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A novel Haemosporida clade at the rank of genus in North American cranes (Aves: Gruiformes)

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ABSTRACT

The unicellular blood parasites in the order Haemosporida are highly diverse, infect many vertebrates, are responsible for a large disease burden among humans and animals, and have reemerged as an important model system to understand the evolutionary and ecological dynamics of host-parasite interactions. The phylogenetics and systematics of Haemosporida are limited by poor sampling of different vertebrate host taxa. We surveyed the Haemosporida of wild whooping cranes (*Grus americana*) and sandhill cranes (*Grus canadensis*) (Aves: Gruiformes) using a combination of morphological and molecular approaches. We identified *Haemoproteus antigonis* in blood smears based on published morphological descriptions. Phylogenetic analysis based on partial cytochrome *b* (cyt*b*) and cytochrome oxidase (coI) sequences placed *H. antigonis* parasites in a novel clade, distinct from all avian Haemosporida genera for which cyt*b* and/or coI sequences are available. Molecular clock and divergence estimates suggest this crane clade may represent a new genus. This is the first molecular description of *H. antigonis* and the first report of *H. antigonis* in wild whooping cranes, an endangered bird in North America. Further sampling of Haemosporida, especially from hosts of the Gruiformes and other poorly sampled orders, will help to resolve the relationship of the *H. antigonis* clade to other avian Haemosporida genera. Our study highlights the potential of sampling neglected host species to discover novel lineages of diverse parasite groups.

1. Introduction

Haemosporida are protozoan parasites that infect diverse vertebrate host tissues and are vectored by dipteran biting flies. The order contains the agents of human malaria and related parasites. Avian Haemosporida (including the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) have been described from a wide range of host species and geographic localities, with most species descriptions based on morphologic characteristics of blood stages and host range. Over 200 species of avian Haemosporida have been described worldwide in hosts belonging to at least 23 orders, however, birds in the order Passeriformes are over-represented in the literature, while most other orders have been poorly studied. Parasites belonging to the genus *Haemoproteus* are vectored by biting midges and hippoboscid flies, *Plasmodium* by mosquitos, and *Leucocytozoon* by simuliid flies, but the specific vectors for the majority of species are unknown (Valkiunas, 2005).

Recently, many researchers have used molecular techniques to detect Haemosporida, although the depth of screening across host taxa remains very heterogeneous. The MalAvi database has been established as a publicly available repository for Haemosporida sequences of the 5′ end of the mitochondrial cytochrome *b* (cyt *b*) gene (Bensch et al., 2009). Molecular studies have shown some Haemosporida species can infect a broader range of host species than previously thought, including host species in different

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families (Beadell et al., 2006; Bensch et al., 2000; Krizanauskiene et al., 2006). While the advent of these molecular techniques have uncovered an unexpected diversity in Haemosporida and their interactions with avian hosts, the increasing use of molecular techniques to identify Haemosporida infections has led to numerous sequences in the GenBank (http://www.ncbi.nlm.nih.gov) and MalAvi databases that are identified only to genus. This underscores the importance of combining molecular and morphologic descriptions to accurately identify species.

Phylogenies based on molecular data can vary widely depending on the species included and the method of analysis (Perkins, 2014). There are wide discrepancies in the literature as to the phylogenetic relationship of Haemosporida genera, depending on the gene sequence(s) analyzed, the method of analysis, and the Haemosporida species included in the analysis. Analysis of parasite cyt *b* sequences from a variety of bird, lizard, and mammal hosts show *Plasmodium* is paraphyletic, forming one clade with *Hepatocystis* and a second clade with *Haemoproteus* (Perkins and Schall, 2002). A recent study by Lutz et al. (2016) also suggests that *Plasmodium* is paraphyletic. In contrast, analysis of four genes showed avian *Haemoproteus* fall into two clades which are sister to *Plasmodium* (Martinsen et al., 2008). Alternatively, phylogenetic analyses of only avian parasites show the subgenus *Haemoproteus* (*Haemoproteus*) as a sister clade to *Plasmodium* and *Haemoproteus* (*Parahaemproteus*) (Santiago-Alarcon et al., 2010; Valkiunas et al., 2014). Additionally, a group of parasites from raptors formed a unique clade not closely related to *Plasmodium* or *Parahaemoproteus* (Outlaw and Ricklefs, 2009). All of these phylogenetic hypotheses, developed with maximum-likelihood or Bayesian techniques, place *Leucocytozoon* as an outgroup to *Plasmodium* and *Haemoproteus*. However, an analysis using relaxed molecular clock methods showed *Plasmodium* as paraphyletic with two major subgroups: mammalian *Plasmodium* and *Hepatocystis*, and avian *Plasmodium*, *Leucocytozoon*, *Haemoproteus*, and *Parahaemoproteus* (Outlaw and Ricklefs, 2011). In contrast, Borner et al. (2016) analyzed a set of 21 nuclear genes, and the resulting phylogeny showed *Plasmodium* as monophyletic and *Leucocytozoon* in a basal position to the rest of the Haemosporida. Further complicating the picture, the majority of researchers in North America use PCR assays targeting the 3′ end of the cyt *b* gene (Fallon et al., 2003; Ricklefs et al., 2005), whereas the majority of researchers in Europe use PCR assays targeting the 5′ end of the cyt *b* gene (Bensch et al., 2009, 2000), with inadequate overlap to compare sequences generated with different assays. While both portions of the gene are represented in GenBank, the MalAvi database consists of only sequences generated with assays targeting the 5′ end (Bensch et al., 2009).

Phylogenetic relationships are greatly influenced by the taxa included in the analysis, and the majority of published avian Haemosporida sequences were recovered from passerine and columbiform hosts, while studies of hosts in other orders are severely lacking. Two crane species (Gruidae, order: Gruiformes) occur in North America, the endangered whooping crane (*Grus americana*) and the abundant sandhill crane (*G. canadensis*). Whooping cranes are restricted to a remnant of their historic range, breeding in pothole wetlands in Wood Buffalo National Park, Alberta and Northwest Territories, Canada, and wintering in coastal salt marshes in and around Aransas National Wildlife Refuge, Texas, USA (Johnsgard, 1983; Wilson et al., 2016). Sandhill cranes have a wider distribution and can be found throughout North America. The mid-continent population of sandhill cranes is sympatric with whooping cranes, however, sandhill cranes are adaptable to a variety of wetland conditions for breeding and prefer ponds near agricultural fields for wintering (Johnsgard, 1983; Kruse et al., 2011). Although microhabitats differ, whooping cranes and sympatric sandhill cranes are likely exposed to similar vector communities, and therefore have similar exposures to Haemosporida infection.

Prior studies of Haemosporida in cranes of North America are based on examination of blood smears and include descriptions of *Haemoproteus antigonis*, *Haemoproteus balearicae*, *Plasmodium-polare*-like, and *Leucocytozoon grusi* in sandhill cranes (Bennett et al., 1975, 1974; Dusek et al., 2004; Lee et al., 1985), and *Haemoproteus antigonis* in a small number of non-migratory whooping cranes in Florida (Forrester and Spalding, 2003). We recently showed a high prevalence of *Haemoproteus antigonis* in the only self-sustaining migratory population of whooping cranes (the Aransas-Wood Buffalo population; AWBP) and in sympatric sandhill cranes, suggesting these two populations are exposed to similar vector communities (Bertram et al., 2016). Associated with our broad surveys of parasites infecting AWBP whooping cranes (Bertram et al., 2015), we identified *Haemoproteus antigonis* on several blood films based on morphology. We also present a phylogenetic analysis of the Haemosporida that challenges the placement of *H. antigonis* in the genus *Haemoproteus*. Instead, our analysis suggests these parasites represent a novel evolutionary lineage of parasites identified in North American cranes, and highlight the importance of sampling neglected vertebrate taxa to resolve the evolutionary relationships of malaria parasites and related Haemosporida.

2. Methods

2.1. Sample collection

Whooping crane blood samples were collected by one of the authors (BKH) as part of an ongoing telemetry and health monitoring study of the AWBP whooping cranes (Pearse et al., 2015). Samples included in this study were collected between December 2009 and February 2014. Pre-fledging juveniles (hatch year, HY, 40–60 days old) were hand captured during the summer at Wood Buffalo National Park (WBNP). Adults (after hatch year, AHY) were captured using a remote triggered snare during the winter at Aransas National Wildlife Refuge (ANWR). Captured birds were manually restrained and blood was drawn from the jugular vein. A blood smear was made immediately after sample collection, air dried, and fixed with methanol within 8 h in the field. An aliquot of whole blood was preserved in Longmire's buffer (0.1 M Tris, 0.1 M EDTA, 0.01 M NaCL, 0.5% SDS, pH 8.0). All whooping crane field techniques were approved by a University of Wisconsin Animal Care and Use Committee (protocol no. V01506-0-10-11).

We collected blood samples from hunter-harvested sandhill cranes at necropsy between November 2012 and January 2014 through relationships with the Texas Parks and Wildlife Department, New Mexico Department of Game and Fish, and private hunting clubs and outfitters. Age (hatch-year or after-hatch-year) was determined based on plumage. All birds were either subjected to necropsy in the field immediately post-harvest or frozen at −20 °C immediately post-harvest and subjected to necropsy in the laboratory at a later date. Each carcass was subjected to a full gross necropsy, at which time we collected a blood sample, either whole blood or blood clot which had pooled in the coelomic cavity. Blood samples were frozen at −20 °C until DNA extraction. Due to clotting and lysis in the blood samples we were unable to make blood smears for sandhill cranes.

2.2. Morphologic detection of Haemosporida

Approximately 2 cm^2 of the red blood cell monolayer on each blood smear was examined at low magnification (500×), and at least 100 fields were examined at high magnification (1250×), as

recommended by Valkiunas (2005). Each blood smear was examined for 15–20 min, and any parasites noted were examined and measured at 1250×. The number of parasites detected was also noted. Morphologic identification of parasites was determined using a published taxonomic key (Valkiunas, 2005).

We received *Haemoproteus antigonis* paratype and voucher blood films from the U. S. National Parasite Collection (#72637, #94499) for comparison with the Haemosporida noted in this study. Blood films and parasites were examined as described above.

2.3. Molecular detection of Haemosporida

2.3.1. DNA extraction

DNA was extracted from 100 µl of whole blood or blood clot using the E.Z.N.A Tissue Extraction kit (Omega Biotek, Norcross, GA) following the manufacturer's instructions for tissue extraction with modifications including an overnight lysis step at 55 °C and elution into 100 µl of elution buffer. Due to clotting in many samples, overnight lysis was necessary to ensure complete lysis of the clot and maximum availability of DNA for the remainder of the extraction. We used a smaller volume of elution buffer than indicated in the manufacturer's instructions to create a higher concentration of DNA and increase the likelihood that rare parasite DNA would be present in the volume of sample used for PCR analysis.

2.3.2. Haemosporida screening

First, *Plasmodium* and *Haemoproteus* infections were detected using a nested PCR reaction targeting an approximately 500 bp region of the 3′ end of the cyt *b* gene. The first PCR reaction used the primers 3932F (Fecchio et al., 2013) and DW4 (Perkins and Schall, 2002) at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix E (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, 0.1 µg/µl BSA, and 1 µl of sample template. The second PCR reaction used the primers 413F and 926R (Ricklefs et al., 2005) at a concentration of 0.2 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR was used as the template. In both rounds of PCR, cycling parameters were as described by Fecchio et al. (2013). A sample collected from a northern cardinal (*Cardinalis cardinalis*) infected with *Plasmodium* was used as a positive control (Medeiros et al., 2013).

To generate a longer portion of the gene, we also used a nested PCR reaction targeting an approximately 700 bp region of the 5′ end of the cyt *b* gene. The first PCR reaction used the primers DW2 and DW4 (Perkins and Schall, 2002) at a concentration of 0.4 µM in a 15 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.15 µl FailSafe Enzyme, and 1 µl of sample template. The second PCR used the primers LeucoF and LeucoR (Sehgal et al., 2006) at a concentration of 0.4 µM in a 20 µl reaction. Remaining reaction components consisted of 1X FailSafe PCR Premix B (Epicentre, Madison, WI), 0.2 µl FailSafe Enzyme, and 2 µl of the product from the first PCR, diluted 1:20. In both rounds of PCR, cycling parameters were as described by Sehgal et al. (2006).

Finally, we used a nested PCR reaction targeting an approximately 900 bp region of the mitochondrial cytochrome oxidase subunit I (coI) gene. The first PCR reaction used the primers *coI*/outerF and *coI*/outerR (Martinsen et al., 2008) at a concentration of 0.3 µM in a 15 µl reaction with remaining reaction components as outlined above. The second PCR used the primers *coI*/nestedF and *coI*/nestedR (Martinsen et al., 2008) at a concentration of 0.3 µM in a 15 µl reaction. Remaining reaction components were identical to the first PCR, except 1 µl of the product from the first PCR, diluted 1:20, was used as the template. In both rounds of PCR, cycling parameters were as described by Martinsen et al. (2008). The same positive control used for cyt *b* PCR reactions was also used for coI PCR reactions.

2.4. Sequencing and phylogenetic analyses

Haemosporida infections were visualized on 1.5% agarose gel, and amplicons of positive samples were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Purified samples were submitted for bi-directional sequencing to Eton Bioscience Inc. (San Diego, CA). Forward and reverse sequences were aligned and agreement between sequences was determined using Clustal W within Mega 6.0 (Tamura et al., 2013). Only samples for which forward and reverse sequences agreed were used in phylogenetic analysis. Samples were considered positive if a DNA sequence was obtained for which the identity matched most closely to a Haemosporida species in GenBank. Chromatographs were examined manually, and sequences with double nucleotide peaks were separated using phasing. For samples with double nucleotide peaks, sequences containing all possible combinations of nucleotides at the base pairs with double nucleotide peaks were created and compared to clean sequences generated in this study and in GenBank. The two sequences which were identical to known sequences or good-quality sequences generated in this study were used in the phylogenetic analysis. For samples which produced a sequence for both cyt *b* assays, sequences were aligned for each sample and a consensus sequence was generated. Sequences for the cyt *b* amplicons overlapped by approximately 400 bp, resulting in an approximately 800 bp consensus sequence. All sequences were compared to known Haemosporida sequences using the BLAST tool in GenBank and were aligned with the closest matches and additional publicly available avian Haemosporida species sequences representative of unique clades in previous studies (Beadell et al., 2006; Hellgren et al., 2007; Ishak et al., 2008; Martinsen et al., 2008; Medeiros et al., 2014; Outlaw and Ricklefs, 2010; Perkins and Schall, 2002). We also compared cyt *b* sequences to previously published avian Haemosporida sequences using the BLAST tool in MalAvi, however the longer sequences available in GenBank were used for phylogenetic analysis. After alignment, sequences were cropped to the same length at the first conserved base-pair closest to each end of the sequence. Samples with poor quality sequences in one or both directions were excluded from phylogenetic analysis. Representatives of all unique sequences produced during this project and utilized in the phylogenetic analysis were deposited in GenBank (Accession #KX223839 – KX223846) (Table 1).

Due to variation in evolutionary rates between haemosporidian clades, methods that allow for rate variation within a data set have a greater effect on tree topology than do models of nucleotide substitution (to a point; that is, that more complex models are always required; see Outlaw et al., 2015). Using BEAST (v.1.7; (Drummond et al., 2012), we reconstructed phylogenetic trees (GTR + I + Γ, Yule process, 10,000,000 generations sampling every 1000 trees) under two sets of priors: one with a strict molecular clock and one with a relaxed (uncorrelated lognormal) molecular clock. After determining that model parameter values were stable (ESS > 200, Tracer v1.6; (Rambaut et al., 2014), we calculated BayesFactors (in Tracer v1.6) to determine which clock model provided a better estimate of the data. Using TreeAnnotator (Drummond et al., 2012), we reconstructed the maximum clade credibility tree and then visualized the tree using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Divergence estimates

Using uncorrected distances, we calculated relative dissimilarity matrices within and between all major groups of parasites from

Table 1
Results of blood film examination and PCR assays targeting avian Haemosporida for 27 AWBP whooping cranes. Hatch year (HY) birds (40–60 days old) were sampled on the breeding grounds, whereas adults (after hatch year, AHY) were sampled on the wintering grounds.

Crane ID	Age	Sex	Blood film	cyt *b* (724 bp)	coI (370 bp)
W01	HY	F	None	0	*Plasmodium*
W02	HY	M	None	*H. antigonis*	*Plasmodium*
W03	HY	M	None	0	0
W04	HY	F	None	*Plasmodium*	0
W05	HY	F	None	0	*Plasmodium*
W06	HY	F	*H. antigonis*	*H. antigonis* (KX223839)	0
W07	HY	M	None	0	0
W11	HY	M	*H. antigonis*	*H. antigonis*	*H. antigonis* (KX223845)
W12	HY	F	None	0	*Plasmodium*
W14	AHY	F	None	*H. antigonis*	*Plasmodium*
W15	AHY	F	None	0	0
W18	AHY	M	*H. antigonis*	*H. antigonis* (KX223840)	*H. antigonis*
W19	AHY	M	*H. antigonis*	*H. antigonis*	0
W20	AHY	F	None	0	0
W21	AHY	F	None	0	*H. antigonis*
W22	AHY	F	None	0	*H. antigonis*
W23	AHY	M	*H. antigonis*	*H. antigonis* (KX223841)	*H. antigonis* (KX223846)
W24	AHY	U	*H. antigonis*	0	*H. antigonis*
W26	AHY	U	None	0	*H. antigonis*
W27	AHY	U	None	*H. antigonis*	0
W28	AHY	U	None	0	0
W30	AHY	U	*H. antigonis*	0	0
W31	AHY	U	None	0	0
W32	AHY	U	*H. antigonis*	0	0
W33	AHY	U	*H. antigonis*	0	*Plasmodium*
W51	AHY	M	None	*H. antigonis* (KX223844)	*H. antigonis*
W55	HY	M	None	*Plasmodium*	*Plasmodium*

the phylogenetic analyses, in order to determine whether the distinctiveness of the crane parasite clade was similar to that between clades of other haemosporidian genera. (Note that our purpose here is not to calculate divergence times [i.e., molecular clock estimates].) The groups we compared were: crane (i.e., *Haemoproteus antigonis*; see below) versus non-crane parasites (all other sequences included in the study), crane parasites versus *Leucocytozoon* parasites, crane parasites versus *Haemoproteus* parasites, crane parasites versus *Parahaemoproteus* parasites, and crane parasites versus *Plasmodium*/*Polychromophilus* parasites.

3. Results

3.1. Morphological screening

We examined blood smears from 27 whooping cranes (Table 1). We noted Haemosporida infection on nine (33.3%) blood smears on microscopy, none of which had morphologic evidence of mixed infection. All infections showed low parasitemia (<1 parasite per 1000 red blood cells). Macrogametocyte description and dimensions noted in this study were compatible with *Haemproteus antigonis* noted on paratype and voucher blood films and with previously published descriptions (Fig. 1, Table 2) (Bennett et al., 1975; Valkiunas, 2005); therefore we conclude that the noted parasite is *Haemoproteus antigonis*.

3.2. Molecular analysis

We screened 61 whooping crane and 102 sandhill crane samples. We obtained 724 bp consensus DNA sequences from the cyt *b* gene of *Haemoproteus antigonis* for 14 samples and 370 bp sequences from the coI gene of *H. antigonis* for 22 samples from both crane species, which were used in the phylogenetic analysis. Additionally, we obtained sequences from the coI gene of *Plasmodium* spp. for 11 samples from both crane species which were also included in phylogenetic analysis. Table 1 shows the results of the PCR assays and GenBank accession numbers for the 27 whooping cranes for which we also had blood smears. Of the nine samples for which we identified *H. antigonis* on blood smear, three had sequences from *H. antigonis* for the cyt *b* or coI gene, three had sequences for both, one had a sequences from the coI gene for *Plasmodium*, and two did not produce a sequence for either gene.

3.2.1. Phylogenetic analyses

The data were best described with a relaxed molecular clock (BayesFactors $\Delta = 37.64$). Phylogenetic relationships between major clades were not well supported for the cyt *b* gene, but generic clades were very well supported with posterior probabilities of 1 in most cases, including the crane parasites (Fig. 2, Fig. S1). *Plasmodium*, *Haemoproteus*, *Parahaemoproteus*, and *Leucocytozoon* were each monophyletic, with *Leucocytozoon* and *Haemoproteus* forming a clade sister to the clade formed by *Plasmodium* and *Parahaemoproteus*. *Haemoproteus antigonis* sequences recovered from sandhill crane samples were identical to *H. antigonis* sequences recovered from whooping crane samples, and *H. antigonis* formed a novel clade at the level of genus, sister to *Plasmodium* and *Parahaemoproteus*. *Haemoproteus antigonis* differed from other Haemosporida included in the analysis at many locations throughout the analyzed region of the gene (Fig. S2). Analysis of the coI gene also supported the placement of *H. antigonis* in a unique clade, however, support values were low (Fig. S3).

3.2.2. Divergence estimates

Uncorrected distances within crane parasites ranged from 0.008 to 0.0014 and that between crane parasites and other genera ranged from 0.075 to 0.198 (Table 3).

4. Discussion

Using a combination of morphologic and molecular methods, we detected for the first time Haemosporida infection in the Aransas-Wood Buffalo population of whooping cranes. Parasites observed on blood smears from AWBP whooping cranes were identified as *Haemoproteus antigonis*, and we provide the first molecular characterization of the species. Sequences recovered from sandhill crane samples were identical to *H. antigonis* sequences recovered

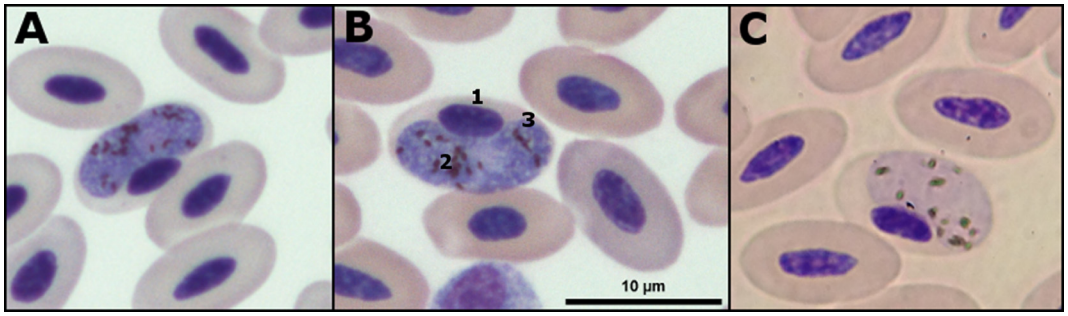

Fig. 1. Crane Haemosporida identified as *Haemoproteus antigonis*. (A and B) Mature macrogametocyte. 1 – Lateral displacement of host cell nucleus. 2 – Pigment granules, usually 20 or less. 3 – Gametocyte slightly encloses, but never completely encircles the host cell nucleus. (C) Mature microgametocyte.

Table 2
Morphometric parameters of gametocytes and host erythrocytes of *Haemoproteus antigonis*. Parasites noted on WHCR blood films in this study and *H. antigonis* noted on paratype and voucher blood films are listed along with previously published *H. antigonis* parameters. Length and width are given in micrometers. NDR is the nucleus displacement ratio, calculated as described in Valkiunas (2005).

Parameter	This study			*H. antigonis* voucher			Bennett et al. (1975)		
	n	mean	sd	n	mean	sd	n	mean	sd
Uninfected erythrocyte	30			30			50		
Length		14	1.1		13.3	1.2		13.7	1
Width		7.6	0.5		7.3	0.7		7.5	0.5
Nucleus length		6.2	0.8		6.2	0.6		6.1	0.6
Nucleus width		2.7	0.4		2.8	0.5		2.8	0.3
Erythrocyte parasitized by macrogametocyte	30			30			50		
Length		14.9	1.1		13.4	1.0		13.6	1.2
Width		8.6	0.7		8.3	0.6		8.5	1.1
Nucleus length		5.8	0.8		6.0	0.5		5.9	0.8
Nucleus width		2.5	0.3		2.6	0.4		2.4	0.4
Erythrocyte parasitized by microgametocyte	9			15					
Length		14.1	1.2		13.6	1.1			
Width		8.2	0.5		8.6	0.8			
Nucleus length		5.2	0.7		5.9	0.5			
Nucleus width		2.7	0.3		2.7	0.3			
Macrogametocyte	30			30			50		
Length		14.1	1.4		13.8	1.3		13	2
Width		4.7	0.5		4.6	0.9		4.8	1.1
Nucleus length		2.5	0.3		3.0	0.4		3.6	0.9
Nucleus width		2.1	0.3		1.9	0.4		3.4	1.1
No. pigment granules		20.5	2.8		18.7	3.7		19.3	4
NDR		0.4	0.2		0.4	0.1		0.4	
Microgametocyte	9			15					
Length		14.5	2.1		14.1	1. 6			
Width		4.6	0.7		4.3	0.6			
Nucleus length				11	6.4	1.5			
Nucleus width				11	2.9	0.6			
No. pigment granules		17.1	2.9		15.0	2.5			
NDR		0.4	0.2		0.5	0.2			

from whooping crane samples. We have reported a high prevalence of *H. antigonis* in whooping cranes and sympatric sandhill cranes detected via PCR, including mixed infections with two strains of *H. antigonis* or with *H. antigonis* and *Plasmodium* (Bertram et al., 2016). In this study, we recovered *H. antigonis* sequences for six (66%) samples for which we also identified *H. antigonis* on blood smear, however, we did not recover sequences for two samples. All infections showed low parasitemia, and parasite DNA may not have been present in the blood sample extracted for DNA or in the aliquot used for PCR for these samples. Additionally, we identified *H. antigonis* on blood smear, but recovered a *Plasmodium* sequence for one sample, indicating mixed infection. Additionally, we recovered DNA sequences from 12 (44%) samples for which we did not identify a parasite on blood smear. This was expected, because PCR can be more sensitive than microscopy, especially when parasitemia is low (Valkiunas et al., 2008).

Our results are similar to previous studies showing monophyly of the common haemosporidian genera (Borner et al., 2016; Martinsen et al., 2008; Santiago-Alarcon et al., 2010; Valkiunas et al., 2014). However, as in these previous studies, we found poor support for relationships between the major clades. The difficulty in resolving the deep phylogenetic relationships among Haemosporida is due in part to poor taxonomic sampling (Perkins, 2014) and uncertainty about the correct out-group for rooting the tree (Outlaw and Ricklefs, 2011). We used an outgroup-free method for our analysis to avoid *a priori* assumptions about root placement, and we included sequences representative of unique clades in previous studies (Martinsen et al., 2008; Outlaw and Ricklefs, 2010; Perkins and Schall, 2002). Unfortunately, many published sequences were only identified to genus, highlighting the need for more studies combining morphological and molecular identification of Haemosporida. This study helps to resolve relationships among Haemosporida by providing molecular information for the morphospecies *H. antigonis*, isolated from the poorly sampled Gruiformes taxon.

Haemoproteus antigonis was not previously represented in either the GenBank or MalAvi database, and our *H. antigonis*

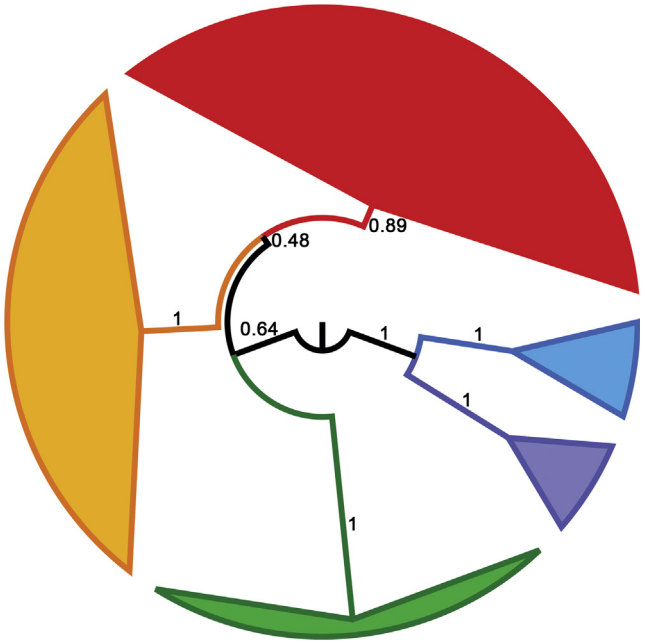

Fig. 2. Phylogenetic relationships between major clades (putative genera). Posterior probability values are indicated on branches. Colors correspond to genera: *Haemoproteus* (blue), *Leucocytozoon* (purple), *Parahaemoproteus* (orange), *Plasmodium* (and close relatives, red), Crane parasites (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Uncorrected (p) distances between crane parasites and other clades (i.e., genera).

Comparison	Uncorrected distances
Cranes-*Leucocytozoon*	0.158–0.197
Cranes-*Haemoproteus*	0.087–0.135
Cranes-*Parahaemoproteus*	0.080–0.113
Cranes-*Plasmodium*/*Polychromophillus*	0.075–0.198

sequences formed a novel clade when analyzed with previously published avian Haemosporida sequences. However, our molecular data cannot support inclusion of the novel clade in the genus *Haemoproteus*. The novel clade forms a polytomy with all Haemosporida. Haemosporida taxonomy based on morphologic characteristics, host species, and geographic location is not always congruent with relationships based on molecular analysis (Perkins, 2014). For example, Martinsen et al. (2007) found that parasites of the subgenus *Plasmodium* (*Giovanolaia*) did not form a monophyletic group, whereas the subgenera *Haemameba*, *Huffia*, and *Bennettinia* were monophyletic. Our results indicate *H. antigonis* is divergent from the rest of the genus *Haemoproteus*, and highlight the need for increased sampling of diverse avian taxa. Many of the avian Haemosporida species described molecularly to date were isolated from passerines and doves, and our novel clade may reflect evolutionary differences between the parasites of these divergent groups. Although the vectors for many avian Haemosporida, including *H. antigonis*, are unknown, the clade might reflect differences in the vector communities encountered by cranes and passerines.

The discovery of new species and even genera is becoming commonplace in haemosporidian research as the field continues to increase the host-taxonomic and geographic breadth of sampling. The phylogenetic uniqueness of the crane parasites compares with other recent discoveries in raptorial birds (Krone et al., 2008; Outlaw and Ricklefs, 2009) and white-tailed deer (Martinsen et al., 2016), to name a few, and is most likely a new genus. However, as in these prior studies, the placement of the putative genus of crane parasites is unclear. This is likely the result of "undiscovered" diversity in neglected host taxa. The phylogenetic tree of Haemosporida parasites is incomplete, and will likely change as we continue to sample more hosts. Further sampling of Haemosporida, especially from poorly sampled host taxa, will help to resolve the relationship of *H. antigonis* to other avian Haemosporida genera, and will help to resolve the deep phylogenetic relationships among haemosporidians.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.12.025.

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