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Studies of Avian Nasal Mites (Acari: Rhinonyssidae and Ereynetidae) and Their Interaction with the Brood Parasite Brown-headed Cowbird (*Molothrus ater*: Icteridae) and Phylogenetic Inferences of the Genus *Ptilonyssus* (Rhinonyssidae) on Different Passerine Hosts Associated to Three States in the US

A dissertation submitted in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy in Entomology

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ABSTRACT

Nasal mites are endoparasites that spend their entire life cycle inside the nasal cavities and respiratory passages of birds. The Brown-Headed cowbird (*Molothrus ater*) (BHCO) is an icterid bird that uses brood parasitism as a reproductive strategy in which it lays an egg in the nest of a different bird species and allows the host to raise its young. Interestingly, nasal mites reported from cowbirds represent the same species found infesting other icterids and others common host groups. In the first study, I examined how diversity and host prevalence might change in a large sample size of BHCO associated with three of the four migratory flyways of North America. I could identify 11 different species of nasal mites and there was an overall prevalence of 89% of infestation from 856 birds. Moreover, prevalence was not different by location or by bird sex, suggesting that patterns of nasal mite infestation in BHCO occur similarly in all locations.

The second study included questions of genetic differentiation in BHCO hosts that are isolated by geographical locations. For this, a phylogenetic analysis was conducted for the two most common nasal mites (*Ptilonyssus icteridius and P. agelaii*) that infest BHCO using the mitochondrial 16S, ITS and COI. We showed that location did not affect the genetic composition of the nasal mites, which suggests a constant movement and mixing of BHCO by which they could be acquiring their nasal mites when socializing in large flocks either during winter or breeding season.

The third study focused on a phylogenetic analysis of the nasal mites from the genus *Ptilonyssus* (Rhinonyssidae). The analysis included different nasal mite species infesting passerine hosts in the US. Sequences from the 16S gene were amplified and relationships showed species of mites with different levels of host specificity with their host and also some variation in species with similar morphology. In conclusion, more studies on these parasites

infesting bird populations are required to understand the biology, taxonomy, and relationships of these nasal mites.

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Special Dedication

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CHAPTER 1. GENERAL INTRODUCTION

1.1 Host-symbiont interactions

Organisms develop unique characteristics that are shaped by their relationships with their environment and other species in that environment. Often, different organisms interact frequently in close association with other species they coexist with, and the interactions may have an important role in the ecology and survival of each other (Mages and Dill 2010). Such a relationship is termed symbiosis. Symbiosis is simply living together, and different types of symbiosis exists–mutualistic, by which both organisms benefit; neutral, by which neither benefits; or negative associations, for which one organism benefits to the detriment of the other– and are found in many kinds of organisms.

Host-parasite interaction is one of the types of symbiosis well studied in many organisms. Many examples of how coevolution of two organisms coexisting have been used as models to understand underlying mechanisms. Such is the example of Pocket gophers (Rodentia: Geomyidae) and their chewing lice (Phthiraptera: Trichodectidae) in which, by isolation in close relationship, both organisms have become well specialized and specific such that these lice only can be found on the gophers and have not been found on any other host (Demastes & Hafner 1993). The system of coevolutionary history among pocket gophers and their chewing lice presents what is known as a parallel cladogenesis that could be described by the Fahrenholz's Rule (the natural classification of some groups of parasites corresponds with that of their hosts) (Gullan & Cranston 2014).

A long-term association between two organisms that present different natural histories could lead to cospeciation, which occurs when host and parasite speciate simultaneously over time. Strict cospeciation results in the phylogenies of host and parasite mirroring each other.

Cospeciation sometimes could happen due to a response to geographical speciation (vicariance) or dispersal (Damastes et al. 2012).

In animals, groups such as birds and their symbionts have been well studied (Crompton 1997, Proctor and Owens 2000). Birds offer suitable environmental conditions to many kinds of symbionts due to adaptation to the power of flight that allows the birds to disperse and reach multiple environments. Birds as hosts provide a body covered in feathers, in which different parts of the bird plumage can serve as a microhabitat for different symbiote species (Gaud and Atyeo 1996). Other parts of the bird's body, such as skin, quills, subcutaneous tissues, down, and respiratory tracts, can also be microhabitats for symbionts (Proctor and Owens 2000). Many symbionts use birds as a host, including endo- and ectoparasites, phoretics, and commensals. Such examples include lice, flies, fleas, ticks, and mites (Proctor 2003), with mites being the most diverse symbionts associated with birds. Approximately 3,000 species of mites, distributed across 40 families, are known as symbionts of birds (Knee and Proctor 2006; Knee 2008).

1.2 Acari in general

Mites (Subclass Acari) are a large group in the class Arachnida. Acari are considered to be the most diverse and abundant group of arachnids and may rival the insects in , diversity, number of environments colonized, and their ubiquity. Approximately 55,000 species have been described but the estimated number is much greater (Walter and Proctor 1999; Skoracka et al. 2015).

Despite their potential diversity, the Acari is a group that has not been studied as greatly as insects. In part, this is a reason that the classification of mites is continually changing. The development of new molecular techniques is helping to classify the phylogenetically related taxa.

Currently, the subclass Acari is divided into two superorders, the Acariformes, also known as Actinotrichida, referring to the sensory setae characteristic of the group (optical setae with actinochitin and trichobotia setae in the legs) and the Parasitiformes (Anactinotrichida) (Walter & Proctor 1999).

The first records of Acari occurred as early as 420 million years before present (MYBP) (Walter & Proctor 1999), and extant families of Acari are represented in the fossil record as early as the middle Devonian (412-354 MYBP) (Walter & Proctor 1999). The oldest mite fossil, *Protacarus crani,* was found in the lower Devonian Chert of Scotland. This species, described by Hirst in 1923 (Evans 1992), is the first record of the suborder Prostigmata (Acariformes). The first appearance of Parasitiformes in the fossil record was dated from the upper Eocene (55-28 MYBP) (Witalinski 2000, Walter & Proctor 1999).

The superorder Acariformes is the most diverse and the most ancestral group of mites, with over 40,000 species described (Walter & Proctor 1999). Species of the Acariformes occupy numerous niches, and can be predators, parasites, ectosymbionts, or phytophages. Two orders are considered from the Acariformes – the Sarcoptiformes, with suborders Endeostigmata and Oribatida (currently includes Astigmata); and the Trombidiformes, with suborders Anystina (currently includes Parasitengona) and Eupodina (Table 1). Acariform mites apart of having specialized setae, they also differ from mites in the Parasitiformes in having the podosoma divided by a suture called the sejugal furrow, which separates the podosomal from legs I-II and III-IV (Evans 1992).

The superorder Parasitiformes contains the ticks and their relatives. This group is represented by the orders, Opilioacarida, Holothyrida, Ixodida, and Mesostigmata. Of these orders, Ixodida are the ticks. Ixodid mites (ticks) are the most well-known due to their size and blood-feeding

habit, and they are recognized as disease vectors. Mesostigmata is the most diverse order of Parasitiformes, with currently 100 families distributed in 12 different suborders, and approximately 8,000 species described (Beaulieu et al. 2011). Mesostigmata mites have diverse lifestyles, including as free-living predators, free-living in the soil litter, rotting wood, dung, carrion, nests or house dust, and also as mites associated with many hosts, such as commensals, predators, mutualists and even phoretics (Klompen et al. 2007).

Mites can be ubiquitous and have been reported from all sorts of environments. Mites have colonized virtually all terrestrial environments. They are important organisms, serving as decomposers, free living predators, and scavengers (Proctor and Owen 1999). In forests, mites such as the oribatid mites, are one of the most important decomposers and are the most abundant group in organic matter (Evans 1992). Acari also occupy aquatic niches, such as freshwater lakes, rivers and springs, and can be important in their role as a bioindicators of freshwater quality (Goldschmidt 2016). Furthermore, they have been able to successfully colonize ocean habitats, and they even have been collected from thermal waters that occur in volcanic regions (Baker 1952).

The morphology of Acari presents the same characteristics as other arachnids. Two body segments (prosoma and opisthosoma), four pair of legs, and a pair of chelicerae as mouth parts. However, in the Acari, the two body segments (prosoma and opisthosoma) are differentiated as gnathosoma or capitulum (mouth parts) and idiosoma (the rest of the body), which is further divided in propodosoma and opisthosoma. A flexible cuticle divides the gnathosoma and idiosoma, showing loss of segmentation (no sutures, no tergites or sternites).

Mite diversification has led to an expansion of adaptations and feeding behaviors. Adaptations to feeding have also allowed mites to utilize vastly different sources of food. Some

mites feed on decaying matter and plant tissue, whereas-other mites are known to be saprophagous and fungivorous. Water mites tend to be predators, scavenger or parasites of other aquatic organisms. Some species of Astigmata and Prostigmata are phytophagous and are considered economically important pests, such as the spider mite *Tetranychus urticae* that feeds on many different kinds of plants (Van Den Boom et al. 2004). Spider mites are fed upon by predatory mites, such as *Phytoseiulus persimilis,* which is a member of the order Mesostigmata, and the predators are used as biological control agents to control the pest mites.

1.3 Paths to Parasitism

The paths to parasitism vary among types of mites. Among plant-feeding mites, the evolution of parasitism might have developed from soil-dwelling mites, such as oribatid mites that began to oviposit in plants. Larvae hatch on plants and begin feeding and adapt their life cycle and overwintering strategy (e.g., gall mites), evolving chelicerae needed for the transition to phytophagous feeding (Krantz & Lindquist 1979).

Parasitism in animal-feeding mites had several possible pathways. One potential evolutionary path to parasitism of vertebrates is the adaptation of some species that used larger animals as a way of transport (phoresy) and, also, used these organisms as hosts by feeding or laying eggs; consequently, the mite larvae would feed on the animal host (Walter and Proctor 1999). Parasitism in mesostigmatid mites that infest vertebrates is thought to have had its beginning in species that switched from predation and evolved to feeding in a host that could survive living with parasites (Fain 1969; Walter and Proctor 1999). The adaptation of chelicerae for piercing and blood feeding allowed mite species to diversify their diet to feed on the blood of vertebrate hosts. Another example is in dermanyssoid and laelapid nest mites that could move

onto the nesting birds for a blood meal, then adapted to stay on the host and became obligate parasites (Dowling & OConnor 2010).

1.4 Nasal mites

Respiratory mites are obligate endoparasites that spend their complete life cycle in the respiratory system of different group of animals. In domestic dogs, the mite *Pneumonyssus caninum,* is an intranasal parasite that tends to cause sinusitis in its hosts. Other groups of mammals with nasal mite that have been studied are bats, known to be infected by members of Trombidiformes (Fain & Lukoshus 1971), different kinds of rodents (Zabludovskaya 1990), and even marine mammals (Fay & Furman 1982). In reptiles, nasal mites, such as laelapids, have been described in the nasal passage of lepidosaurs (reptiles with overlapping scales, such as snakes or lizards), (Fajfer 2012). Members of the family Ereynetidae (Prostigmata) can occur in the respiratory passages of toads and frogs, as well as being intranasal parasites known in birds and mammals (Krantz 1978).

Mites living in the nasal cavities of birds belong to several unrelated families of both Acariformes and Parasitiformes. In North America, nasal mites are known from four families: Rhinonyssidae (Mesostigmata), Ereynetidae (Prostigmata: Speleognathinae), and two families of Astigmata, the Turbinoptidae (Turbinoptinae) and Cytoditidae (Knee et al. 2008; Beaulieu et al. 2011). Nasal mites in birds have been studied the most for years and still new reports are recorded on new species and new host records (Knee 2017; Dimov 2018; De Rojas et al 2020; Hilario-Perez and Dowling 2020).

Surveys of nasal mites have been conducted worldwide (Fain 1964; Bell 1996; Mascarenhas et al. 2018; De Rojas et al. 2020). In North America, nasal mite surveys in birds have been

conducted from different parts of the United States and Canada (Strandtmann 1948, 1951; Pence 1973 Spicer 1987; Knee et al. 2008; Hilario-Perez and Dowling 2020). These studies have been sources of information to understand the diversity of nasal mites and the prevalence of their infestation, which can vary from 20 to 40% in avian populations infested.

Rhinonyssidae (Mesostigmata) is the most diverse family of nasal mites. The family is currently classified into 11 genera with approximately 600 known species (De Rojas et al. 2020). This is a group of slow-moving, blood-feeding mites that live in the intranasal passage of birds and can remain sustained embedded in nasal mucus. Some species, such as *Sternostoma tracheacolum*, may invade the bird's lung and cause harm to the birds (Lawrence 1948). Generally, rhinonyssids do not cause significant harm to the host, but there have been some documented cases in which nasal mites have caused irritation to the nasal epithelium (De Rojas et al. 2002). Species of the genus *Ptilonyssus* are common parasites in birds in the order Passeriformes (Strandtmann 1948; Knee 2008). The genus *Ptilonyssus* is known to be the most diverse genus of nasal mites. Similarly, the genus *Sternostoma* is nearly as diverse.

Historically, descriptions of nasal mite species have been based on morphological characters. More recent studies, particularly of the Rhinonyssidae, have used molecular methods to try to understand phylogenetic relationships of different mites, demonstrate evidence of cryptic species or develop taxonomic inferences (Navajas and Fenton 2000, De Rojas et al 2001; 2002; Morelli and Spicer 2007). Within Rhinonyssidae, some of the genera used for different studies have been *Tinaminyssus*, *Sternostoma*, *Rhinonyssus*, and *Ptilonyssus*. De Rojas et al. (2002) studied phylogenetic relationship of nasal mites to discriminate among closely related species regarded as a "complex," such as the "*T. melloi*" complex or the "*P. sairae*" complex. Morelli and Spicer

(2007) studied *P. sairae* infesting different hosts and found some evidence of cospeciation that might be happening in these mites and their host.

Molecular studies have also been used in other groups of mites that are closely related to rhinonyssids, such as the poultry red mite (*Dermanyssus gallinae*), which is an obligatory blood feeding ectoparasite of poultry and has been well studied using different phylogenetic markers (Marangi et al. 2009; Karp-Tatham 2020). Moreover, phylogenetic studies have tried to understand at large scale the super family Dermanyssoidea or the whole Parasitiformes, using 18S and 28S nuclear rRNA gene sites (Dowling and OConnor 2010; Klompen et al. 2007), whereas others have used Internal Transcriber Spacer (ITS) and the mitochondrial 16S of the rDNA with different group of mites to infer phylogenetic relationships (De Rojas et al 2001; 2002).

A phylogeny is an attempt to visualize the evolutionary history of a particular group of organisms and represents a hypothesis. Phylogenetic studies of nasal mites may help inform patterns of host specificity, and host specificity may help resolve a phylogeny. Host specificity in nasal mites that parasitize birds varies by order, family, genus, or even to species of birds. Host specificity is somewhat difficult to determine in wild birds because transmission of nasal mites from the host is still unclear. Transmission has been suggested to occur either horizontally, in which mites move among hosts when birds are socializing in flocks, or vertically, in which nasals mites crawl out to the face of the bird and transfer from parent to offspring when feeding their young.

1.5 Brown-headed cowbird

Like other blackbirds, brown-headed cowbird (*Molothrus ater* Boddaert, 1783) (hereafter, BHCO) is a member of the family Icteridae, which also includes grackles, meadowlarks, orioles, and bobolinks. BHCO is an obligate brood parasite (cowbird females lay eggs in the nests of other bird species that serve as hosts) that has been reported to parasitize more than 220 species of birds, although only 144 species have been recorded to successfully rear the cowbird young (Dufty 1985, Briskie et al. 1990). The range of species parasitized by BHCO includes many orders of birds, but most successes are when parasitizing species of the order Passeriformes. Unsuccessful attempts at parasitism include blue-winged teal, ferruginous hawks, red-headed woodpeckers and ruby-throated hummingbirds (Ehrlich et al. 1988)

Some populations of BHCO are migratory, breeding in the northern parts of North America with winter migration into the southern U.S. and Mexico, whereas other BHCO populations are local residents in southern parts of North America (Sibley 2000; eBird 2021). Most migratory birds in North America follow four north-south flyways (U.S. Fish and Wildlife Service 2021), which were first classified by wildlife managers noting the movement of waterfowl. The four primary North American flyways are largely defined by geological features. The Pacific and Atlantic flyways are bounded on one side by the respective oceans and largely by mountains along the other side. The Pacific flyway is between the Pacific Ocean and Rocky Mountains, whereas the Atlantic flyway occurs between the Atlantic Ocean and Appalachian Mountains. The Mississippi flyway occurs mostly between the Appalachian Mountains to the east, westward to the dry Great Plains, and the Central Flyway is located from the Rocky Mountains to the west, eastward through the Great Plains. The bordering feature between the Central and Mississippi flyway is the arid-humid boundary that runs approximately along the 100th meridian west, with

the semi-arid Great Plains to the west and the more humid states to the east. Those BHCO that are migratory occur in all four of the flyways, and migratory birds can overlap in wintering locations with resident individuals that stay year-round.

1.6 Brown-headed cowbird as host to nasal mites

The brood parasite BHCO is, itself, a host to parasitic nasal mites. A previous study of nasal mites (Hilario-Perez and Dowling 2018) included examining nasal mites from 126 individuals of BHCO, including nasal mites that were common among species of social bird hosts, including members of the Icteridae, and nasal mites from solitary birds. Bird hosts that are social may be able to transmit nasal mites horizontally among individuals, and this is especially possible where migratory and resident birds overlap in winter. With the wide range of hosts infested by the BHCO and its broad dispersal throughout North America, transmission of their nasal mite symbionts could also occur by vertical transmission (from parent to offspring). Both horizontal and vertical transmission could affect diversity of these nasal mite symbionts on their cowbird hosts.

Sometime parasitic species, such as nasal mites that have different levels of host specificity, might differentiate and become specific with their hosts. These genetic differentiations could also lead to the formation of cryptic species, in which species of nasal mites that appear morphologically identical could be genetically different (Bickford et al 2007).

This dissertation examined nasal mites infesting brown-headed cowbirds, to try to understand relationships between the hosts and parasites. The study includes three parts:

1. (Chapter 2) I performed a survey of nasal mites infesting individuals of the BHCO from different geographical locations. The aim of this study was to study the diversity of nasal mites

infesting BHCO and how these might vary from hosts collected in different migratory flyways of North America.

2. (Chapter 3) I developed a phylogenetic study of some specimens of nasal mites from the BHCO hosts. The aim of this part of the study was to examine whether these mites might differ genetically and, if so, how they differ among geographic locations. For this part of the study, I used molecular analysis on two species (*Ptilonyssus icteridius* and *P. agelaii*) that are common and abundant as nasal parasites of BHCO. In addition, I also performed a molecular analysis with the mitochondrial cytochrome C oxidase subunit I gene (COI), which is used as a barcode for identifying relationships of many species (Li et al 2010), and I created phylogenetic trees by using maximum likelihood analysis.

3. (Chapter 4) For the third study, I conducted a phylogenetic analysis of specimens of *Ptilonyssus* mites with the aim to determine phylogenetic inferences on this genus of nasal mites. For this study I used ITS, 16S and COI as target gene sites for the amplification, and maximum likelihood analysis to understand relationship and to develop phylogenetic tree.

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1.8 Table 1.

Table 1. Classification (Superorder, Order, Suborder) of Class Acari.

Superorder	Order	Suborder
Acariformes (Actinotrichida)		
	Sarcoptiformes	
		Endeostigmata*
		Oribatida (includes Astigmata)
	Trombidiformes	
		Anysitina
		Eupodina (Prostigmata)
Parasitiformes (Anactinotrichida)		
	Mesostigmata	
		Monogynaspida (Includes Sejida)
		Trigynaspida
		Gamasina
	Ixodida	
	Holothyrida	
	Opilioacarida	

*Known to be a paraphyletic group Krantz & Walter 2009

CHAPTER 2. Survey of avian nasal mites infesting *Molothrus ater* individuals from different geographic locations in the US.

2.1 Birds as host

Birds represent good example of hosts for many species of symbionts including lice, flies, and mites, with an array of life histories and adaptations in close association with birds. Among these different symbionts, mites are the most diverse and abundant with 40 families and ~3000 species (Proctor and Owens 2000).

Avian mites can occupy many parts of the body, such as face, nasal passages, quills, feathers, legs, on skin or subcutaneous, and even inside the tracheal tissue and lungs, all of which serve as individual microhabitats (Phillips 2000; Hilario-Perez and Dowling 2020). Symbiotic mites exploit the different microhabitats by feeding on oil, pollen and fungi that attach to feathers, or secretions on the bird skin, blood, and tissue (Proctor and Owen 2000).

2.2 Nasal mites

Nasal mites are one example of these symbionts. These are regarded as obligate intranasal parasites due to the blood-tissue feeding behavior. Effects associated with nasal mites in birds are irritation of the nasal passages, anaemia from loss of blood, or transmission of pathogenic organisms (Knee and Proctor 2006).

As parasites of North American birds, four unrelated families have been recognized as nasal mites: Rhinonyssidae (Mesostigmata), Ereynetidae (Prostigmata, in Speleognathinae), and two families belonging to Astigmata—Turbinoptidae and Cytoditidae (Knee et al. 2008; Beaulieu et al. 2011).

Respiratory mites inhabit different niches inside nasal passage from the superficial cutaneous part of the nasal cavity to the lungs of some species, such as *Sternostoma tracheacolum* Lawrence, 1948, which infests the lungs of canaries (Lawrence 1948, Fain 1994).

Rhinonyssidae is the most diverse family of nasal mites. The family is currently classified into 11 genera with approximately 600 known species (De Rojas et al. 2020)**.** Host specificity in Rhinonyssidae varies among genera. Some genera of mites infest different bird orders, (*Ptilonyssus* infesting Passeriformes, *Tinaminyssus* in Columbiformes) whereas others are genera restricted to families and even to species, such as the genus *Rhinoecius*, in which each species of mite will infest a single species of Strigiformes (Strandtmann 1948; Knee 2008).

Mechanisms of transmission are still unclear. The primary mode of transmission for nasal mites has been hypothesized to be vertically from the host parents to the young during feeding (Murray 1966). Another hypothesis is that birds can obtain nasal mites horizontally when they socialize in groups, by preening, or courtship billing (Amerson 1967).

2.3 Brown headed cowbird BHCO (*Molothrus ater* Boddaert, 1783)

The brown-headed cowbird, *Molothrus ater* (Passeriformes: Icteridae), is widespread across North America. Brown-headed cowbird (hereafter, BHCO) is the most abundant species of cowbird in the United States and the most studied in the genus (Dufty 1982). Some populations of BHCO are migratory, breeding in the northern parts of North America with winter migration into Mexico, whereas other BHCO populations are local residents in both southern and northern parts of North America (Sibley 2000).

Cowbirds are well known to be obligate brood parasites and generalists, with more than 200 species of birds from different orders of birds as hosts, including orders, such as,

Passeriformes, Falconiformes, Galliformes, and Charadriiformes (Shaffer et al. 2019). As brood parasites, cowbird females lay eggs in the nests of other species of birds that will serve as foster parents (hosts).

Due to high levels of parasitism and the diversity of hosts, and the fact that the BHCO is also a host for a variety of symbionts acquired by different means of transmission, BHCO are good models for the study of host-parasite relationship.

2.4 Nasal mites shared by the cowbirds and other hosts

Currently, the list of nasal mites that have been found in BHCO includes seven species, six Rhinonyssidae (*Ptilonyssus agelaii* Fain and Aitken, 1964; *P. japuibensis* Castro, 1948; *P. icteridius* Strandtmann and Furman, 1956; *P. richmondinae* George, 1961; *Sternostoma strandtmanni* Furman, 1957; and *S. tracheacolum* Lawrence, 1948) and one Ereynetidae (*Boydaia quiscali* Clark, 1960) (Pence, 1975; Knee and Galloway, 2017; Hilario-Perez and Dowling 2018). Some of the mites found in BHCO have been found also infesting other icterids, for instance, *P. icteridius, P. japuibensis* and *Boydaia quiscali* have been described from red-wing black birds and grackles, which are birds that socialize and flock together in groups (Pence 1975), suggesting a possible horizontal transmission of mites by physical contact. However, *P. icteridius* have been also found in solitary individuals, such as orioles, which may suggest vertical transmission from parent to offspring (Hilario-Perez and Dowling 2018).

2.5 Assumptions and hypotheses

In the study from 120 cowbirds individuals in Texas (Hilario-Perez and Dowling 2018) we saw a low diversity of nasal mites infesting BHCO, with just five nasal mite species

identified, with 66.6% prevalence and with the same species of nasal mites that are commonly found in other icterids.

In this study we wanted to survey nasal mites in individuals of BHCO covering a larger sample size and individuals located in different regions associated with the migratory flyways of North America. We also wanted to determine if the diversity of nasal mites in cowbirds varies by location.

We examined the variation of mites by location of the infestation, by sex of the birds and whether multiple infestations of nasal mites might vary within individuals. The diversity of nasal mite species might shed light on whether cowbirds acquire nasal mites horizontally by flocking or vertical from parent to offspring. For this, we observed whether there were the same patterns of infestation and diversity or if they varied among locations.

MATERIAL AND METHODS

2.6 Bird collection

This study of bird nasal mites was based on 856 Brown-headed Cowbirds (*Molothrus ater*) (hereafter BHCO) from five states in the United States. Six BHCO were salvaged from window strikes in Conway, Arkansas (hereafter AR), and 120 BHCO belonged to a collection from the laboratory of Dr. Than Boves, Arkansas State University (ASU), originally collected at Ft. Hood, Texas (TX1). Birds from the trapping programs in four states were collected from March to May, 2014-2017: 455 birds from Simi Valley, California (CA); 133 birds from Fort Hood, Texas (TX2); 92 birds from Waupun, Wisconsin (WI); and 50 birds from Mormon Island, Nebraska (NE). Sexes of the birds from the four trapping programs were noted. The collecting locations were assigned to three North American migratory flyways. Birds from California were

assigned to the Pacific flyway, Nebraska and Texas were assigned to the Central flyway, and Wisconsin and Arkansas were assigned to the Mississippi flyway.

2.7 Processing Mites

Bird specimens were maintained and stored in freezers maintained at -20°C at the ASU laboratory (TX1) or a laboratory at the Arkansas Experiment Station, University of Arkansas, Fayetteville (all remaining specimens) until processed. Individual birds were removed from the freezer and allowed to reach room temperature. The respiratory passages of each bird were flushed using a mixture of warm water with ethanol in a 3:1 ratio. A 5cc syringe was used to push the mixture through the nasal passages. Typically, four consecutive flushes were made, using hard liquid pressure applied to each nostril and alternating flushes of the nasal cavities on both sides. The flushed liquid was collected in a 9 cm Petri dish and was examined for nasal mites using a Leica MZ16 stereomicroscope at 20-25x magnification. Mites from each bird were collected and stored in a vial of 70% ethanol for later identification.

For identification, mites were slide-mounted for observation under the compound microscope. Mites were cleared in 85% lactic acid at 70°C for one hour. They were placed on a microscope slide in a drop of Hoyer's mounting medium and, once positioned correctly, a cover slip was put in place. Identification of both larval and adult mites was made using diagnostic keys (Pence 1975; Knee & Proctor 2006). Vouchers of mite species were deposited in the Acarology Collection in the Department of Entomology and Plant Pathology at the University of Arkansas**.**

2.8 Counting Mites and Calculating Prevalence

Numbers of nasal mites were counted under the stereomicroscope. We assessed the numbers of mites in each bird. Exact counts were made for 10 or fewer mites per bird; estimates to the nearest five were made when birds contained more than 10 mites. Numbers of uninfested birds and birds that were infested by nasal mites were used to calculate infestation prevalence for the total sample and by each collection. We calculated infestation prevalence by sex for male and female BHCO at four of the collection locations. Infested birds were classified as being either infested by one species of mite or had co-infestations by two or three species of mites.

RESULTS

The six collections from five U.S. states produced a total of 856 Brown-headed Cowbird (BHCO) individuals (Table 2). California represented the largest sample with 455 BHCO, followed by Texas, in which the two collections totaled 253 individuals. Smaller numbers of BHCO were in the WI, NE and AR collections (Table 2).

Examinations showed that 764 BHCO were infested by nasal mites, giving a prevalence of 89.3% infested (Table 2). Prevalence for five of the collections was similar, ranging from 88-100%. However, one of the two collections from Texas (TX1) yielded only 76 of 120 BHCO infested (63.3%) (Table 2). Assigning the collections to U.S. migratory flyways, 97 of 98 birds (99.0%) from the Mississippi flyway were infested, versus 428 of 455 (94.1%) in the Pacific flyway and 239 of 303 (78.8%) from the Central flyway (Table 2).

Numbers of nasal mites per infested bird were counted for four of the six collections. Numbers ranged from 1 mite per bird to several birds containing more than 100 mites. Percentages of infested birds containing 10 or fewer mites, representing light infestations, or

more than 40 mites, representing heavy infestations, were calculated and compared among collections. A total of 247 of the 765 infested birds (32.3%) were infested by fewer than 10 mites. Percentages of infested birds with 10 or fewer mites from each of the collections ranged from 25.0% (CA) to 66.3% (TX2). Percentages of infested birds with 40 or more mites ranged from 1.7% (TX2) to 18.2% (NE) (Table 2).

Sex of the BHCOs were listed only for bird samples received from the four trapping programs (Table 3). The number of males summed to 360 BHCO versus 370 females collected. Only 4 of 133 birds in the TX2 collection were males, whereas only 3 of 92 birds in the WI collection were females. Birds in the California collection were 56.2% male, whereas Nebraska birds were 78% female. Both female and males yielded similar prevalence of infestation of nasal mites with an average of 94.4% of nasal mites infesting males and 92.4% of females infested (Table 3). BHCO sex was also listed by infestation in single individuals and co-infestations. In BHCO males, single infestations of nasal mites totaled 305 of 360 individuals infested, whereas 35 male birds presented co-infestations of nasal mites. Among female BHCO, 286 of 370 individuals were infested by one species of mite and 56 were co-infestations (Table 3).

There were 669 birds infested by one species of nasal mites (Table 4). These nasal mites belonged to nine species, eight of which belonged to the family Rhinonyssidae and one species to the family Ereynetidae (Table 4). The eight species belonging to the Rhinonyssidae included four species in the genus *Ptilonyssus* and four species in the genus *Sternostoma*. The most frequently collected species overall and in all locations was *P. icteridius*, which was found in 610 out of the 671 single infestations, followed by *P. agelaii*, which infested 47 BHCO. Six species were represented by a total of three or fewer mites, and there were 6 specimens of the one species of the Ereynetidae, *Boydaia quiscali*, which was found in three of the five locations

(Table 4). Five species were represented by one individual each (Table 4), and for *P. richmondinae* and *S. strandtmani*, were the only specimens from these species. One species missing from single infestations was *S. sialiphilus*, which was found only in both 2-species and 3-species co-infestations.

There were 84 occurrences of co-infestation by two species of nasal mites, which were found in every collection (Table 5). The most common co-infestations of BHCO found were 31 instances of *P. icteridius* & *B. quiscali*, which we counted from four of the five locations. Also, the combination of *P. icteridius* & *P. agelaii* was found in 29 birds from four of the collections, 18 of which occurred in the California collection. One species, *S. sialiphilus*, was found when in a co-infestation with *P. icteridius*, which occurred in four birds from three states (Table 5). One co-infestation with *P. icteridius* included an individual of *Sternostoma* that could not be identified to species (Table 5).

There were 11 occurrences of BHCO having co-infestation by three different species of nasal mites and these occurrences were distributed in six triple co-infestations (Table 6). The 11 triple-coinfestations included seven mite species plus one unidentified mite. The most abundant included the co-infestation by *P. icteridius*, *P. agelaii* and *B. quiscali*, which occurred in 6 birds from three locations. The remaining five triple co-infestations all occurred in individual birds. *Ptilonyssus icteridius* occurred in 10 of the 11 triple co-infestations, *B. quiscali* in 9 triple co-infestations and *P. agelaii* in eight (Table 6).

DISCUSSION

2.9 Mite identifications

In this study eleven species of nasal mites were identified from Brown-headed cowbird (BHCO), in which three of the eleven nasal mites represent new records for the cowbird and there was an unidentified species.

The most common and abundant mite infesting BHCO was *Ptilonyssus icteridius.* This mite was found in every location in 697 out of the 856 birds examined, including single and co-infestations. These large collections of this mite might suggest that every BHCO may always be infested by *P. icteridius* in nature. This nasal mite is largely specific to species in the family Icteridae but has also been infrequently recorded from other bird families (Strandtmann and Furman 1956; Knee and Galloway 2017). Other nasal mites that were commonly found were *P. agelaii*, found in 91 birds and *Boydaia quiscali*, which was collected from 52 birds.
Three species found that have not been reported before for BHCO were from the genus *Sternostoma—Sternostoma pirangae, S. hutsoni* and *S. sialiphilus*. Each of these three nasal mites have been reported from a single host and unrelated the BHCO. *S. pirangae* was found in *Piranga rubra* L. 1758: (Cardinalidae); *S. hutsoni* from *Catharus ustulatus* Nuttall, 1840 (Turdidae); and *S. sialiphilus* collected from *Sialis sialis* L. 1758 (Turdidae) (Pence 1975). *Sternostoma sialiphilus* and *S. hutsoni* were found only in Nebraska and Wisconsin, and *S. pirangae* was found in five birds from three states. Each of these three bird species shows different level of flocking, from being solitary to socializing in large flocks, such as *S. sialis*, which would suggest different routes for nasal mite transmission. In addition, these three species of birds are also hosts of female BHCO (Lowther 2018). The diversity of species showed some differences in nasal mites found infesting birds possibly representing migratory versus local

populations of BHCO. Although small numbers of the BHCO populations do not migrate and may remain locally in temperate North America, many BHCO tend to migrate before winter (ebird.org 2021).

There was a one individual of a species of *Sternostoma* that could not be identified to species level, and it might represent a new record or even a new species. More specimen vouchers from this host might be needed for examination as well as a need for a more suitable identification key to determine this species. One other specimen could not be identified because it was a single specimen that was not in good condition. It might not represent a true nasal mite since it is not similar in appearance to any of the families considered to be true nasal mites in American avifauna.

2.10 Mite prevalence

In this study, a total of 856 brown-headed cowbird individuals yielded sufficient data to try to identify patterns. Overall, the study found 89.3 % prevalence of nasal mites, and five of the six collections had prevalence of at least 88%, exceeding the 66% prevalence from our previous study of nasal mites infesting BHCO (Hilario-Perez and Dowling 2018). The study of nasal mites of BHCO by Clark (1963), found up to 55 % of BHCO from Pennsylvania to be infested with nasal mites, well below the 89.4% prevalence we found.

Sexes of the birds, when identified, varied by locations, especially in the collections made from Texas and Wisconsin, dominated by females and males, respectively. Collected birds from California had similar numbers of male and female BHCO. Infestation levels did not differ by sex of the host, ranging from 75-100%.

Only cowbird samples from the laboratory collection at ASU (Texas 1) did not yield a similar percent prevalence as the other locations, producing 63.3% prevalence. A factor influencing the results of nasal mites found in the carcasses might be the technique for collection or the freshness and the condition of the birds. Birds obtained by trapping programs were sent immediately after trapped and sacrificed, thus, having fresher specimens, whereas the birds in the ASU laboratory collection were maintained for few years after collection, and it is unknown whether the specimens were manipulated in any way. However, all birds in the present study were treated identically for mite collection. The birds from the Clark (1963) study were collected by dissection of the bird hosts, but it is not known if fresh specimens were used.

The prevalence of infestation by BHCO was different from those in surveys of nasal mites in non-brood parasites, in which prevalence of nasal mites in hosts ranged from 15 to 40 % (Domrow 1969; Pence 1973; Spicer 1987; Knee et al. 2008). Among passerine birds, other species of Icteridae and also warblers (Parulidae) and sparrows (Emberizidae) produced 29.1% prevalence of nasal mites (Hilario-Perez and Dowling 2021). The prevalence of nasal mites in the present study appears to be a factor that occurs in bird groups that tend to be gregarious and in great numbers like the BHCO. This idea suggests that gregarious birds that are very abundant and flock together might have similar kind of mite species and large infestations (Strandtmann 1958). BHCO show similar characteristics as other organisms that socialize, in which those species that socialize in large groups correlate with abundant transmission of parasites and diseases (Poiani 1992).

The levels of infestation found in the present study pose the question of whether every BHCO bird might always contain mites infesting the nasal passages or tracheal system and, if no mite is found, if the absence could be due to mites crawling out of the birds at the time of death

and before the birds were collected. We used nasal flushing as method of collecting, which is a useful technique for thoroughly collecting nasal mites rapidly, but the technique does not account for mites that may have been deep in the tracheal system. Dissection of the head may yield better recovery of nasal mites, although it is a more time consuming and complicated technique (Wilson 1964; Pence 1973; Spicer 1987), and this technique does not recover mites lower in the tracheal system.

2.11 Migratory flyways

BHCO, which is distributed throughout the U.S., is known both to migrate before and after winter, and also have individuals that stay locally year-round (ebird.org 2021). Thus, it would be expected to see mixing of individuals and, with that, transfer of their symbionts, including parasites. The 'migratory connectivity' is the set of pathways that species from the same species use to migrate to same locations for breeding and wintering (Pagenkopp 2008). Studies of the parasites and diseases that birds carry often may be used for studying these patterns for migratory birds (Pagenkopp et al 2008).

Results of nasal mites infesting BHCO associated with migratory flyways appeared to be similar in prevalence and did not present a clear difference between in prevalence among geographical locations. Birds from Wisconsin and Nebraska represented two flyways (Central and Mississippi) and northern populations of BHCO. Those birds showed no differences in infestations compared to southern populations, such as California or Texas. Transfer of symbionts among populations might occur when BHCO are gathered in large flocks. BHCO are known to form large flocks with other blackbirds, even during breeding season (Tonra 2011).

2.12 Multiple infestations

All locations had co-infestations of multiple species of nasal mites infesting BHCOs. This study presented BHCOs with more multiple infestation than in previous surveys of different bird species (Clark 1963, Spicer 1987, Knee 2008, Hilario-Perez and Dowling 2018, 2020). In addition, this study found triple infestations of nasal mites infesting BHCO at the same time, which have never been found in studies of BHCO (Clark 1963; Fain 1964; Hilario-Perez and Dowling 2018). In two birds, the triple co-infestations included three different genera per bird.

Co-infestations by nasal mites of female birds from two collections stood out. In the Nebraska collection, 13 of 39 females (33.3%) were co-infested, and the Texas 2 collection showed 24 of 129 (18.6%) females infested with multiple nasal mites. In contrast, 18 of 199 (9.0%) female birds from California had multiple infestations. For males, the greatest rate of co-infestation (with more than 12 birds) was from Wisconsin, with 12 of 89 (13.4%) co-infestation rates. Male birds from California had 21 of 256 (8.2%) co-infested.

For co-infestations, questions of sequence of infestation or coexistence are raised. *Ptilonyssus icteridius* was found in 90.9% of all single-species infestations and 91.7% of co-infestations, suggesting it may be an initial colonizer. In contrast, *Boydaia quiscali* was found in 53 birds, but only 6 (11.3%) were single-species infestations, suggesting it may be more likely to infest a bird already infested by another mite species. Coexistence of multiple species may be enhanced if the mites occupy different areas of the respiratory system. Nasal mite species such as the genus *Ptilonyssus* tend to exploit the nasal passages. Other species, such as *Boydaia* (Ereynetidae), may exploit different parts of the nasal cavities and tracheal tissue (Fain 1994). Dissection of the complete respiratory system may be necessary to resolve this question.

2.13 Conclusions

In this study we covered larger samples and wanted to determine variation by location and by migratory flyways of North America. We could not see variation among populations in the prevalence of infestation of nasal mites. This would suggest that the prevalence of infestation of BHCO would not change depending on samples from different locations. In this study, we were not able to find fresh specimens of BHCO from the East Coast that represent the Atlantic flyway. However, we could predict that similar levels of infestation prevalence would be found, and we could add new records of more nasal mites and the relationships to different hosts.

The diversity of nasal mites was more variable from those locations that represented northern regions such as Wisconsin and Nebraska which could suggest some isolation and acquiring of nasal mites from different hosts. It would be interesting to compare nasal mites in large samples of other icterids compared to BHCO nasal mites. Also, collection of nestling of the cowbirds could enlighten on the transmission mechanisms, whether BHCO are acquiring nasal mites horizontally or vertically.

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2.15 Tables 2 to 6

Table 2. Numbers of brown-headed cowbirds (BHCO) collected, numbers infested by nasal mites, prevalence, numbers of bird with 10 or fewer mites, numbers of birds with 40 or more mites, migratory flyways of North America and infestation prevalence for flyways.

Location	Number BHCO	Number infested	Prevalence (%)	Birds with <10 mites	Birds with >40 mites	Migratory flyway	Prevalence (%) for flyway
Arkansas	6	6	100.0	*	*	Mississippi	99.0
Wisconsin	92	91	99.0	46	8		
Texas 1	120	76	63.3	*	*	Central	78.8
Texas 2	133	119	89.4	79	2		
Nebraska	50	44	88.0	15	8		
California	455	428	94.1	107	20	Pacific	94.1
Totals	**856**	**764**	**89.3**	**247**	**38**		

* Means not calculated

Table 3. Total numbers of male and female brown-headed cowbirds with infestations by nasal mites of one species (single infested) or more than one species (co-infested) and percent prevalence of infestation by sex, from collections made in four states in the United States.

Location	Male Cowbirds				Female Cowbirds			
	Total	Single Infested	Co- Infested	Prevalence (%)	Total	Single Infested	Co-infested	Prevalence (%)
Texas 2	4	3	0	75.0	129	92	24	89.9
Wisconsin	89	76	12	98.8	3	2	1	100
California	256	218	21	93.4	199	171	18	95.0
Nebraska	11	8	2	90.9	39	21	13	87.2
Totals*	**360**	**305**	**35**	**Avg=93.1**	**370**	**286**	**56**	**Avg=92.4**

Table 4. Species of nasal mites in the families Rhinonyssidae and Ereynetidae, and the numbers collected, from male and female brown-headed cowbirds infested by one mite species, in samples from California, Nebraska, Wisconsin, Texas (two collections), and Arkansas.

	Location										
Family and Species	**California**		**Nebraska**		**Wisconsin**		**Texas 1**	**Texas 2**		**Arkansas**	**Totals**
Rhinonyssidae											
	Male	Female	Male	Female	Male	Female	*	Male	Female	*	
Ptilonyssus icteridius	207	163	6	18	73	2	54	3	79	5	610
Ptilonyssus agelaii	10	8		2	3		17		7		47
Ptilonyssus richmondinae							1				1
Ptilonyssus japuibensis	1						1				2
Sternostoma pirangae				1							1
Sternostoma hutsoni									1		1
Sternostoma tracheacolum									1		1
Sternostoma strandtmanni			1								1
Ereynetidae											
Boydaia quiscali			1						4		5
Total Infested Birds	389		29		78		73	95		5	669

*Sexes not identified.

Table 5. Species of nasal mites, and the number of occurrences of two-species co-infestations, from male and female brown-headed cowbirds, in samples from California, Nebraska, Wisconsin, Texas (two collections), and Arkansas.

	Location										
Species	**California**		**Nebraska**		**Wisconsin**		**Texas 1**	**Texas 2**		**Arkansas**	**Totals**
Two-species Co-infestation											
	Male	Female	Male	Female	Male	Female	*	Male	Female	*	
Ptilonyssus icteridius & *P. agelaii*	7	11		3			1		6	1	29
Ptilonyssus icteridius & *P. japuibensis*	1	1			1						3
Ptilonyssus icteridius & *Boydaia quiscali*	6	2	1	4	6		1		11		31
Ptilonyssus agelaii & *Boydaia quiscali*	2	2					1		2		7
Ptilonyssus icteridius & *Sternostoma pirangae*		1		2					1		4
Ptilonyssus icteridius & *Sternostoma hutsoni*				1							1
Ptilonyssus icteridius & *Sternostoma tracheacolum*	2				2						4
Ptilonyssus icteridius & *Sternostoma sialiphilus*		1		1	1	1					4
Ptilonyssus icteridius & *Sternostoma* sp.				1							1
Totals	36		13		11		3	20		1	84

Table 6. Species of nasal mites, and the number of occurrences of three-species co-infestations, from male and female brown-headed cowbirds, in samples from California, Nebraska, Wisconsin, Texas (two collections), and Arkansas.

	Location										
Species	**California**		**Nebraska**		**Wisconsin**		**Texas 1**	**Texas 2**		**Arkansas**	**Totals**
Three-species Co-infestation											
	Male	Female	Male	Female	Male	Female	*	Male	Female	*	
Ptilonyssus icteridius, P. agelaii & Boydaia quiscali	3			1					2		**6**
Ptilonyssus icteridius, P. agelaii & Sternosoma hutsoni					1						1
Ptilonyssus icteridius, Boydaia quiscali & Sternosoma hutsoni									1		1
Ptilonyssus icteridius, Boydaia quiscali & Sternosoma tracheocolum					1						1
Ptilonyssus icteridius, Boydaia quiscali & unidentified mite									1		1
Ptilonyssus agelaii, P. japuibensis & Sternostoma sialiphilus			1								1
Totals	3		2		2		0	4		0	11

CHAPTER 3. Genetic relationship among specimens of nasal mites (*Ptilonyssus icteridius* and *P. agelaii*: Rhinonyssidae) infesting specimens of the brown-headed cowbird (*Molothrus ater* Boddaert, 1783) from different geographic locations in the US.

INTRODUCTION

Host-symbiote relationships are studied in order to understand different phylogenetic and biological questions regarding co-adaptation. Relationships among these organisms are developed through continued intimate interaction in time. Geographical barriers and isolation sometimes allow symbiotic relationships to become more specific within each other and often diverge to consequently share coevolution, in which the host phylogeny mirrors that of their symbionts.

3.1 Birds and their symbionts

Birds offer ideal characteristics for an array of symbionts. Birds have a body covered in plumage and are able to disperse long distances by air and have access to different environments. Birds and their symbionts have been frequently studied. However, many of these studies have been focused on the negative effects of parasites caused to their hosts (Proctor 2003). Symbionts can inhabit the body of birds by exploiting different microenvironments inside and outside the bird's body.

The most-studied symbionts include insect parasites such as, lice, flies and fleas. However, mites, because of their minute size and rapid development, inhabit multiple niches, which makes them very diverse (Proctor and Owen 2000). Many symbionts develop different life histories acting as commensals such as feather mites, ectoparasites such as ticks, endoparasites such as respiratory mites or even some examples of mutualistic relationships (Walter and Proctor 1999).

3.2 Cowbirds to study nasal mites

The brown-headed cowbird (BHCO) (*Molothrus ater* Boddaert, 1783) is a member of Icteridae, which also includes blackbirds, grackles, meadowlarks, orioles, cowbirds, and bobolinks. Some BHCO are migratory, breeding in the northern parts of North America with winter migration into Mexico, whereas other BHCO populations are local residents in north and southern parts of North America (Sibley 2000). BHCO have the characteristic of being a well-studied obligate brood parasite that use more than 240 of different species of birds as hosts.

BHCO tend to socialize and flock together in large groups with other species of birds from their same family or even unrelated species such as European starlings (Sternidae). With the history of symbiont transmission among birds, the gene flow between populations moving through migratory routes and the diversity of hosts parasitized by cowbirds, it would be interesting to study some of these parasite groups infesting cowbirds in natural populations.

Nasal mites are endoparasites that live and spend their life cycle inside the nasal passage of birds, with some mite species even reaching the lungs of some species of birds (Fain and Hyland 1962). Nasal mites may exhibit different levels of host specificity. Species of nasal mites may be specific to particular bird orders, found infesting different families or restricted to particular genera of birds (Fain 1964). Nasal mites may be more prevalent and similar in birds that are closely related and socialize (Strandtmann 1948) and more specific to species that are solitary, for instance nasal mites of the genus *Rhinoecius* Cooreman, in which 12 species of mites each parasitize a single species of owl (Strigiformes).

In the BHCO, two species of nasal mites that most commonly occur are *Ptilonyssus icteridius* and *P. agelaii*, which also occur in other icterids (Clark 1963; Pence 1975).

Ptilonyssus icteridius is the most common and abundant mite in cowbirds with high prevalence whereas *P. agelaii* is less common and less abundant. *Ptilonyssus agelaii* is part of the "*sairae* complex" (based on *P. sairae*), which includes species with similar morphology, such as, *P. japuibensis* that have also been reported from BHCO (Hilario-Perez and Dowling 2018).

Since BHCO infest hosts all across North America and they host a diversity of mites, it would be interesting to examine whether these species of nasal mites show some level of specificity with their host. It also would be interesting to determine if mites differ genetically depending on the geographical location. If there were differentiations, those could lead to the formation of cryptic species, which helps to explain how these parasites become specific with their hosts.

In this study we examined the nasal mites *P. icteridius* and *P. agelaii* infesting BHCO from different parts of the United States. We used phylogenetic analysis to identify possible clades of mites that could represent geographic isolation of BHCO. We also wanted to see if these populations of BHCO would be geographically isolated (i.e., west coast birds do not mix with birds found in Texas and neighboring states) therefore, the mites in these birds should also be geographically isolated since they need birds to come together so they can transfer hosts.

MATERIAL AND METHODS

3.3 Bird collection

Molothrus ater (Brown headed cowbirds; BHCO) were obtained through donations by control trapping programs and research laboratory collections throughout the US from 2014-2017. Locations included in this study were, Simi Valley, California; Fort Hood Texas; Waupun,

Wisconsin; Mormon Island, Nebraska; and a collection of cowbirds from northeast Texas and Louisiana from Louisiana State University (LSU).

3.4 Nasal mite collection

The BHCO were maintained and stored in a -20°C freezer until processed. The sample for mites, the respiratory passages of the birds were flushed using a mixture of warm water with ethanol. A 5cc syringe was used to push four consecutive flushes creating strong water pressure through each nostril, alternating flushes on both sides of the nasal cavities. The flushed liquid was collected in a 9 cm Petri Dish and was examined for nasal mites using a Leica MZ16 stereomicroscope at 20-25x magnification. Nasal mites were collected and stored in vials of 70% ethanol.

3.5 Molecular analyses

DNA was extracted from individual mites using Qiagen DNeasy® Tissue Kit following manufacturer's recommendations with a slight modification including piercing the body of the mite using a minutin pin and incubating the samples at 55°C overnight, rather than the suggested 1-4 hours. Final elutions were made using 100 µl of ddH_2O. Samples were stored at -20°C until genetic analysis. The COI barcoding fragment (Hebert et al. 2003), cytochrome oxidase 1 (COI), was amplified with primers designed in the lab to more universally amplify mites: HCOInew 5′-DAR RAT RTA NAC YTC DGG RTG NCC AAA RAA YCA RAA WA - 3′ and LCOInew 5′ - TGA ATW TWY TCN ACW AAT CAY AAA GAY ATY GGA ACW MT - 3′ (modified from Folmer et al. 1994). The mitochondrial large subunit (16S) was amplified using primers and protocols from De Rojas et al. (2002). The Internal Transcriber Spacer (ITS) was also amplified using primers from De Rojas et al. (2007).

All PCR reactions were performed in a total volume of 25 µl containing 2 µl of mite DNA, 13.65 µl of ddH_2O, 2.5 µl 10X Buffer, 2.5 µl dNTPs, 1.75 µl 50 mM $MgCl_2$, 1.2 µl each of 10 µM primers, and 0.2 µl Platinum Taq (Invitrogen). PCR protocols for each gene were as follows: 16S - denaturation of samples for 2 min at 94°C followed by 35 cycles of 94°C for 20s, 56°C for 30s, and 72°C for 45s, followed by a final extension step of 10 min at 72°C; COI - denaturation of samples for 2 min at 94°C followed by 35 cycles of 94°C for 50s, 48°C for 30s, and 72°C for 1 min, followed by a final extension step of 10 min at 72°C.

Reaction results were visualized on a 1% agarose gel and purified using the PureLink™ PCR purification kit (Invitrogen). In some cases when secondary bands were present, the remaining sample was run on a 2% low melting point agarose gel, the target band excised with a sterilized scalpel, and the sample purified with a PureLink™ Quick Gel Extraction kit (Invitrogen). Samples were sent to MacrogenUSA for sequencing using the PCR primers.

3.6 Phylogenetic analysis

All obtained COI, ITS and 16S sequences from this study were aligned using MAFFT online (Katoh et al. 2019) using the default Auto settings. The alignment was output as a Phylip file and analyzed using RAxML (Stamatakis 2014) through the CIPRES portal (https://phylo.org). *Pellonyssus reedi* Zumpt & Patterson, 1952 was set as the outgroup. The tree was visualized with the Interactive Tree of Life v5 (https://itol.embl.de/).

RESULTS

3.7 Sequences

All obtained sequences for COI, ITS and 16S sequences yielded characters of 414, 624 and 832 in the alignments.

Sequences for the different *Ptilonyssus* ranged from 392 bp to 555 bp. The mitochondrial 16S yielded the majority of sequences with nasal mite species presenting up to 553 bp. The ITS sequencing presented species with 392 bp. The barcode mitochondrial gene COI was the one that yielded less species with a total of 16 sequences in which produced species with numbers of 456 bp.

3.8 Phylogenetic analysis

All three gene regions presented distinct clades separating *Ptilonyssus icteridius* and *P. agelaii.* The 16S tree identified three different clades of nasal mites. Two major clades differentiating both *Ptilonyssus icteridius* from *P. agelaii* (Figure 1). One clade corresponded to three species of *P. agelaii* that might represent genetically different and separated from the rest.

In the 16S *P. icteridius* clade, it appears to have a group formed by species of *P. agelaii* from California that appears to be a sister group to *P. icteridius* species and separated from the *P. agelaii* clade.

The ITS tree, which was the second most numerous in sequences had results similar to the 16S tree. From this tree, two distinct clades separated genetically *P. icteridius* from *P. agelaii.* As the 16S tree, three species of *P. agelaii* that appear to be genetically different and formed a clade (Figure 2).

The COI yielded less sequences and yet it identified two clades separating *P. icteridius* and *P. agelaii* clades (Figure 3).

3.9 Diversity by locations

All the nasal mites from the five locations (CA, WI, LSU, TX, NE) were distributed within the tree. No clades showed differentiation on the relationship of genetics of nasal mite species and locations. The same results repeated among the three phylogenetic trees with all locations distributed all over the trees.

DISCUSSION

In our analysis, 16S and ITS genes sites presented the majority of sequences for building the phylogenetic trees inferences. For this reason, our discussion will be based on these two trees for this study.

The goal of this study was to observe how gene flow and distribution of BHCO were affecting their nasal mites and if BHCO dispersal could lead to the specialization and genetic differentiation of the nasal mites. Two *Ptilonyssus* species, *P. icteridius* and *P. agelaii*, were commonly found in BHCO, so the population genetics of both species was examined. Despite sampling BHCO from multiple regions of the US (e.g., California, Nebraska, Texas, Wisconsin, Louisiana), the analysis of mite sequences did not present genetic differences that could represent isolation by geographical regions of BHCO. On the contrary, geographical location seemed to be well distributed all over the trees suggesting that BHCO are likely frequently mixing and flocking during the winter or breeding seasons.

The two phylogenetic trees showed similar results with nasal mites from different geographical locations clustered together genetically forming clades of related species with no differentiation. Similar results were documented by De Rojas et al. (2002) in which *Tinamynssus meloi* (Rhinonyssidae) from different geographical locations presented similar genetic relationships. These results suggest that geographic location or association to migratory flyways does not seems to be a factor of nasal mite genetic differentiation and instead we might be looking at an array of mixing, moving, and gene flow among BHCO and their symbionts distributing all across North America.

Our data show a great distribution of BHCO throughout the transcontinental range with mixing of populations, which might suggest possible transfers of nasal mites by horizontal transmission when BHCO are in large flocks. Usually, BHCO tend to form big flocks with other black birds such as the redwing (*Agelaius phoeniceus*) and brewer (*Euphagus cyanocephalus)*. The behavior of BHCO seems to be of gregarious, being commuters and spending most of the time in large groups (Strantdmann 1958). Even during the breeding season, females spend a couple of hours early in the morning looking for nests and then will join a large group for the rest of the day and roosting in flocks during the night (Tonra 2011). It would seem that overwintering and gathering in flocks could be the most likely frequently encountered mechanism for nasal mite transmission and movement among hosts; however vertical transmission from parent to host could also be a possibility because of nasal mites frequently found from gregarious birds have also been reported from solitary birds (Hilario-Perez and Dowling 2018).

BHCO typically have monogamous mating behavior and mating is regulated by the host species, the distribution of the species nests, the proportion of males, and how many males and females might be in a BHCO population (Strausberger and Ashley 2003). If nasal mites could

opportunistically transfer during the breeding season, we would likely be able to see some patterns of geographical isolation due to mating system behavior, but our data is suggesting otherwise. The BHCO has wintering migration ranges widespread throughout North America, typically traveling to northern parts of Mexico and the southern United States (Al-jabber 2003). During winter, BHCO congregate in large flocks, when intra or interspecific transmission of symbionts is more likely to happen. In addition, transmission of parasites seems to be an obvious cost of sociality. Poiani (1992) suggested that ectoparasite transfer and contagiousness tend to increase in social passerines. Whether BHCO are migrating or staying local at a particular location, certain factors might affect the dynamic of the population and transmission of parasites, such as geneflow, competition for resources, predator avoidance, mating success and parasite load (Brown et al. 2017). A small population of non-migratory BHCO have been reported to stay year-round during winter migrations (ebird 2021). What we do not really know is the connectivity and how the mixing of BHCO that are breeding, or wintering is occurring (Tonra 2011).

BHCO seem to carry the same species of nasal mites as other icterids that socialize in large groups, but also appear to have mites that have been reported from solitary species (Hilario-Perez and Dowling 2018). The social behavior of BHCO could suggest a mechanism of how nasal mites, such as *P. icteridius* and *P. agelaii* that can be prevalent in other icterids and transmit between individuals (Pence 1975). We decided to use these two *Ptilonyssus* mites for this study because they represent the most common and abundant species infesting BHCO (Hilario-Perez and Dowling 2018). Both species were genetically differentiated within the trees, forming separated clades in all of the trees.

Ptilonyssus icteridius is a nasal mite highly prevalent (50.8% infestation from 126 BHCO reported in Hilario-Perez and Dowling 2018) in BHCO and appears to have some level of host specific to Icteridae, especially among the more social species that congregate as observed in many bird groups within Passeriformes (Poiani 1992). For instance, *P. icteridius* has been reported from the western tanager (Cardinalidae), which is a bird that can migrate and socialize in groups (Pence 1975). *Ptilonyssus icteridius* has even been reported infesting bird hosts from different continents, for example, the red-headed bunting (Emberiza bruniceps:Emberizidae) is a species found in Iran in which *P. icteridius* has been reported (Proctor and Owen 2000; Knee et al 2008; Moodi et al. 2014; Bernardon et al 2018). According to our phylogenetic trees, *P. icteridius* found in the five locations sampled showed no genetic signatures indicating geographical isolation. This would imply that although BHCO may be geographically isolated during the summer months (e.g., California and Texas populations), these populations mix when they move to the overwintering sites. *Ptilonyssus icteridius* is a highly prevalent mite in cowbirds and these results suggest that the mites actively move between hosts while the birds are flocking. *Ptilonyssus* in general are considered to be slow-moving mites (Proctor and Owen 2000); however, they clearly have the ability to actively move between hosts, possibly while the birds are roosting overnight. These mites are also viviparous, with gravid females giving birth to fully developed larvae (personal observation), which allows a newly transferred female to quickly establish a local population within the bird.

For the nasal mite *P. agelaii*, 16S and ITS yielded the same results with individuals forming a large clade of related mites with no genetic differences regarding geographical locations. *Ptilonyssus agelaii* has been reported from species of Icteridae, including the BHCO (Pence 1975). This mite is highly prevalent in BHCO and other icterids, and our data suggest

that, like *P. icteridius*, this mite may actively transfer between hosts during flocks. *Ptilonyssus agelaii* is morphologically similar to *P. japuibensis*, which also has been reported from BHCO (Hilario-Perez and Dowling 2018) and along with *P. agelaii* is considered a member of the "*sairae* complex" of morphologically similar mites. Morelli and Spicer (2006) presented some evidence of differentiation in individuals of *P. sairae* when infesting different hosts. Thus, the possibility of genetic variation of these avian parasites could occur depending on the time, ecology and the behavior of the host and parasite during their relationship.

Both 16S and ITS trees presented a clade of three individual that were morphologically identified as *P. agelaii* but appear to be genetically different. These results suggest nasal mites that might represent an evidence of species differentiation or may show and what could represent a cryptic species. Further analysis might be needed to find more species that could support this clade of species.

We wanted to see how gene flow between the different cowbird populations occurs by looking at the two most common nasal mite species infesting the same host bird. The lack of knowledge on the transmission of nasal mites among hosts sometime makes it difficult to determine how these mechanisms of host-parasite specialization are really happening and a combination of different factors might be involved, such as ecology, fitness, migration and mating behavior of the host. Our study supports the transmission of nasal mites during wintering stages where birds congregate in big flocks. It would be interesting analyze populations that represent the southeastern parts of the US, because the BHCO is known to overwinter in southern Florida. As a result, we might see some genetic signs of geographic isolation in the southeast because these populations are most likely to not migrate to the same overwintering grounds as the BHCO populations in this study. This could suggest new records of nasal mites,

different diversity and the probability of finding genetically different species that represent cryptic speciation. Unfortunately, BHCO from the southeastern US were unavailable for this study.

Further investigation on the gene flow of nasal mite species and their hosts is needed to better understand phylogenetic relationship among nasal mites and their hosts. Examination of phylogenetic relationships on nasal mites infesting BHCO and other members from the same bird family could expand on the knowledge of the diversity, host specificity and coevolution with their hosts. Since geographical location does not seem to be a factor in genetic diversification of these nasal mites, it would be interesting to study this phylogenetic interaction within a family of bird hosts. There are other brood-parasite species of Icteridae, many locations not yet surveyed, other non-brood parasite bird populations that represent particular communal systems and solitary systems and it would be interesting to examine the symbionts they carry study question on host-symbiont interaction.

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3.11 Figures 1 to 3

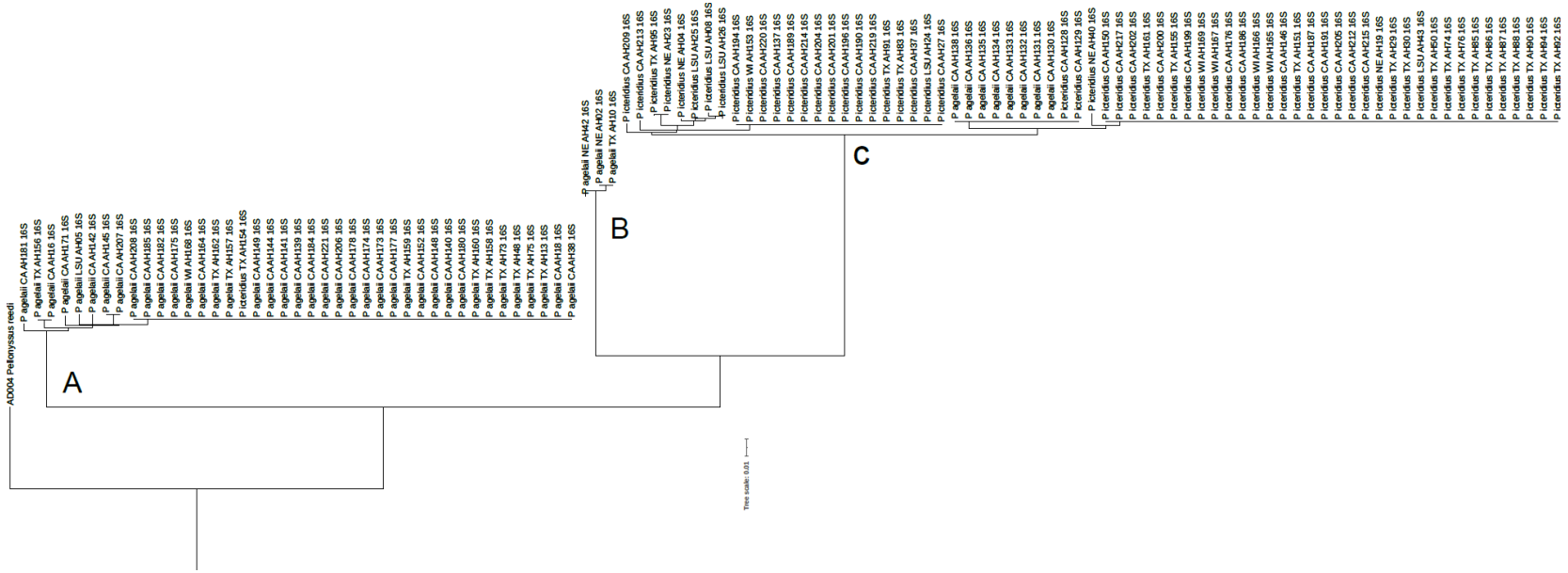

Figure 1. Phylogenetic tree of specimens of *Ptilonyssus icteridius* (represented in Clade C) and *P. agelaii* (represented in Clade A), inferred from 16S sequences and rooted with *Pellonyssus reedi*. Clade B represented by three *P. agelaii* species.

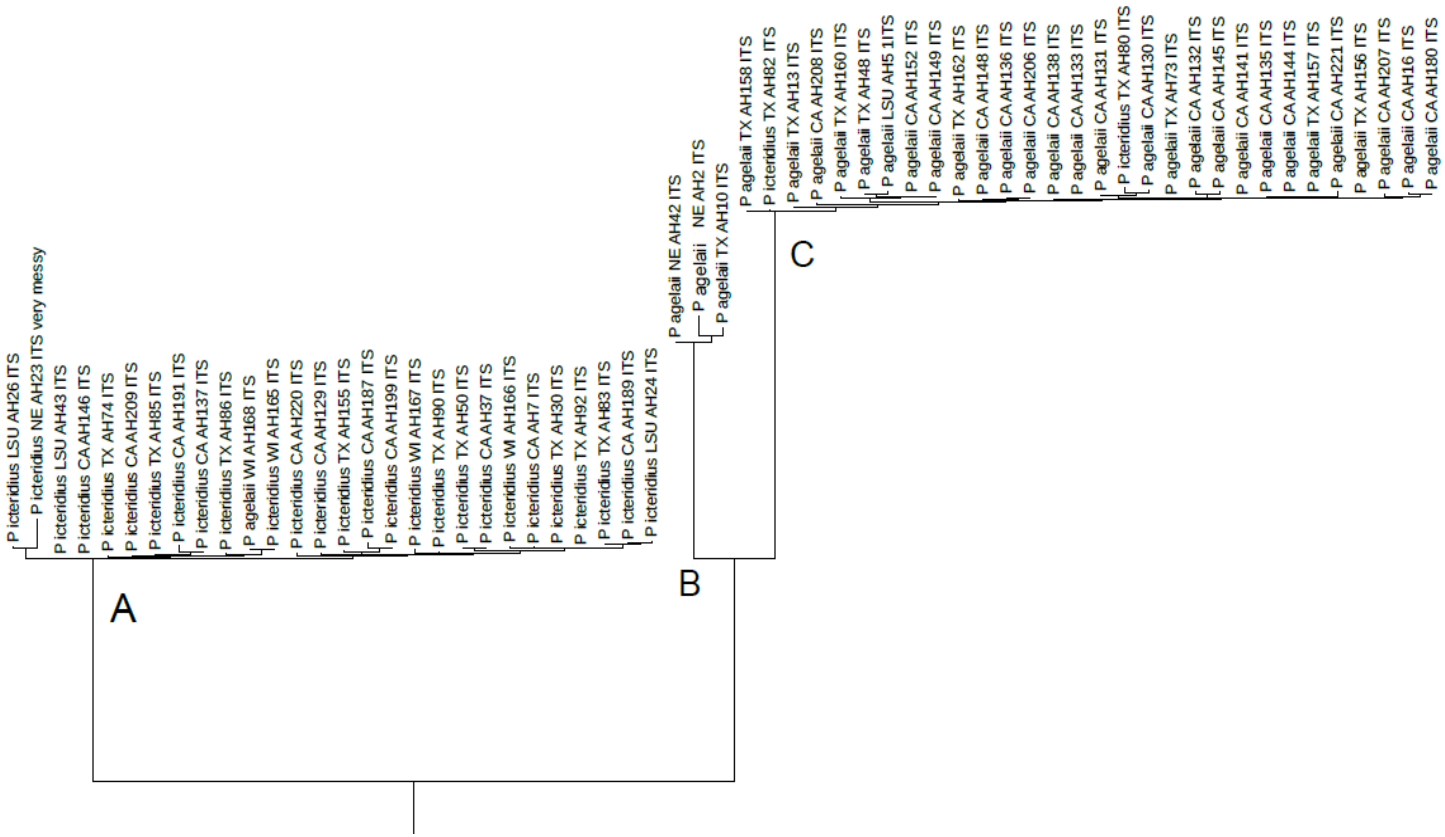

Figure 2. Phylogenetic tree of specimens of *Ptilonyssus icteridius* (Clade A) and *P. agelaii* (Clade C), inferred from ITS sequences. Clade B represented three *P. agelaii* species.

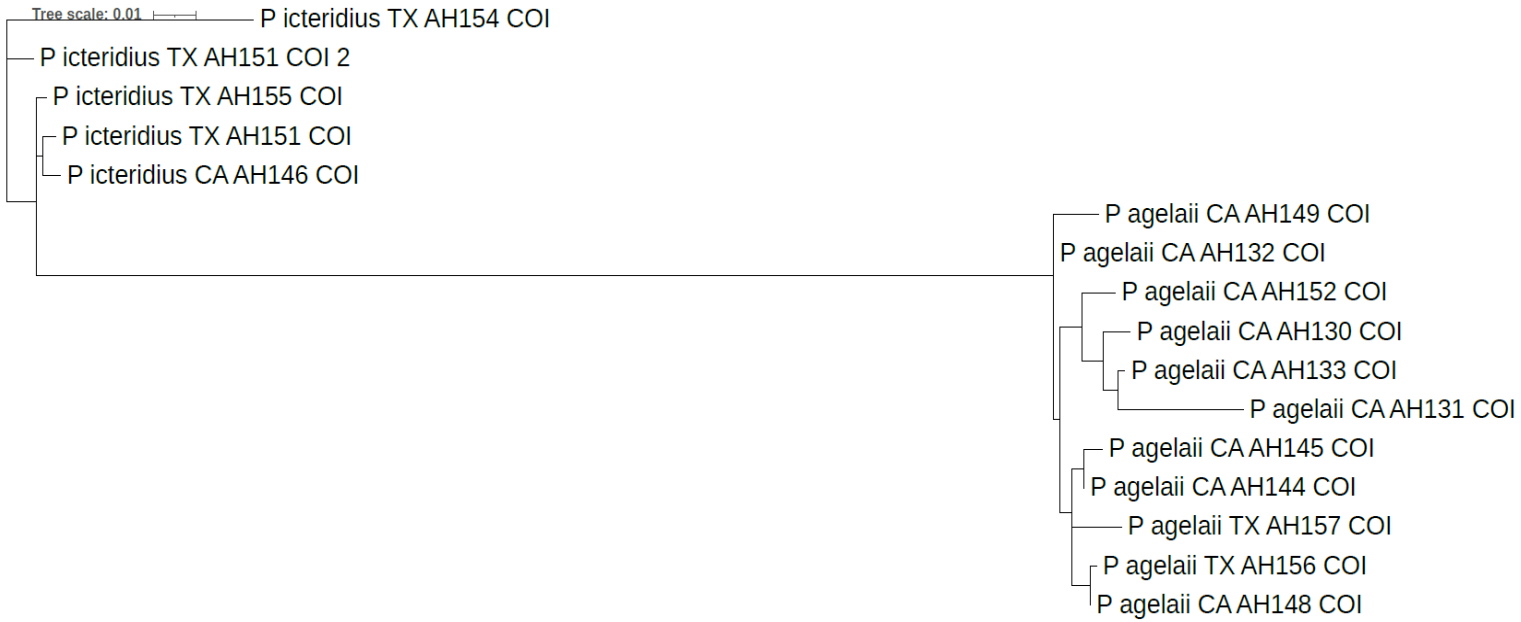

Figure 3. Phylogenetic tree of specimens of *Ptilonyssus icteridius* and *P. agelaii*, inferred from COI sequences.

CHAPTER 4. Phylogenetic relationship of species from the genus *Ptilonyssus* associated to different species of passerine birds from three states in the US.

4.1 Rhinonyssidae

Mites in the family Rhinonyssidae (Parasitiformes: Mesostigmata) live and spend their entire life cycle inside the nasal passage of birds (Vitzthum 1935, Fain et al. 1974). Among the multiple lineages known to inhabit bird nasal passages, Rhinonyssidae is considered to be the most diverse. Currently, the family includes approximately 600 species distributed among 11 genera: *Larinyssus* Strandtmann, 1948; *Locustellonyssus* Bregetova, 1965; *Mesonyssus* Fain, 1960; *Ptilonyssoides* Vitzthum, 1935; *Ptilonyssus* Berlese et Trouessart, 1889; *Rallinyssus* Strandtmann, 1948; *Rhinoecius* Cooreman, 1946; *Rhinonyssus* Trouessart, 1894; *Sternostoma* Berlese et Trouessart, 1889; *Tinaminyssus* Strandtmann et Wharton, 1958; and *Vitznyssus* Castro, 1948. (Veiga et al, 2020).

It is thought that rhinonyssid mites radiated from mite species that once were parasites of vertebrates (Strandtmann, 1948). It has been suggested that they might have evolved from ectoparasites of the family Macronyssidae (Mesostigmata) (Domrow 1969). Macronyssidae is a family of mites that evolved in close association with bats and later colonized other vertebrate groups, such as reptiles and birds (Knee et al. 2008). Rhinonyssidae and Macronyssidae might be a monophyletic group with species having morphological similarities and sharing the same kind of deutonymphs that do not feed until the next stage (Dowling and OConnor 2010).

4.2 Effects on hosts

Rhinonyssids do not significantly harm the host. However, mites feeding on the blood and tissue can lead to irritation, anaemia, or transmission of pathogenic organisms (Knee and Proctor 2006). Some species of mites may invade the tracheae, lungs, and air sacs of birds, such as the

mite *Sternostoma tracheacolum* Lawrence, 1948, which in large numbers can lead to more severe problems like bronchial dilation and even death of the birds (Lawrence 1948; Tidemann et al. 1992).

Most studies of host-parasite relationships have focused on parasitic mites affecting humans or animals of economic importance, such as *Dermanyssus gallinae* De Geer, 1778 (Dermanyssidae), which is a blood feeder of many birds including passerines and domestic poultry. Other studies have focused on *Varroa destructor* Anderson & Trueman, 2000, (Varroidae), which is a major parasite of honeybees worldwide (Guzman-Novoa et al. 2010).

4.3 Studies on Rhinonyssidae

Most of the previous work on rhinonyssids has been taxonomic in nature, focusing on morphological descriptions, producing keys for genera and species identification, and compiling information on host records and their prevalence (Fain 1957; Domrow 1969; Pence 1975). Most of the taxonomy for these mites has been determined based on morphological features, so it is difficult to analyze the systematic relationship of these parasites. Many mites that are closely related and parasitize closely related species of birds may show similar morphological characters that make them difficult to accurately identify species. Consequently, authors have gathered these groups into "species complexes" such as the "*sairae* group" of *Ptilonyssus* (Pence and Casto 1976), "*coniventris* group" of *Rhinonyssus* (Fain 1963), or "*melloi* group" of *Tinaminyssus* (Fain 1962) (De Rojas et al. 2007).

Molecular examination of rhinonyssids has been sporadic and very few studies inform on the phylogenetic relationship of these mites. However, some studies by De Rojas et al. (2002), examined the phylogenetics of closely related species of the genera *Tinamynisus* and

Sternostoma by using ribosomal DNA sequences, internal transcribe spacer (ITS1, ITS2) and also using the mitochondrial 16S rDNA (De Rojas et al. 2001; Ubeda et al. 2003). Additional studies have examined the "species complexes" and cospeciation among the nasal mite *Ptilonyssus sairae* Castro, 1948 and different birds hosts (De Rojas et al. 2007; Morelli and Spicer 2007).

4.4 Genus *Ptilonyssus* Berlese & Trouessart, 1889

Mites of the genus *Ptilonyssus* are endoparasites that lives in the nasal cavity of birds. This is the most diverse genus of rhinonyssids, with more than 130 species recorded (Pence 1975, Dimov 2012) and are most commonly found associated with Passeriformes, but also found associated with Caprimulgiformes, Falconiformes, and Apodiformes.

The first species *Ptilonyssus echinatus* Berlese & Trouessarat 1889 was described from the Barn swallows (*Hirundo rustica* L. 1758), and is a mite widely spread and commonly found in swallows (Fain et al. 1974). In Passeriformes, *Ptilonyssus* mites tend to infest birds that are closely related and sometimes socialize in groups, which has resulted in many morphologically similar mites associated with Passeriformes (Strandtmann 1956). Based upon the published literature, *Ptilonyssus* species seem to exhibit some degree of host specificity to host family level. For instance, *Ptilonyssus icteridius* Strantdmann and Furman, 1956 and *P. agelaii* Fain and Aitken, 1964 are associated with many species of Icteridae, whereas *P. sairae* is a mite commonly on species of the closely related families Emberizidae and Parulidae (Pence 1975, Hilario-Perez and Dowling 2020).

4.5 Molecular work on *Ptilonyssus*

Only a few studies regarding phylogenetic aspects of *Ptilonyssus* have been carried out. These studies on rhinonyssids have been limited in taxonomic scope, often focusing on a particular "species complex" or a set of mites associated with specific hosts in attempts to examine coevolutionary patterns (De Rojas et al. 2001, 2002; Ubeda et al. 2003; Morelli and Spicer 2007). More information is crucial to understand host-parasite relationship in regard to specificity, species level differentiation, and phylogenetic relationship among the closely related species of nasal mites.

The objective of this study was to use DNA sequence data to examine the phylogenetic relationships of mites within the genus *Ptilonyssus*.

MATERIAL AND METHODS

Nasal mites were obtained from birds reported in Hilario-Perez and Dowling (2020). Additionally, a few nasal mite specimens were used from the Dowling laboratory collection at the department of Entomology and Plant Pathology from the University of Arkansas. Passerine host species included in this study corresponded to three different states in the US (Arkansas, Illinois and Texas). A list of species included in the phylogenetic analysis is found in Table 7.

4.6 DNA extraction

DNA was extracted from individual mites using Qiagen DNeasy® Tissue Kit following manufacturer's recommendations with a slight modification including piercing the body of the mite using a minutin pin and incubating the samples at 55°C overnight, rather than the suggested 1-4 hours. Final elutions were made using 100 µl of ddH_2O. Samples were stored at -20°C until

genetic analysis. When possible, remaining mite cuticle was recovered and slide mounted as a voucher.

4.7 PCR amplification and sequencing

Three genes were targeted for this study: ITS, 16S, and COI. The entire ribosomal internal transcribed spacer (ITS) region including ITS1, 5.8S, and ITS2, was amplified through PCR using primers from Navajas et al. (1999). The mitochondrial large subunit (16S) was amplified using primers from De Rojas et al. (2001) and the barcoding fragment (Hebert et al. 2003), cytochrome oxidase 1 (COI), was amplified with primers designed in the lab to more universally amplify mites: HCOInew 5′- DAR RAT RTA NAC YTC DGG RTG NCC AAA RAA YCA RAA WA - 3′ and LCOInew 5′ - TGA ATW TWY TCN ACW AAT CAY AAA GAY ATY GGA ACW MT - 3′ (modified from Folmer et al. 1994).

All PCR reactions were performed in a total volume of 25 µl containing 2 µl of mite DNA, 13.65 µl of ddH_2O, 2.5 µl 10X Buffer, 2.5 µl dNTPs, 1.75 µl 50 mM $MgCl_2$, 1.2 µl each of 10 µM primers, and 0.2 µl Platinum Taq (Invitrogen). PCR protocols for each gene were as follows: ITS – denaturation of samples for 2 min at 94°C followed by 35 cycles of 94°C for 30s, 54°C for 30s, and 72°C for 2 min, followed by a final extension step of 10 min at 72°C; 16S - denaturation of samples for 2 min at 94°C followed by 35 cycles of 94°C for 20s, 56°C for 30s, and 72°C for 45s, followed by a final extension step of 10 min at 72°C; COI - denaturation of samples for 2 min at 94°C followed by 35 cycles of 94°C for 50s, 48°C for 30s, and 72°C for 1 min, followed by a final extension step of 10 min at 72°C.

Reaction results were visualized on a 1% agarose gel and purified using the PureLink™ PCR purification kit (Invitrogen). In some cases when secondary bands were present, the remaining

sample was run on a 2% low melting point agarose gel, the target band excised with a sterilized scalpel, and the sample purified with a PureLink™ Quick Gel Extraction kit (Invitrogen). Samples were sent to MacrogenUSA for sequencing using the PCR primers.

4.8 Phylogenetic analysis

All obtained sequences from this study were aligned by individual gene using MAFFT online (Katoh et al. 2019) using the default Auto settings. The alignment was output as a Phylip file and analyzed using RAxML (Stamatakis 2014) through the CIPRES portal (https://phylo.org). Two macronyssid species, *Steatonyssus occidentalis* and *Pellonyssus reedi* were used as outgroups. The trees were visualized with the Interactive Tree of Life v5 (https://itol.embl.de/). Additionally, all three gene datasets were combined for a final analysis using the previously mentioned approach.

RESULTS

4.9 Sequences and Phylogenetic analysis

The 16S rDNA sequencing yielded a total of 31 good quality sequences. For ITS and COI we were only able to obtain 19 sequences each. However, the tree topologies recovered from phylogenetic were similar for all three genes. Many specimens were not identifiable to species either because the cuticle was lost or badly damaged during DNA extraction or the specimens keyed to species in a specific couplet, but had some differing characteristics.

The phylogenetic analysis identified different three primary clades within *Ptilonyssus* labeled as A, B, and C (Figure 4). Clade A included species of *P. pirangae*, *P. icteridius, P. hirsti*, *P. morofskyi,* and a species of *Sternostoma*. Clade B includes species from the sairae complex,

including *P. ludivicianus*, specimens near *P. ludivicianus*, *P. sairae*, and what appears to be 2-3 species near *P. sairae*, but not perfectly matching the description of that species. Clade C includes *P. taxostomae*, *P. euroturdi* and an unidentified *Ptilonyssus*. *Ptilonyssus vireonis* and three specimens of a species close to *P. vireonis* are sister to the remaining *Ptilonyssus* species and labeled as group D.

DISCUSSION

Avian nasal mites are regarded as parasites due to their habits of being blood and tissue feeders and potential vectors for diseases in bird wildlife populations (Proctor and Owen 2000). The evolution of rhinonyssids might have its origin in morphological adaptations to parasitism. In the case of endoparasites, a regressive or degenerative phenomena might have happened, which means the reduction or loss of organs and the production of new structures to adapt to interior environments, such as reduction of chaetotaxy in Rhinonyssidae and suckers to avoid being dislodged from the host (Fain 1969). It has been suggested that Rhinonyssidae evolved from within Macronyssidae, which includes ectoparasites of mammals, reptiles, and birds (Strantdmann 1948). Based upon morphological characteristics, the transition to birds may have first started with a switch to reptiles, followed by a switch to birds (Fain 1969, Knee 2008).

Within Rhinonyssidae, *Ptilonyssus* and *Sternostoma* are the genera most commonly known for infesting passerine birds (Pence 1975). Passeriformes is the most diverse of the bird orders, including 60% of birds species (Johansson et al. 2008). The fast generation time and the ability of mites successfully to transfer among hosts, makes them highly prevalent in some bird groups. Our results indicate that *Sternostoma* evolved from within *Ptilonyssus*, which has never been suggested before. *Ptilonyssus* has evolved as an endoparasite in the nasal cavities, with

modifications like other rhinonyssids, such as a reduction of chaetotaxy, body lightly sclerotized and with modified claws for staying embedded in the nasal mucus (Proctor and Owen 2000). *Sternostoma* on the other hand, is considered the most derived because some species migrate into the lungs and have more reduced morphology (chaetotaxy, solenidiotaxi, chelicerae, peritreme and tristosternum) (Fain 1969). However, it is not unreasonable to suggest that *Sternostoma* represents a lineage of *Ptilonyssus* that invaded further into the tracheal system and as a result, evolved more reduction of features. Further rhinonyssid diversity needs to be added into a phylogenetic analysis with our dataset to better test the origins of *Sternostoma.*

In this study we examined phylogenetic relationships of *Ptilonyssus* by looking at some of the species from the genera. Since there have been so few studies on rhinonyssid evolution, we have very few expectations about the evolutionary history of *Ptilonyssus* species. However, morphology has suggested some likely groupings.

Species in clade A, *P. icteridius, P. pirangae, P. morofkyi* and *P. hirsti* all look similar and share the same morphological characteristic of two dorsal plates, a podosomal plate similar shape, an opisthosomal plate present longer than wide and similar type of chelicerae (Pence 1975). *P. morofkyi* differ in having sternal plate more rectangular than longer than wide (Pence 1975).

All four of these *Ptilonyssus* species parasitize families of birds in the infraorder Passerida and have some overlapping host use. *P. icteridius* is a mite that is common in host species of the families Passeridae, Icteridae and Cardinalidae (Hilario-Perez and Dowling 2020). *P. pirangae* is frequently reported from families such as, Cardinalidae, Emberizidae and Paridae, which are also common hosts of *P. hirsti*. The association with Paridae, which is in a different infraorder and phylogenetically disparate from Passerida (Oliveros et al. 2019) is the only outlier in the

hypothesis that these mites use phylogenetically related hosts. *Ptilonyssus morofkyi* is usually found in species of Parulidae, Fringillidae, Passeridae and Emberizidae (Pence 1975). These *Ptilonyssus* mites appear to be common among these different hosts, which might suggest that similar mites tend to infest closely related hosts. This idea agrees with the statement of birds that socialize in groups tend to have more similar infestation of mites and more host specific species occur on more solitary species of birds (Strandtmann 1958).

The *Sternostoma* sp. included in the analysis also seemed to be related to the same clade of *P. icteridius* and related to *P. morofkyi*. It would be interesting to know the species and see the host specificity of both species to see the true relationship.

Clade B represents the *P. sairae complex* and includes 6-8 closely related species, many of which we could not confidently identify further than similar to *P. sairae*. Included within this "complex" clade were some identifiable mites, such as *P. ludovicianus,* which has previously been considered as a member of the *sairae* complex (Pence and Casto 1976). One specimen we believe to be *P. sairae* based upon 97% genetic similarity in ITS sequence to published sequences is also included in the tree. Previous authors have suggested that this group might represent a single mite with very low host specificity or a group mites infesting different hosts with high host specificity (Morelli and Spicer 2007). Morelli and Spicer (2007) suggested that their analyses identified some level of strict cospeciation between bird hosts and *P. sairae* mites and suggested that cryptic speciation is a phenomenon that might be happening among this group of similar mites in wild populations. Due to our inability to confidently put species ID on most of the specimens included in this clade, we cannot say much about the "*sairae"* complex, other than that based upon genetic differentiation, it does appear that the complex includes multiple closely related species that tend to be more specialists than generalists. Much more investigation into

this complex is necessary to understand how these nasal mite group relate and differentiate genetically within their hosts.

Clade C included *P. euroturdi* and *P. taxostomae,* which share morphological characters such as a pair of spine-like setae in the posterior part of podosomal plates and the same form of the chelicerae (Pence 1975). Also, in this group there was an unidentified species sister to *P. euroturdi* but clearly genetically different. Additionally, this mite morphologically differed from all known *Ptilonyssus* species, suggesting this mite is possibly a new species. This species was collected from a brown-headed cowbird, which as a brood parasite has the potential to acquire nasal mites from the host parents, explaining why it is different from the most commonly reported species *P. agelaii* and *P. icteridius*. Further examination of new specimens would be required to support this mite as a new species.

Sister to clades A-C is *P. vireonis* (D), which is a species that appears to show some level of host specificity with the family Vireonidae, hence the name *P. vireonis* (Pence 1975, Knee et al. 2008, Hilario-Perez and Dowling 2020). One specimen identified as *P. vireonis* (AH24) and collected from a yellow-throated vireo, shows genetic differentiation from the other *P. vireonis* specimens and may represent a new species or possibly genetic variation within the species. Taxon sampling was too low to adequately assess typical genetic intra- and interspecies variation.

Both ITS and COI yielded 19 sequences, about half of what was obtained for 16S. Other researchers have also had trouble reliably obtaining ITS and COI from Rhinonyssidae for reasons unknown (Greg Spicer pers. comm.). In order to avoid issues of missing sequences in the dataset, we analyzed each gene dataset separately, but did not do a combined dataset phylogenetic analysis. However, although sampling was much lower, for the taxa that did

overlap between the three genes, both the ITS and COI trees corroborated groupings found in the 16S tree. The mitochondrial 16S rDNA gene has been shown to be useful in different studies of phylogenetic relationship on mites, identification of closely related species, including those within genera of Rhinonyssidae (De Rojas et al 2001). However, it has been suggested that it is not useful for comparing distant related taxa due to the rapidly evolving rate and a high level of homoplasy as a consequence (Whitfield and Cameron 1998).

While we do not have broad enough taxon sampling to elucidate the full phylogeny of *Ptilonyssus*, our phylogeny does corroborate the idea that the "*sairae"* complex is diverse and likely consists of multiple species that are fairly host specific. One of our main issues in this study was our ability to identify specimens based upon morphology. For many of the voucher specimens left over after DNA extraction, the cuticles were badly damaged or missing entirely. Additionally, several of the hosts appeared to have multiple species of *Ptilonyssus*, so we could not be confident that the slide mounted specimens from unextracted specimens were the same species as those we extracted DNA from. Further analyses will require a different handling and extraction methodology to ensure we can recover high quality, identifiable voucher specimens. Also, the existing identification keys appear to have problems and lack some taxa, so there is a need for updated, expanded, and illustrated diagnostic keys for nasal mites.

Future phylogenetic studies on *Ptilonyssus* should include a broader diversity of species and include more representatives of *Sternostoma*, as our current phylogeny indicates that the genus likely evolved from within *Ptilonyssus*. Host-specificity also could play a role in understanding how these mites are related to each other and with their hosts and a larger analysis will better help elucidate this. Lastly, more intensive sampling within the *sairae* complex seems necessary

to better delimit species, understand the cryptic diversity present, and patterns of host association.

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4.11 Table 7

Table 7. List of nasal mites, Passeriformes host and locations from three states (AR, IL, TX) used for the phylogenetic analysis.

Nasal mite	Host	Voucher Number	Location
Ptilonyssus pirangae	Summer tanager	AH1	Springdale, AR
P. pirangae	White throated sparrow	AH2	Conway, AR
P. morofskyi	Song sparrow	AH4	Craighead Co., AR
P. ludovicianus	Indigo bunting	AH5	Craighead Co., AR
P. sairae complex	White throated sparrow	AH6	Rock Island, IL
P. ludovicianus	Indigo bunting	AH7	Rock Island, IL
P. sairae	Song sparrow	AH8	Craighead Co., AR
P. sairae complex	Eastern Towhee	AH9	Rock Island, IL
P. sairae complex	Black and white warbler	AH10	Rock Island, IL
P. sairae complex	Morning warbler	AH11	Rock Island, IL
P. sairae complex	Tennessee warbler	AH12	Rock Island, IL
P. hirsti	Black and white warbler	AH13	Rock Island, IL
P. hirsti	Grey cat bird	AH14	Saline Co., AR
P. icteridius	House sparrow	AH16	Conway, AR
P. taxostomae	Brown thrasher	AH17	Craighead Co., AR
P. sp. unidentifable	Brown-headed cowbird	AH18	Fort Hood, TX
P. icteridius	Brown-headed cowbird	AH19	Fort Hood, TX
P. pirangae	White throated sparrow	AH21	Rock Island, IL
P. icteridius	Brown-headed cowbird	AH22	Fort Hood, TX
P. nr. acrocephali	Scarlet tanager	AH23	Rock Island, IL
P. vireonis	Yellow-throated vireo	AH24	Rock Island, IL
P. nr. vireonis	Red eye vireo	AH25	Rock Island, IL
P. nr. vireonis	Red eye vireo	AH26	Rock Island, IL
P. nr. vireonis	Nashville warbler	AH27	Rock Island, IL
P. sairae complex	Nashville warbler	AH28	Rock Island, IL
P. icteridius	Brown-headed cowbird	AH29	Rock Island, IL
P. nr. acrocephali	Black and white warbler	AH30	Rock Island, IL
*P. euroturdi**		AJ250115.1	
*P. euroturdi**		NC_045208.1	
*P. taxostomae**		AD592	
Sternostoma sp.*		AD515	

*Means specimens from Dowling's laboratory

4.12 Figure 4

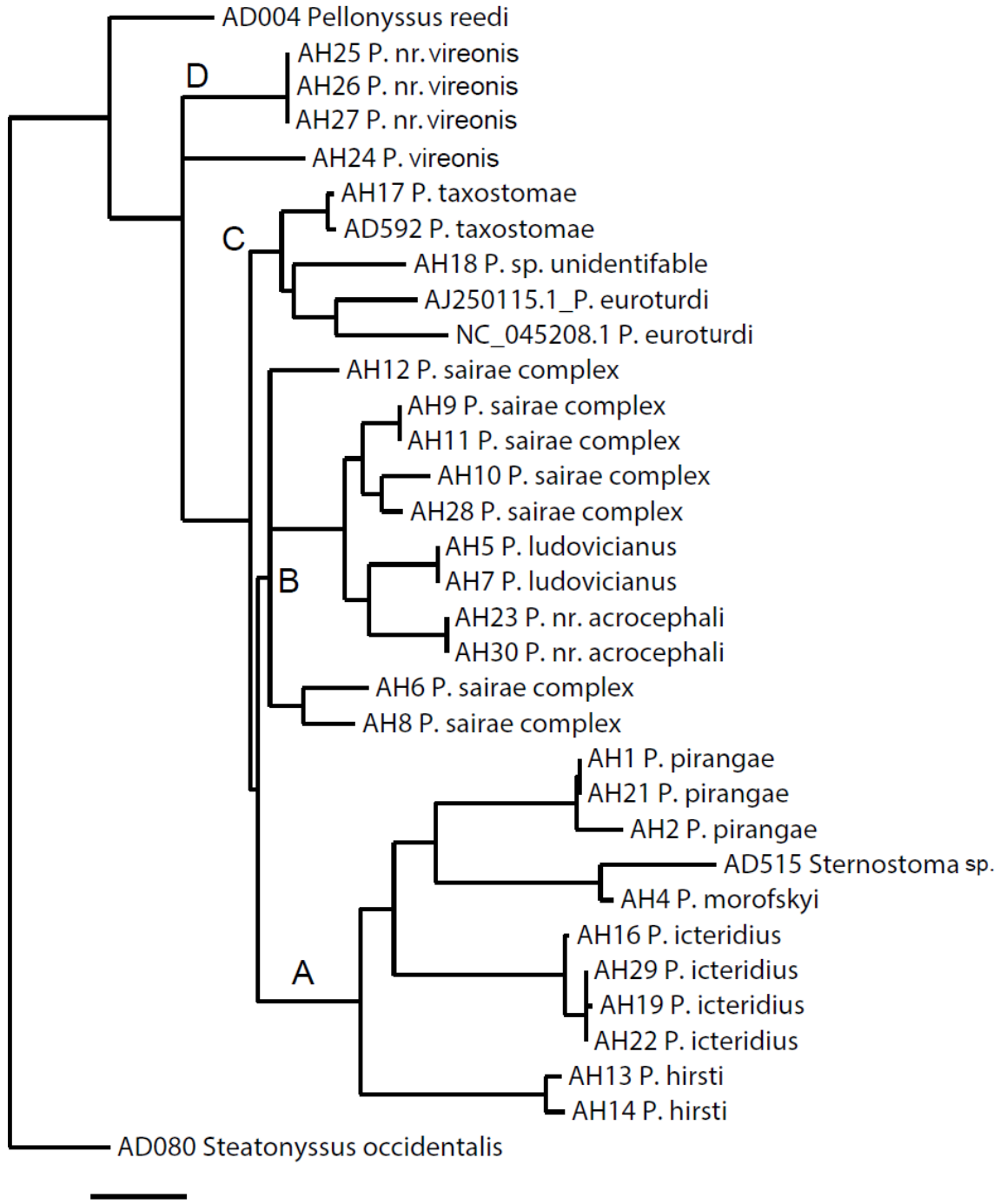

Figure 4. Phylogenetic tree of the *Ptilonyssus* mites 16S rDNA sequences with *Pellonyssus reedi* as an outgroup and rooted with *Steatonyssus occidentalis*.

CHAPTER 5. GENERAL CONCLUSION

In this study we studied diversity, biology and evolutionary relationships of nasal mites in interaction with their bird hosts. We wanted to understand how nasal mite infestation happens in host individuals that use brood parasitism as a reproductive strategy, such as the BHCO that uses other birds as hosts. Our hypothesis was to observe diversity of nasal mites since BHCO infests several hosts and might be acquiring nasal mites from different species, thus having a great diversity of nasal mites. Also, we could see whether nasal mite infestation patterns might differ by geographical location and if location might have consequences in the composition and diversity of nasal mites. Our results presented a different picture where BHCO present similar diversity of nasal mites species that also occur in other icterids. Our results showed that horizontal transmission is likely to be the mechanism that is frequently used by nasal mites because the gregarious behavior of BHCO. We also could think of other factors that might prevent prevalence and host specificity of these less common nasal mites species in BHCO, such as niche competition or fast developmental rate of species, which would contribute for better adaptation to the host of those more abundant nasal mites. Less common nasal mites were found occurring in northern populations suggesting some kind of isolation and maybe a different mechanism of transmission among hosts that are more solitary and could be transferring from parent to offspring.

In our survey of nasal mites infesting BHCO, we studied how prevalence varies by location by studying bird samples from different regions. We learned that populations of BHCO might disperse all around North America and have similarly high prevalence of infestation.

Further research could focus on the study of nasal mites infesting other members from Icteridae, since BHCO tends to be infested by the same mites. It would be interesting to see how

diversity and prevalence varies in other icterids and how host specificity plays a role when looking at infestation at family level.

We wanted to sample as many birds as possible that could be assigned to the four migratory flyways as a parameter of isolation since BHCO populations are widespread all over the country and also show short migration patterns. For a future study, we could try to gather samples that could represent eastern populations, including those further south that tend to spend the entire year and do not migrate during the breeding season. We were not able to find bird specimens that could represent the Atlantic migration flyway and we should also study how prevalence of infestation could be compared to other flyways already surveyed.

Since transmission seems to be a factor of opportunism and it is likely to happen in different ways, study of BHCO's nestling could enlighten on the transmission of mites among birds. It would be interesting to see whether BHCO do have different species of nasal mites when they are young compared to later in life, which is a question that remains to be answered.

In our second study we saw how BHCO move and disperse among different regions and how geneflow might be happening by studying genetic relationship among two of the most common nasal mites found in BHCO. We could see that no isolation of BHCO is happening depending on locations and nasal mites infesting BHCO appear to be genetically similar from different regions showing no differentiation or evidence of isolation. This could mean that at some point these populations connect and likely gathering in large social flocks during winter and breeding seasons.

Further investigation on population genetic studies on the BHCO might be recommended to better understand how geneflow really changes by looking at populations from different regions

that were not included in this study. As we mentioned before, eastern populations might need to be considered where it is likely that we could see some isolation patterns in southeastern BHCO.

This study also could be conducted using other species commonly collected from BHCO such as *Boydaia quiscali* to expand more clades that could support the idea of geographic locations not influencing genetic differentiation. Investigation at family level also applies for these kind of study of population genetic among icterid hosts.

Lastly, in our third study we saw preliminary results of how phylogenetic relationship of nasal mites within the genus *Ptilonyssus* happen. As we were able to see, *Ptilonyssus* are host specific to Passeriformes species and with species of nasal mites also varying in their degree of host specificity within the genus or even families of bird hosts.

Our phylogenetic study did not cover enough species of *Ptilonyssus* to infer on the evolution of the whole genus. However, our results corroborate the "*sairae* complex" as being a group of closely related nasal mites with similar morphological characters that can be very diverse and with some degree of host specificity within their hosts. A complete phylogenetic study with nasal mites from this complex, covering the most common bird hosts in larger scale than previous studies might enlighten on these questions of diversification and differentiation of mites, which helps understanding questions such as cryptic cospeciation.

Other clades in our phylogenetic analysis represented species with some level of host specificity to hosts from closely related family of birds such as Parulidae, Passeridae or Emberizidae. Also supporting the idea that closely related groups of birds that also commute tends to have similar species and infestation prevalence of nasal mites. Others nasal mites that appeared to be sister to other *Ptilonyssus* species were found to be frequently occurring in a

single family such as Vireonidae, also suggesting some variation in the degree of host specificity within the genus.

Further analyses on the phylogenetics of nasal mites are required in order to support the clades found in our tree. For future studies it would be recommended to acquire good vouchers in order to have confident identifications. An extended and updated key for nasal mites covering current identifications compiled is needed to have better morphological identifications. Also, it would be important to extend this kind of study to species of *Sternostoma,* which also is host specific to Passeriformes.

In this study we could see that both ITS and 16S genes were useful for the study of closely related species of nasal mites, although 16S seemed to be the one that yielded more sequences. This study ensure how 16S amplification is a useful tool for inferring phylogeny of closely related species of nasal mites.

In conclusion, there is much to do concerning the study of nasal mites, the amount of hosts that are not yet surveyed just in the United States is a case of study. Expanding in different areas of study, such as biology, taxonomy, ecology, and evolutionary history of these arachnids would be valuable information for workers of these topics, such as acarologists, parasitologists, biologist, ornithologists, and even the general public.