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# Genetic sequence data reveals widespread sharing of *Leucocytozoon* lineages in corvids

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**Abstract** *Leucocytozoon*, a widespread hemosporidian blood parasite that infects a broad group of avian families, has been studied in corvids (family: Corvidae) for over a century. Current taxonomic classification indicates that *Leucocytozoon sakharoffi* infects crows and related *Corvus* spp., while *Leucocytozoon berestneffi* infects magpies (*Pica* spp.) and blue jays (*Cyanocitta* sp.). This intrafamily host specificity was based on the experimental transmissibility of the parasites, as well as slight differences in their morphology and life cycle development. Genetic sequence data from *Leucocytozoon* spp. infecting corvids is scarce, and until the present study, sequence data has not been analyzed to confirm the current taxonomic distinctions. Here, we predict the phylogenetic relationships of *Leucocytozoon* cytochrome *b* lineages recovered from infected American Crows (*Corvus brachyrhynchos*), yellow-billed magpies (*Pica nuttalli*), and Steller's jays (*Cyanocitta stelleri*) to explore the host specificity pattern of *L. sakharoffi* and *L. berestneffi*. Phylogenetic reconstruction revealed a single large clade containing nearly every lineage recovered from the three host species, while showing no evidence of the expected distinction between *L. sakharoffi* and *L. berestneffi*. In addition, five of the detected lineages were recovered from both crows and magpies. This absence of the previously described host specificity in corvid *Leucocytozoon* spp. suggests that *L. sakharoffi* and *L. berestneffi* be reexamined from a taxonomic perspective.

**Keywords** Hemosporidian parasite · *Leucocytozoon* · Host specificity · Corvid

## Introduction

Leucocytozoids are abundant blood parasites that exclusively infect birds. They are known to infect a wide range of avian host species across a wide diversity of habitats (Valkiūnas 2005). Many *Leucocytozoon* species are pathogenic to both wild and domestic birds, so understanding their diversity and pathogenicity could have vital conservation implications (Morii 1992; Hunter et al. 1997; Smith et al. 1998; Sehgal et al. 2006a, b; Bunbury et al. 2007). As with other avian blood parasites, leucocytozoids must utilize a vector and host to complete their life cycle; however, the specificity of the host-vector-parasite interaction of *Leucocytozoon* species remains the least studied of the three major avian hemosporidian parasites (*Haemoproteus*, *Plasmodium*, *Leucocytozoon*). Leucocytozoids are vectored by blood-sucking black flies (Diptera: Simuliidae), and with over 1800 recognized black fly species, *Leucocytozoon* has a large number of potential vectors (Crosskey and Howard 2004; Hellgren et al. 2008). An investigation of black fly blood meals revealed that individual species tend to target either mammals or birds and that ornithophilic species preferentially target large and abundant host species (Malmqvist et al. 2004). Based on these criteria,

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the authors conclude that corvids are an expected host for black flies.

Recent studies have suggested that corvids may be an important reservoir for avian blood parasites (Kim and Tsuda 2010; Leclerc et al. 2014). Corvids (family: Corvidae) are large, gregarious birds that are well adapted to life around humans (Johnston 2001). Increasing urbanization and sprawl favors birds that are able to exploit anthropogenic resources, thereby contributing to increased global populations (Marzluff et al. 2001; Marzluff and Neatherlin 2006). The expansion of cities has reinforced the importance of understanding the effects of urbanization on the ecology of wildlife-parasite interactions (Bradley and Altizer 2007). Knowledge of blood parasite-bird interactions in urban areas is characterized by contradictory findings on parasite prevalence, diversity, and host impact (reviewed in Delgado-V and French 2012). Urban-exploiting corvids represent an important host for further study of blood parasite ecology in an increasingly urbanized world.

Much of the current understanding of *Leucocytozoon* parasites infecting corvids is either outdated or incomplete. Over the past century, many studies have documented *Leucocytozoon* in corvids; however, these studies have relied solely upon blood smear or tissue sample examinations, detecting a range of prevalence from 2.7 to 36.4 % (Morgan and Waller 1941; Jones 1968; Murata 2002; Leclerc et al. 2014). In contrast, a recent study using molecular techniques detected both high prevalence (93.6 %) and a high diversity of *Leucocytozoon* parasites in corvids (Yoshimura et al. 2014). This and other studies revealed that leucocytozoids exhibit genetic diversity exceeding that observed in other blood parasites (i.e. *Plasmodium* and *Haemoproteus* spp.) (Martinsen et al. 2006, 2008; Sehgal et al. 2006a, b; Jenkins and Owens 2011). The detection of genetic diversity has led to the recent characterization of a cryptic species complex of the avian blood parasite *Plasmodium homocircumflexum*; in this case, the newly discovered species differs from its morphologically identical counterpart species in multiple ways, namely species-level genetic differences and increased pathogenicity in experimentally infected birds (Palinauskas et al. 2015). Mounting evidence suggests that *Leucocytozoon* could harbor undiscovered cryptic species as well (Bensch et al. 2004; de León and Nadler 2010; Lotta et al. 2013). A reexamination of *Leucocytozoon* in diurnal raptors, for example, yielded probable cryptic speciation, although morphological distinctions were later observed (Sehgal et al. 2006a; Valkiūnas et al. 2010). It is essential, therefore, to clarify the relationships among potential cryptic species infecting corvids.

The majority of *Leucocytozoon* species are specific only to the level of host family or order (Valkiūnas 2005). In contrast, there are two *Leucocytozoon* species specific to the corvid host family. Both described over a century ago, *Leucocytozoon sakharoffi* Sambon, 1908, was initially

distinguished from *Leucocytozoon berestneffi* Sambon, 1908, based on how completely the host cell nucleus surrounds the parasite (Bennett and Peirce 1992). This initial morphological difference, however, did not bear out under further analysis. Subsequent studies revealed that the two parasite species could not be microscopically differentiated based on morphology of gametocytes or host cell (Ramisz 1962; Bennett and Peirce 1992; Valkiūnas 2005). However, other distinguishing features emerged to allow for the continued separation of the two corvid leucocytozoids: species differences in hepatic merogony, pre-patent period, and host transmissibility (Clark 1965; Khan and Fallis 1971; Valkiūnas 2005). *Leucocytozoon* might exhibit host specificity among the corvids: *Leucocytozoon* spp. found in American Crows and Common Ravens (*Corvus brachyrhynchos*, *Corvus corax*) were not experimentally transmitted to a blue jay (*Cyanocitta cristata*), and vice versa (Khan and Fallis 1971). In addition, the meronts described from the liver of *C. cristata* were determined to be identical in form and size to those found in magpies (*Pica* sp.) (Clark 1965; Valkiūnas 2005). Therefore, the original taxonomic description remains in place, with two *Leucocytozoon* parasites specific to the corvid family: *L. sakharoffi* infecting birds from the genus *Corvus*, and *L. berestneffi* restricted to corvids from the *Cyanocitta* and *Pica* genera (Valkiūnas 2005). This classification, however, is not universally accepted within the field of avian parasitology. A contradictory stance was taken by Bennett and Peirce (1992), who, after careful review of multiple historical studies, determined *L. berestneffi* to be a junior synonym of *L. sakharoffi*.

In this study, we used genetic data to clarify discrepancies in the literature regarding *Leucocytozoon* host specificity in corvids. Using molecular techniques and analyses, we investigated the specificity and diversity of *Leucocytozoon* in populations of American Crows (*C. brachyrhynchos*), yellow-billed magpies (*Pica nuttalli*), and Steller's jays (*Cyanocitta stelleri*). We used a fragment of the cytochrome *b* gene (cyt *b*) from the mitochondrial DNA (mtDNA) to infer the phylogenetic relationship of *Leucocytozoon* sequences obtained from these populations. We hypothesized that distinct phylogenetic clustering of *Leucocytozoon* lineages according to host species would reflect the taxonomic separation of *L. sakharoffi* and *L. berestneffi*. Our study is the first to use *Leucocytozoon* cyt *b* haplotypes from corvid populations to investigate host specificity in this avian family.

## Methods

### Study area and sampling

Blood samples were collected from 198 American Crow nestlings from 82 nests in Davis, CA, in 2013 ($n = 110$ nestlings

from 45 nests) and 2014 ($n = 88$ nestlings from 37 nests). The study site spanned the urban campus of the University of California, Davis, into the surrounding campus-owned agricultural areas (e.g., vineyards, pasture, and row crops: described in Townsend and Barker 2014). Nests were situated on lateral tree branches and accessed by boom lift. Birds were sampled 10–28 days after hatching. Adult crows were captured in Davis, CA, using Australian drop-in crow traps in January of 2014 ($n = 16$ crows) and 2015 ($n = 44$ crows). Adults were captured from a large, overwintering urban flock (>7000 birds), comprising both residents and migratory birds (Hinton et al. 2015). Blood samples were taken from the jugular vein of the crows using 27 gauge ½ inch needles and 1-mL syringes. Samples were placed immediately in Queen's lysis buffer (Seutin et al. 1991) at an approximate rate of 50-μL blood to 1-mL buffer and kept at room temperature until extraction. All field work pertaining to American Crows was performed under protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Permit Number: 16897).

Yellow-billed magpie samples ($n = 44$) were collected from California's Central Valley from 2004 to 2010 and were subsampled at random from a previous study (Ernest et al. 2010). Steller's jay samples were graciously donated from specimens at the Museum of Vertebrate Zoology, University of California, Berkeley. All Steller's jays were collected from El Dorado County, CA, in November 2001.

## Parasite detection and sequencing

DNeasy Blood and Tissue extraction kits (Qiagen, Valencia, CA) were used to extract genomic DNA from blood samples from American Crows and yellow-billed magpies. DNA was recovered from liver samples of Steller's jays using the same extraction technique. Successful DNA extraction from crow samples was confirmed through genotyping, as described in Townsend et al. (2009). DNA extraction success in magpie and jay samples was verified by amplification of the brain-derived neurotrophic factor (Sehgal and Lovette 2003). We screened for *Leucocytozoon* blood parasites using PCR amplification of a partial sequence of the parasite mtDNA cyt *b* gene. A nested PCR was used to detect *Leucocytozoon* spp. using the primers NF/NR3-FL/R2L as described in Hellgren et al. (2004), with a variation in the temperature profile of the second, nested PCR reaction. That is, we used a step-down approach, with annealing temperatures of 58, 56, 54, and 52 °C for 3 cycles each, and the remaining 23 cycles at 50 °C. Each PCR reaction was run in 25-μL volumes, accompanied by a positive control which was previously verified by sequencing and microscopy, and a negative control in which purified water was used in place of DNA template. The PCR amplicons were visualized on a 1.8 % agarose gel using ethidium bromide staining under ultraviolet light.

PCR samples with parasite cyt *b* amplification were purified with ExoSAP (United States Biochemical Corporation, Cleveland, OH) according to the manufacturer's directions. We identified lineages by sequencing the fragments bidirectionally using the BigDye version 1.1 sequencing kit (Applied Biosystems, Inc., Foster City, CA) on an ABI PRISM 3100™ automated sequencer (Applied Biosystems).

## Phylogenetic analyses

All sequences were edited in Sequencher 4.9 (GeneCodes, Ann Arbor, MI). If the chromatograph of any sequence showed one or numerous double peaks, infection status was categorized as mixed. No attempt was made to parse out the individual sequences of mixed infections due to the possibility that more than two parasites were present in a sample; samples with mixed infections were scored as *Leucocytozoon* positive, but not included in any further genetic analyses. Remaining sequences were aligned by eye in MacClade 4.08a (Maddison and Maddison 2005). The sequence dataset was condensed to eliminate redundant sequences and then subject to a BLAST search to ensure that a parasite of the expected genus was accurately amplified. After trimming primers, a fragment length of 476 bp was obtained. Any unique sequence, differing by one or more nucleotide from other recorded sequences, was considered to be a distinct *Leucocytozoon* lineage. However, while each lineage is considered separately when determining diversity data and phylogenetic predictions, no assumption of species-level difference is implied by distinct lineages and should not be viewed as such.

The dataset used for phylogenetic reconstruction consisted of 28 *Leucocytozoon* lineages detected in corvids and 8 reference sequences of *Leucocytozoon* species downloaded from the MalAvi (Bensch et al. 2009) database or GenBank, each trimmed to 456 bp to ensure consistency of sequence length. The corvid lineages include our American Crow, yellow-billed magpie, and Steller's jay samples from California, as well as nine *Leucocytozoon* lineages recovered from crow species (*Corvus corone*; *Corvus macrorhynchos*) in Japan (Yoshimura et al. 2014). A cyt *b* sequence from *Leucocytozoon mathisi* was downloaded from GenBank (DQ177252) and used as an outgroup. We analyzed the dataset in MrModeltest v3.7 (Posada and Crandall 1998) to determine which nucleotide substitution model was appropriate based on the Akaike Information Criterion (Huelsenbeck and Crandall 1997). Trees were constructed using a Bayesian inference performed in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). The GTR+G model was implemented, and two Markov chains were run simultaneously for 10 million generations; trees were sampled every 200 generations, resulting in 50,000 trees. After a burn-in period of 25 %, the remaining 37,500 trees were used to calculate posterior probabilities. To estimate bootstrap values, and to check for

congruence of phylogenetic relationships across multiple approaches, a maximum likelihood (ML) approach was implemented in PAUP version 4 (Swofford 2002). A thorough bootstrap analysis using 1000 replicates was used to determine support of individual branches in the program RAxML (Stamatakis 2006). The Bayesian majority consensus tree and maximum likelihood phylogram were viewed and edited with FigTree 1.4 (Rambaut 2012) and Adobe Illustrator.

## Results

### Prevalence and diversity

Using PCR detection methods, the prevalence of *Leucocytozoon* parasites in American Crow samples over a 3-year period (*n* = 258) was 57.4 %. Infection prevalence remained relatively consistent across years, ranging from 50.0 to 62.7 %. Of the 44 yellow-billed magpie blood samples selected randomly from the population detailed in Ernest et al. (2010), 54.5 % (24) were infected with one or more *Leucocytozoon* lineages. One hundred percent of the Steller's jays sampled (*n* = 20) harbored a *Leucocytozoon* lineage.

Partial cyt *b* mtDNA sequencing revealed 13 distinct *Leucocytozoon* lineages in the American Crow population

(Table 1), 10 of which are a novel cyt *b* lineage not previously recorded in GenBank; three lineages have been recovered recently from black fly blood meals in Colorado (Murdock et al. 2015). Five lineages accounted for 89.1 % of single infections in the population. In the yellow-billed magpie population, we identified nine distinct cyt *b* lineages. Interestingly, five of these lineages are identical to a lineage also detected in the American Crow population. Of the four lineages discovered solely in magpies, three were previously unrecorded; one lineage (YBMag.4), however, had been isolated from a different corvid (Western Scrub Jay, *Aphelocoma californica*) in California (unpublished, GenBank no. KJ584594). We detected two novel *Leucocytozoon* lineages in the Steller's jay samples. Neither of the lineages detected in Steller's jays were identical to the lineages infecting American Crows or yellow-billed magpies. Mixed infections were recorded in 25.7 % of crow, 20.8 % of magpie, and 35 % of jay samples, respectively. All lineages have been deposited in GenBank under accession nos. KU842391–KU842409.

### Phylogenetic and genetic distance analyses

Both Bayesian inference (Fig. 1) and ML predictions (not shown, but bootstrap values imported to Bayesian tree) suggest that 26 of the 28 corvid lineages fall within a large clade

**Table 1** The diversity and overall prevalence of *Leucocytozoon* partial cyt *b* lineages (476 bp) recovered from American Crow, yellow-billed magpie, and Steller's jay populations

| GenBank no. | Lineage | American Crow *n* = 258 | YB magpie *n* = 44 | Steller's jay *n* = 20 |
|---|---|---|---|---|
| KU842391 | AmCrow.1 | 21 | 0 | 0 |
| KU842392 | AmCrow.2.YBMag.5 | 9 | 1 | 0 |
| KU842393 | AmCrow.3 | 31 | 0 | 0 |
| KU842394 | AmCrow.4 | 2 | 0 | 0 |
| KU842395 | AmCrow.5 | 21 | 0 | 0 |
| KU842396 | AmCrow.6.YBMag.6 | 3 | 1 | 0 |
| KU842397 | AmCrow.7.YBMag.7 | 1 | 7 | 0 |
| KU842398 | AmCrow.8 | 1 | 0 | 0 |
| KU842399 | AmCrow.9 | 2 | 0 | 0 |
| KU842400 | AmCrow.10.YBMag.8 | 16 | 1 | 0 |
| KU842401 | AmCrow.11 | 1 | 0 | 0 |
| KU842402 | AmCrow.12.YBMag.9 | 1 | 1 | 0 |
| KU842403 | AmCrow.13 | 1 | 0 | 0 |
| KU842404 | YBMag.1 | 0 | 1 | 0 |
| KU842405 | YBMag.2 | 0 | 5 | 0 |
| KU842406 | YBMag.3 | 0 | 1 | 0 |
| KU842407 | YBMag.4 | 0 | 1 | 0 |
| KU842408 | SteJay.1 | 0 | 0 | 10 |
| KU842409 | SteJay.2 | 0 | 0 | 3 |
| | Mixed infections | 38 | 5 | 7 |
| | Total prevalence | 57.4 % | 54.5 % | 100 % |

Note that five lineages are shared by crows and magpies
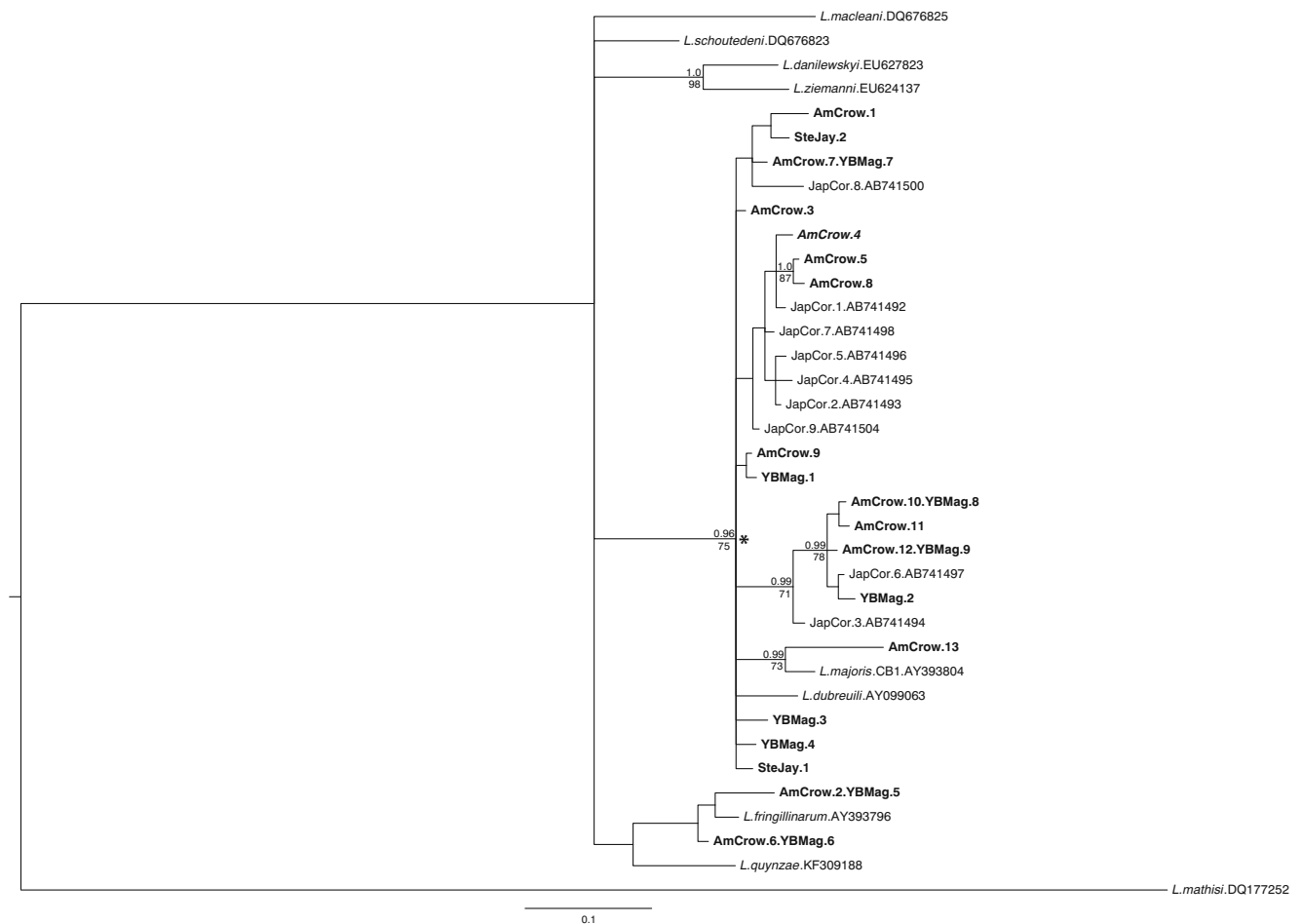
**Fig. 1** Consensus tree displaying *Leucocytozoon* phylogenetic relationships as predicted by Bayesian inference, using GTR + G substitution model in MrBayes v3.1.2. Posterior probabilities >0.95 (above branch) and maximum likelihood bootstrap values (calculated in RAxML) >70 (below branch) are indicated. Lineages detected in the current study are indicated in *bold*. Sequences displaying GenBank accession numbers are either corvid lineages from a study in Japan (lineages named JapCor) or reference *Leucocytozoon* spp. downloaded from GenBank. *L. mathisi* is used as an outgroup. Node of clade which contains 26 of 28 corvid lineages likely predicting a single corvid *Leucocytozoon* sp. is marked with *asterisk*

with strong nodal support. However, the relationships between individual lineages within this clade remain mostly unresolved, displaying low nodal support. Furthermore, no pattern emerges that suggests a species-level distinction between *Leucocytozoon* spp. recovered from crows versus magpies and jays; lineages originating from each host species are interspersed throughout the large corvid clade. Also contained within this clade are two reference sequences (*Leucocytozoon majoris*; *Leucocytozoon dubreuili*).

Interestingly, according to our predicted phylogenies, two *Leucocytozoon* lineages recovered from both American Crow and yellow-billed magpie samples fall outside this large clade and appear to be most closely related to the *Leucocytozoon fringillinarum* reference sequence. A distance matrix (Table 2) confirms that these two corvid lineages (AmCrow.2.YBMag.5 and AmCrow.6.YBMag.6) are genetically similar to one another (displaying a distance of 2.2 %), while their average distance from all other corvid lineages is 7.9 %. The 17 other

corvid *Leucocytozoon* lineages found in our study, which cluster together in the phylogeny, typically display genetic distances between 0 and 5.0 % from one another.

## Discussion

Our results imply a lack of species-level host specificity previously believed to differentiate *L. sakharoffi* from *L. berestneffi*. While previous studies had relied mainly on morphological descriptions of blood stages, our study was one of the first to look at a genetic marker in corvid *Leucocytozoon* infections. The use of molecular techniques allowed further taxonomic inference where previously microscopic investigation dominated. Recent studies have revealed a hitherto undetected diversity of haplotypes among hemoproteid species, stressing the importance of molecular and phylogenetic

**Table 2** Genetic distance matrix of *Leucocytozoon* cyt *b* (456 bp) lineages detected in American Crows, yellow-billed magpies, and Steller's jays

| Lineage | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AmCrow.1 | – | | | | | | | | | | | | | | | | | | |
| AmCrow.2.YBMag.5 | 8.9 | – | | | | | | | | | | | | | | | | | |
| AmCrow.3 | 2.8 | 8.1 | – | | | | | | | | | | | | | | | | |
| AmCrow.4 | 3.9 | 8.3 | 2.5 | – | | | | | | | | | | | | | | | |
| AmCrow.5 | 4.4 | 8.3 | 2.5 | 0.9 | – | | | | | | | | | | | | | | |
| AmCrow.6.YBMag.6 | 8.4 | 2.3 | 7.0 | 7.0 | 7.0 | – | | | | | | | | | | | | | |
| AmCrow.7.YBMag.7 | 2.3 | 8.7 | 0.9 | 3.5 | 3.5 | 7.5 | – | | | | | | | | | | | | |
| AmCrow.8 | 4.9 | 8.4 | 2.7 | 1.1 | 0.2 | 7.0 | 3.7 | – | | | | | | | | | | | |
| AmCrow.9 | 2.3 | 8.1 | 0.9 | 3.5 | 3.4 | 7.0 | 1.3 | 3.7 | – | | | | | | | | | | |
| AmCrow.10.YBMag.8 | 4.7 | 9.4 | 3.9 | 4.1 | 4.1 | 8.1 | 4.2 | 3.9 | 4.4 | – | | | | | | | | | |
| AmCrow.11 | 4.4 | 9.2 | 4.1 | 3.9 | 3.9 | 7.8 | 4.4 | 3.6 | 4.7 | 0.2 | – | | | | | | | | |
| AmCrow.12.YBMag.9 | 4.2 | 8.9 | 3.9 | 3.7 | 3.6 | 7.6 | 4.2 | 3.4 | 4.4 | 0.4 | 0.2 | – | | | | | | | |
| AmCrow.13 | 7.1 | 10.0 | 6.2 | 5.4 | 4.9 | 9.1 | 6.5 | 4.9 | 6.3 | 4.9 | 4.6 | 4.4 | – | | | | | | |
| YBMag.1 | 2.5 | 8.4 | 1.1 | 3.7 | 3.7 | 7.3 | 1.6 | 3.9 | 0.2 | 4.7 | 4.9 | 4.7 | 6.5 | – | | | | | |
| YBMag.2 | 5.2 | 9.7 | 3.9 | 4.2 | 4.1 | 8.4 | 4.2 | 3.9 | 4.9 | 0.9 | 1.1 | 0.9 | 5.4 | 5.2 | – | | | | |
| YBMag.3 | 2.7 | 7.3 | 1.8 | 3.9 | 3.7 | 7.3 | 2.3 | 3.9 | 1.3 | 5.1 | 4.9 | 4.7 | 6.3 | 1.6 | 5.7 | – | | | |
| YBMag.4 | 2.5 | 8.7 | 1.1 | 3.2 | 3.2 | 7.6 | 1.6 | 3.4 | 1.1 | 3.9 | 4.2 | 3.9 | 6.5 | 1.4 | 4.4 | 2.0 | – | | |
| SteJay.1 | 2.5 | 7.9 | 1.1 | 2.3 | 2.2 | 6.8 | 1.6 | 2.5 | 1.1 | 3.6 | 3.4 | 3.2 | 5.4 | 1.4 | 4.1 | 1.6 | 1.4 | – | |
| SteJay.2 | 1.6 | 8.8 | 2.0 | 3.7 | 4.2 | 8.1 | 1.6 | 4.4 | 2.0 | 4.2 | 3.9 | 3.7 | 6.4 | 2.3 | 4.7 | 2.0 | 1.8 | 1.8 | – |

Values were calculated using the GTR + G substitution model. Distance is given as a percentage

analyses in species delineation (Perkins and Schall 2002; Beadell et al. 2004; Martinsen et al. 2008; Dimitrov et al. 2010; Oakgrove et al. 2014). Likewise, our molecular detection methods revealed both a high *Leucocytozoon* infection prevalence across corvid species (ranging from 54.5 to 100 %) and high lineage diversity; 19 distinct lineages (15 of which are novel) were detected.

Five *Leucocytozoon* lineages overlapped host species (i.e., these distinct haplotypes were detected in both American Crows and yellow-billed magpies). This finding suggests that crows and magpies—hosts currently thought to be parasitized by different *Leucocytozoon* species—could share lineages of *Leucocytozoon*. However, detection of a parasite lineage by PCR does not necessarily confirm infection in the individual. A positive PCR outcome can result either from successful sporozoite infection and replication (Valkiūnas et al. 2009), or failed infection where sporozoites are introduced but fail to develop, perhaps due to parasite-host incompatibility (Levin et al. 2013). Therefore, it is difficult to make definitive conclusions about *Leucocytozoon* host specificity. However, 11 of the 19 singly infected magpie samples (57.9 %) revealed lineages also recovered from crow samples. With a majority of the magpie lineages overlapping with crow lineages, it is difficult to imagine that failed infections accounted for all detections. Viable sporozoites typically exist in a bird's bloodstream for 1–3 days, a short time frame for detections (Valkiūnas 2005). Therefore, we believe that the majority of

our detections were from successful infections and that American Crows and yellow-billed magpies share at least some lineages of *Leucocytozoon*.

If crows and magpies indeed share lineages, this finding would contradict the current classification of *Leucocytozoon* in corvids. At present, *L. sakharoffi* and *L. berestneffi* are considered host specific within corvids, according to their original taxonomic classification in 1908; *L. sakharoffi* infects the *Corvus* genus, while *L. berestneffi* infects magpies (*Pica* spp.) and jays (*Cyanocitta* spp.). This determination of host specificity is based upon the parasite's (i) transmissibility amongst host species, (ii) length of pre-patent period of gametocyte development, and (iii) morphology of hepatic meronts (Clark 1965; Khan and Fallis 1971; Valkiūnas 2005). The current classification was reinforced when Khan and Fallis (1971) studied *Leucocytozoon* in American Crows (*C. brachyrhynchos*), common ravens (*C. corax*), and blue jays (*Cyanocitta cristata*), and found that *Leucocytozoon* initially found in *Corvus* spp. could not be experimentally transmitted to blue jays, and vice versa. Furthermore, the authors found that the minimum pre-patent period for infection is 92 h in jays (*n* = 3), while for crows (*n* = 2) and ravens (*n* = 1), it is at least 24 h longer. However, pre-patent period can vary widely within a *Leucocytozoon* sp. amongst its various hosts: e.g., the pre-patent period of *L. sakharoffi* in *Corvus frugilegus* is 192 h (Baker 1971), a sharp contrast to the 116 h recorded by Khan and Fallis. In addition to the supposed pre-patent period

differences, the reliability of distinguishing *L. sakharoffi* and *L. berestneffi* by the morphology of hepatic meronts is tenuous. Valkiūnas (2005) catalogs these differences in his comprehensive review of avian hemosporidia, while noting that hepatic meronts found in one Eurasian magpie (*Pica pica*) were identical to those found in *C. cristata*. However, when describing hepatic meronts in yellow-billed magpies (one of our study species), Clark (1965) specifically emphasized that his findings actually reflect those observed in *L. sakharoffi* found in crows (Wingstrand 1947).

Despite the possible unreliability of two of the three distinguishing features in *L. sakharoffi* and *L. berestneffi* (pre-patent period; morphology of hepatic meronts), we cannot refute the experimental evidence of transmissibility of *Leucocytozoon* in crows and jays recorded by Khan and Fallis (1971). It should be noted, however, that a few details of the transmissibility study cast ambiguity on the results. First, the age of the bird receiving the parasite transmission varied by species: Raven and crow recipients were immature birds, while jay recipients were adults. Studies have shown that host age can affect both *Leucocytozoon* infection prevalence and immune response and sensitivity (Taft et al. 1994; Remple 2004; Forrester et al. 2001). Second, very few experimental details are included about the two black fly species (*Simulium aureum* and *Prosimulium decemarticulatum*) used to harbor and potentially transmit the infections. There is limited knowledge about the natural hosts of individual *Leucocytozoon* spp., and inconsistencies could be present regarding host-vector-host transmission pathways. Finally, their study did not include magpie species, leaving open the possibility that while *Leucocytozoon* host specificity in corvids exists, it could be inaccurate as currently classified.

A differing viewpoint contends that *Leucocytozoon* host specificity is lacking within corvids. A 1992 study by Bennett and Pierce presents a taxonomic review of leucocytozoids of the Corvidae family. The authors argue that early morphological descriptions, upon which the distinction of *L. sakharoffi* and *L. berestneffi* is based, were in fact comparing different stages of the parasite life cycle. This and other early studies (e.g., Coatney and West 1938; Wingstrand 1947) led the authors to conclude that *L. berestneffi* should be considered a synonym of *L. sakharoffi*. Also of note is a comparative study of the macrogametocytes of leucocytozoids occurring in Common Raven, Carrion Crow, and magpie, which did not reveal separation by host species (Ramisz 1962). In the course of our study, therefore, microscopic investigation of infected blood smears was de-emphasized; blood films collected for our study were incomplete and of inconsistent quality, which prevented reliable parasite detection capabilities, and we were unable to obtain tissue stage samples. However, for morphospecies comparisons, blood smears were not likely to have been informative, as the *Leucocytozoon* species we encountered are morphologically indistinguishable

in the blood stages (Valkiūnas 2005). Instead, we relied solely on molecular techniques for parasite detection.

Our genetic distance and phylogenetic reconstruction analyses yielded two interesting trends. First, two lineages found in both crows and magpies were seemingly distinct from the other lineages, which could indicate they are in fact from a different *Leucocytozoon* sp. They were closely related to *L. fringillinarum*, a generalist parasite that has been recovered from over 200 host species (Valkiūnas 2005). Second, there was no genetic distance differentiation between our remaining 17 lineages. If a distinction between *L. sakharoffi* and *L. berestneffi* was evident, we would expect high distance values from crow lineages to both magpie and jay lineages but low distance to other crow lineages. Furthermore, we would expect low distance between magpie and jay lineages. These results, however, did not materialize (Table 2). For example, lineage AmCrow.1 ($n=21$) displays distance values ranging from 2.3 to 7.1 % divergence from other lineages detected in crows while displaying distance values below 3.0 % from five of six lineages detected solely in magpies or jays; in fact, the lineage most genetically similar to AmCrow.1 was detected in Steller's jays. Bayesian and ML inferences indicated that the majority of lineages detected in corvids forms a large and mostly unresolved clade. The results of our distance and phylogenetic analyses do not support the current nomenclature separating *L. sakharoffi* and *L. berestneffi*.

Two reference *Leucocytozoon* species (*L. majoris*; *L. dubreuili*) are contained within what we have speculated to be the probable *Leucocytozoon* corvid clade. The inclusion of these two generalist species with the corvid lineages is unexpected and warrants further assessment. One possible explanation for our findings is that the partial cyt *b* sequence was of insufficient phylogenetic robustness to accurately approximate the fine-scale species-level distinction we were testing. Previous studies have examined nuclear and/or apicoplast genes in addition to mitochondrial genes to gain a clearer picture of hemosporidian diversity (Bensch et al. 2004; Martinsen et al. 2008; Hellgren et al. 2014). As such, we attempted to amplify both the *msp1* and *ama-1* nuclear genes, and though each had been successfully amplified in recent studies on avian *Plasmodium* spp. (Hellgren et al. 2014; Lauron et al. 2014), this approach was unsuccessful with *Leucocytozoon*. Unfortunately, obtaining multiple genetic markers is a significant challenge in avian hemosporidian research (reviewed in Perkins 2014). However, Bensch et al. (2004) concludes that a 500-bp fragment of cyt *b* provides a phylogenetic signal as robust as the complete cyt *b* gene or the DHFR-TS nuclear gene. A second potential explanation for our results is that the evolutionarily recent speciation of *L. sakharoffi* and *L. berestneffi* is yet to be reflected in their respective cyt *b* sequence. However, a study on the

relationships of corvid genera does not indicate that *Corvus*, *Pica*, and *Cyanocitta* spp. are recently diverged or even closely related within the corvid family (Ericson et al. 2005). Therefore, if these host-specific *Leucocytozoon* parasites have recently evolved, they are doing so independently of host speciation.

A final explanation is that *L. sakharoffi* and *L. berestneffi*, which are indistinguishable morphologically and undifferentiated genetically, are in fact part of one large corvid-parasite complex. Our data suggest that there is no definitive distinction in the *Leucocytozoon* species found in crows, magpies, and jays. It is clear, therefore, that further molecular studies are needed, ideally involving longer and/or additional genetic sequences from multiple genomes. In addition, a study attempting to replicate the transmissibility experiment of Khan and Fallis (1971) would be of immense value. A new experimental study should involve members of all three focal corvids at the center of the *L. sakharoffi* versus *L. berestneffi* classification issue and should ideally be a consistent age in order to minimize age-specific immune response differences. These further studies would help clarify the host-parasite specificity in the rapidly expanding corvid family.

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