailed deer in New York (Verocai et al., 2012; McFrederick et al., 2013). *Onchocerca gutturosa* and *O. lienalis* affect cattle in the United States while *O. lupi* has been reported in dogs (Ferenc et al., 1986; Hassan et al., 2015). No COI sequences were homologous to any of these species when compared in GenBank or Barcode of Life. Although of interest, it is unlikely that mature filarioid nematodes were present in the mosquitoes as *Onchocerca* species are normally transmitted black flies and biting midges (Mullens and Durden, 2018). Studies evaluating the vector competence in *Ae. aegypti* for *Onchocerca* species reported that development occurs until the L3 stage in the thorax but die before transmission (Zielke, 1977; Zielke et al., 1977). Due to the high sensitivity of the PCR assay, however, DNA from some undeveloped form may have been detected when mosquitoes were processed for extraction. In general, more work is needed to explore the relationships of these filarioid nematodes with potential mosquito hosts and other protocols should be evaluated as the two protocols used may not have detected all species present (Hamer et al., 2013).

4.3. Haemosporida

This was also the first study to evaluate mosquito species involved in haemosporida transmission in Oklahoma, the first to report an avian *Plasmodium* species in *Cx. coronator*, an invasive mosquito species in the United States (Noden et al., 2015, Bradt et al., 2018), and the first to identify a haemosporida species associated with white-tailed deer in Great Plains-collected *Anopheles* mosquitoes (Martinsen et al., 2016). *Plasmodium* species have been identified in regional birds, including the red-shouldered hawk, mourning dove, eastern meadowlark and red-winged blackbird (Janovy, 1964) and *Haemoproteus* spp. and *Leucocytozoon* spp. were also detected in birds in the Oklahoma City zoo (Halpern and Bennett, 1983) as well as small birds in western Oklahoma

(Lewis et al., 1975). However, mosquitoes in the region have never been tested for haemosporida parasites.

In the current study, Haemosporida-positive pools were only detected in green, urban and suburban park areas where wildlife and birds would provide mosquito blood meals (Bradt et al., 2019). The limited numbers of *Culex* sp. collected in 2017 may have been a result of changing the types of traps used (Carlson et al., 2015). In 2016, the CDC gravid traps, mainly used to collect *Culex species*, BG Sentinel traps and CDC light traps were replaced in 2017 by *Aedes*-focused GAT traps and BG Sentinel traps. In 2017, species diversity and the numbers of *Culex* decreased dramatically with the change of trap types with a predominate collection of *Aedes albopictus* and *Aedes aegypti* (Jordan, 2019). The change of those two factors most likely contributed to the lack of infected pools detected in 2017 (Carlson et al. 2015). The addition of CO_2 with BG Sentinel traps, as occurs in Europe (Schoener et al., 2017; Übleis et al., 2018; Martínez-de la Puente et al., 2019), may have enhanced the collection of *Culex* spp. in the study.

Based on the NCBI Blast results, two groups of haemosporidian parasites appear to be present in Oklahoma – species identified in birds and others identified in white-tailed deer. DNA associated with avian malaria parasite species were found in mosquito species most likely to feed on birds (*Cx. pipiens, Cx. tarsalis*, and *Cx. coronator*) while DNA associated with ungulate malaria species were identified in mosquito species most likely to feed on mammals (*Ps. columbiae, An. quadrimaculatus, An. punctipennis*) (Edman, 1971). It is likely that *Anopheles* species are involved with *Plasmodium* transmission to deer as *An. punctipennis* has been linked with *P. odocoilei* (Garnham and Kuttler, 1980; Martinsen et al., 2016) but the role of *Ps. columbiae* is unclear as it has not been identified as a competent vector for *Plasmodium* species. Also, *Cx. pipiens* has been identified as competent vector for *P. cathemerium* (Huff, 1965). *Haemoproteus* spp. are commonly found in mosquitoes that have fed on birds, but these species are not transmitted by mosquitoes (Carlson et al., 2015; Gutiérrez-López et al., 2016). Instead, they are transmitted by hippoboscids (Valkiūnas et al., 2013). While interesting to consider, the vector competence of most haemosporidian species has not been experimentally confirmed. PCR amplifies DNA from any form of development within the mosquito but without experimental infection and transmission, it is possible over interpret the results. Experimental haemosporidian infections in several mosquito species have demonstrated abortive sporogonic development does occur, producing no infectious parasites in the salivary glands (Valkiūnas et al., 2013; Carlson et al., 2015; Gutiérrez-López et al., 2016; Bernotienė et al., 2019).

In conclusion, this study identified novel filarioid nematode and haemosporidian DNA in diverse mosquito species across 10 cities in the southern Great Plains. While these parasite-vector relationships may not be considered as important as those related to public health, there is a need to identify what other parasites are inhabiting the mosquito species within particular regions. As recent studies in Europe have reported, filarioid nematodes, known (*Setaria* sp) or unknown, are circulating in mosquitoes and wildlife in varied environments (Czajka et al., 2012; Kemenesi et al., 2015; Übleis et al., 2018; Martínez-de la Puente et al., 2019; Netherlands et al., 2020). Some may have an impact on the health of livestock or cervids while others may provide a means for an arbovirus to escape a midgut barrier and develop to infectious levels in the salivary glands (Vaughan et al., 2007; Vaughan and Turell, 2017). Others are focused on the haemosporidian parasites in wildlife, but little is known about the relationships between malaria parasites in mammals (eg. White-tailed deer, cattle) and their mosquito vectors (Martinsen et al., 2016). By focusing on parasite-mosquito relationships across a wide geographic area, we gain a better understanding of the relationships which contribute to the most risk to humans and animals and may develop novel ways by which to mitigate the risk of transmission.

CRediT authorship contribution statement

Bruce H. Noden: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **David L. Bradt:** Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Jordan D. Sanders:** Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105735.

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