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Description and molecular characterization of novel *Leucocytozoon* parasite (Apicomplexa: Haemosporida: Leucocytozoidae), *Leucocytozoon polynuclearis* n. sp. found in North American woodpeckers

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Abstract We describe *Leucocytozoon polynuclearis* **n. sp.** (Haemosporida: Leucocytozoidae) from two North American woodpeckers, the northern flicker (*Colaptes auratus* Linnaeus) and white-headed woodpecker (*Dryobates albolarvatus* Boie, 1826), based on the morphology of its blood stages and portions of the mitochondrial cytochrome *b* gene. The most distinctive features of *Leucocytozoon polynuclearis* **n. sp.** development are the triangular-shaped host cell nuclei and position of host cell nuclei above gametocytes. This parasite inhabits thrombocytes. *Leucocytozoon squamatus* Nandi, 1986, the only other *Leucocytozoon* species detected from Picidae birds, lacks features that are commonly found with *L. polynuclearis* **n. sp.** infections. Phylogenetic analysis identified DNA lineages associated with *L. polynuclearis* **n. sp.** and showed that this parasite is more closely related to other North American *Leucocytozoon* species than to *L. squamatus*, whose initial description was from infected Old World Picidae species. Although there are reports of *L. squamatus* in North American Picidae species, these detections were based only on microscopic examinations, remain genetically non-characterized, and might be misidentifications with regards to *L. polynuclearis* **n. sp**. Available parasite distribution data indicate that *L. polynuclearis* **n. sp.** infects Picidae species throughout North America and *L. squamatus* distribution probably is restricted to Old World Piciformes birds.

Keywords *Leucocytozoon polynuclearis* **n. sp.** · molecular and morphological characterization · Picidae · new species

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Introduction

Avian haemosporidian parasites (Apicomplexa, Haemosporida) have been used as model organisms for scientists studying human malaria since the 1880s, as they allow for the investigation of the basic biological properties and natural ecology of the group (Valkiūnas, 2005). Fully understanding the phylogenetic relationships among avian haemosporidians can contribute to the accurate reconstruction of trait evolution in members of this group that infect humans (Galen et al., 2018). The question of how to taxonomically classify haemosporidian parasites has been

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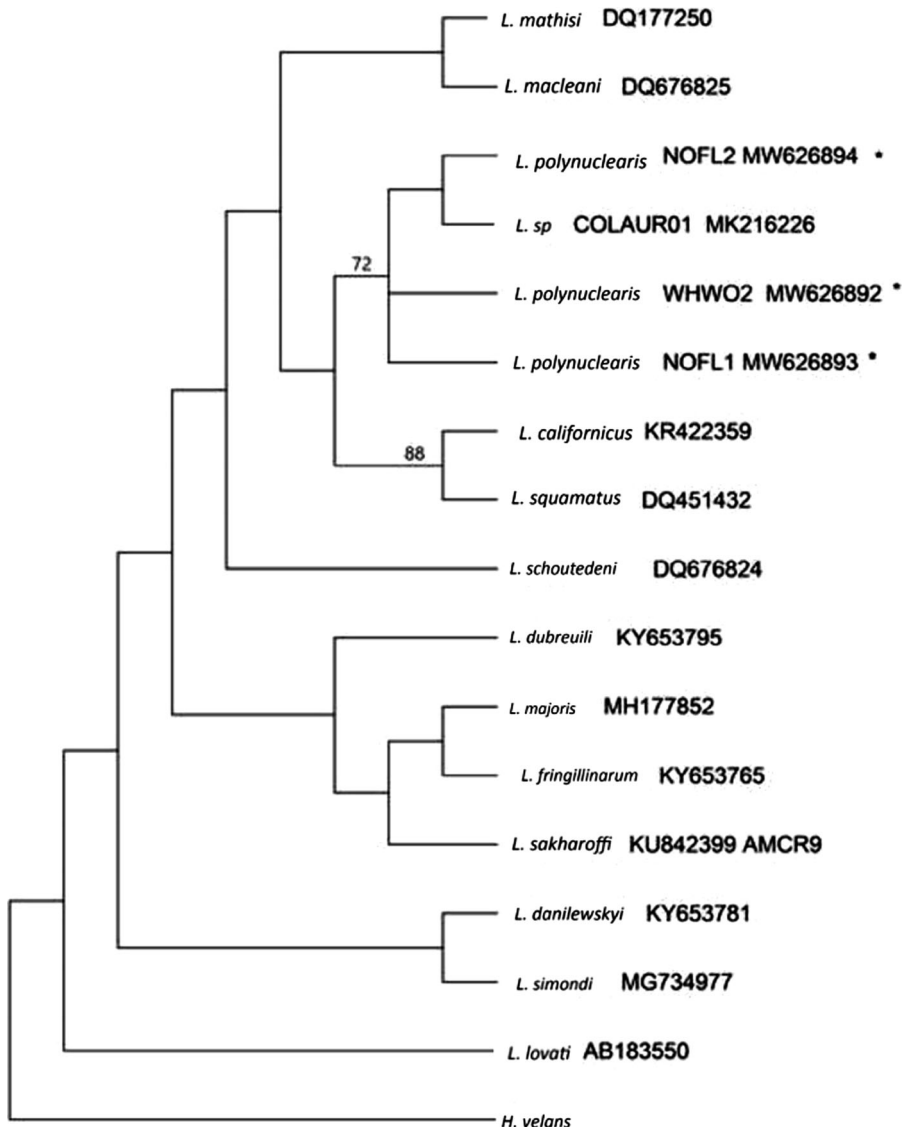


Fig. 1 Consensus tree displaying *Leucoytozoon polynuclearis* **n. sp.** phylogenetic relationships as predicted by Maximum-likelihood inference, using GTR + Γ substitution model in PAUP* v.4.0a.b161. Maximum-likelihood bootstrap values > 70 are indicated. Lineages detected in the current study are indicated with an asterisk *

controversial since their discovery (Galen et al., 2018; Valkiūnas and Atkinson, 2020). Most species of avian haemosporidians were initially classified based on the morphological characters seen in infected host cells from blood smears as well as limited information about their vertebrate host specificity (Valkiūnas, 2005; Martinsen et al., 2006). Introduction of molecular data in parasite diagnostics revealed enormous genetic diversity of wildlife haemosporidians and raised additional questions related to cryptic speciation (Nilsson et al., 2016; Galen et al., 2018). Multiple studies (Martinsen et al., 2006; Lotta et al., 2019) report cryptic species, i.e., species that look similar morphologically but are genetically distinct. Due to vertebrate host specificity and the resulting restriction of parasite distributions by taxonomic order of vertebrate hosts, the natural host range of haemosporidians still remains helpful in species identification although no longer the predominant taxonomic

characteristic (Valkiūnas, 2005; Valkiūnas and Ashford, 2002).

Investigations of parasites of the genus *Leucocytozoon* are limited when compared to the other avian haemosporidian genera *Plasmodium* and *Haemoproteus* (Bensch et al., 2009). This is partly due to an insufficient development of their molecular characterization and limited ability of available general primers in distinguishing many *Leucocytozoon* species (Himmel et al., 2019; Lotta et al., 2019). This limitation is of concern because some species, such as *L. smithi* Laveran and Lucet, 1905 in domestic turkeys (*Melagris gallapavo* Linnaeus), *L. simondi* Mathis and Léger, 1910 in many species of ducks and geese, *L. caulleryi* Mathis and Léger, 1909 in domestic chickens (*Gallus gallus* Linnaeus), and *L. struthionis* Walker, 1912 in ostriches (*Struthio camelus* Linnaeus), often cause disease and can have high rates of mortality resulting in economic losses (Valkiūnas, 2005). Basic questions such as the number of extant species lack answers. Louse flies (Simuliidae) transmit the majority of investigated *Leucocytozoon* species, with only one known species (*L. caulleryi*) vectored by biting midges (Ceratopogonidae) (Atkinson et al., 2008). As in all haemosporidians, sporozoites initiate exo-erythrocytic development (tissue meronts) in various organs; the latter produce merozoites, which initiate development of gametocytes in blood cells. The gametocytes are infective to vectors and are important stages for distinguishing *Leucocytozoon* species in blood preparations but remain undescribed in the majority of detected genetic lineages. This is a prominent obstacle in the development of parasite taxonomy and biodiversity research, particularly during co-infections.

Prior to this study, there had only been one *Leucocytozoon* species worldwide found in Picidae (woodpeckers), *L. squamatus* Nandi, 1986 (Valkiūnas, 2005). *L. squamatus* was originally detected in the Asian scaly-belled green woodpecker (*Picus squamatus* Vigors) in India (Nandi, 1986). Bennett et al. (1993) identified *L. squamatus* in North American northern flickers (*Colaptes auratus* Linnaeus) by comparing the original description and morphometrics of the species and concluded that all records of *Leucocytozoon* in the North American family Picidae belonged to this species. This study also identified additional records of *L. squamatus*-similar gametocytes in sub-Saharan Africa and southeast Asia (Bennett et al., 1993).

The original description of *L. squamatus* considered the changing conventions of parasite species taxonomy in the 1980s (Nandi, 1986). This was a shift from the idea of "one host-one parasite" that had been prevalent during the first part of the 20th century, to the notion that species specificity occurs at the host familial level (Nandi, 1986). Nandi (1986) indicated that morphologically *L. squamatus* resembled *L. majoris* Laveran, 1902. The author further espoused that if future studies showed that leucocytozoid parasites could infect different orders, *L. squamatus* and *L. majoris* could be the same species based on their morphological similarities (Nandi, 1986). Numerous experimental studies (see review in Valkiūnas, 2005, p.76) showed that *Leucocytozoon* species markedly vary in vertebrate host specificity, but the same species usually cannot infect birds belonging to different orders. Molecular sequence data supports this and indicates an exceptionally rare likelihood that the same *Leucocytozoon* lineages could be found in birds belonging to different orders (Bensch et al., 2009). Importantly, the exceptionally rare reports of *Leucocytozoon* lineages in birds of different orders have never been supported by the observation of gametocytes, an invasive parasite stage for vectors, indicating abortive (incomplete) development and a dead end infection (Valkiūnas and Atkinson, 2020). Thus, morphologically similar parasites in birds belonging to different orders likely are different parasite species.

There have been insufficient efforts in taxonomically describing *Leucocytozoon* parasites in birds belonging to Picidae. Here we report a morphologically unique *Leucocytozoon* parasite first identified in the white-headed woodpecker (*Dryobates albolarvatus* Boie, 1826) (Picidae), describe its gametocytes, report its host cells, and molecularly characterize and determine its phylogenetic relationships. There are reports of this parasite lineage in multiple species of woodpeckers in the western United States, but its gametocytes are reported and described here for the first time.

Materials and methods

Blood sampling field methods

Collection of samples from live woodpeckers took place during the breeding season in June-July 2016 and May-July 2017 in the Eastern Cascade Mountains (46.694731, -121.080742) in Yakima County, Washington. Nest searches of known white-headed woodpeckers and black-backed woodpeckers (*Picoides arcticus* Swainson) territories occurred throughout the nesting season. To increase sample size, northern flicker and hairy woodpecker (*Dryobates villosus* Linnaeus) nests located during these searches were included. Targeted mist-nets positioned in front of the nest cavity were used to capture adults. When trapping nestlings, nets positioned in place after the adult had fed the nestlings to limit the amount of time the adults were kept away from foraging. Nestlings were an estimated 1-4 days from fledging (18-25 days old) when sampled, and removed from the nest used the hole saw method (Ibarzabal and Tremblay, 2006). Weight and measurements collected from all birds, as well as banding, were conducted in compliance with the Ornithological Council Guidelines for the Use of Wild Birds in Research (Fair et al. 2010) and U.S. Department of Agriculture, Forest Service, Institutional Animal Care and Use Committee (Proposal number 2016-007).

Each bird had 25-50 µl of blood collected via brachial venipuncture with a sterile 27-gauge needle. Samples were stored in a lysis buffer (10mM Tris-HCL pH 8.0, 100 mM EDTA, 2% SDS) at ambient temperature while in the field and preserved at -20 °C in the laboratory until further processing (Sehgal et al. 2001). Two to three blood smears from each bird were prepared in the field using established techniques (Bennett, 1970; Valkiūnas, 2005), fixed in methanol, stained with Giemsa, and examined microscopically following established protocols in the laboratory (Valkiūnas et al., 2008).

Molecular analysis

DNA extraction from collected blood samples used the commercial DNA extraction kit Wizard SV Genomic DNA Purification System (Promega Corporation, Madison, WI) and followed the manufacturer's protocol. Confirmation of successful DNA extraction was done using PCR with primers that amplified the brain-derived neurotrophic factor (Sehgal and Lovette, 2003). The commonly used nested PCR protocol was used to screen samples for a partial sequence of the mitochondrial cytochrome *b* (cyt *b*) gene from *Plasmodium* and *Haemoproteus* using the primers HaemNF/HaemNR2-HaemF/HaemR2 (Bensch et al., 2000; Waldenström et al., 2004) and from *Leucocytozoon* using primers NF/NR3-FL/R2L (Hellgren et al., 2004). Each reaction used negative (ddH_2O)

Table 1 Nucleotide differences between mitochondrial cytochrome *b* lineages closely related to *Leucocytozoon polynuclearis* shown in Figure 1

	L. californicus (KR422359)	*L. polynuclearis NOFL2 (MW626894*)*	*L. sp. COLAUR01 (MK216226)*	*L. polynuclearis WHWO1 (MW62892*)*	*L. polynuclearis NOFL2 (MW62893*)*
L. californicus (KR422359)	X	16	18	18	17
L. polynuclearis (MW626894)*	16	X	1	2	1
L. polynuclearis (MK216226)	18	1	X	2	1
L. polynuclearis (MW62892)*	18	2	2	X	1
L. polynuclearis (MW62893)*	17	1	1	1	X

Table 2 Morphometric parameters of mature gametocytes and host cells of *Leucocytozoon* polynuclearis n. sp. from the blood of the Northern flickers *Colaptes auratus*

Feature	Measurements (μm)[a]
Macrogametocyte	
Length	9.6-14.0 (11.8 ± 1.2)
Width	8.6-11.4 (9.8 ± 0.6)
Area	69.0-110.0 (90.4 ± 11.8)
Parasite nucleus	
Length	2.8-5.2 (3.8 ± 0.6)
Width	1.9-3.8 (2.8 ± 0.5)
Area	5.8-10.8 (8.6 ± 1.6)
Host-cell nucleus	
Length	6.5-17.5 (11.1 ± 3.1)
Width	2.5-7.4 (4.4 ± 1.2)
Area	21.5-43.5 (31.8 ± 6.2)
Host-cell parasite complex	
Area	69.0-156.50 (123.3 ± 22.8)
Microgametocyte	
Length	8.6-13.7 (10.6 ± 1.3)
Width	6.9-10.6 (8.9 ± 1.2)
Area	56.4-96.5 (76.5 ± 12.0)
Parasite nucleus[b]	
Length	–
Width	–
Area	–
Host-cell nucleus	
Length	7.8-17.7 (11.8 ± 2.8)
Width	2.4-6.6 (4.0 ± 1.3)
Area	23.1-44.5 (32.0 ± 6.5)
Host-cell parasite complex	
Area	75.6-150.2 (112.5 ± 20.9)

[a]Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

[b]Nuclei of microgametocytes are markedly diffuse and their boundaries hardly distinguishable, so were difficult to measure.

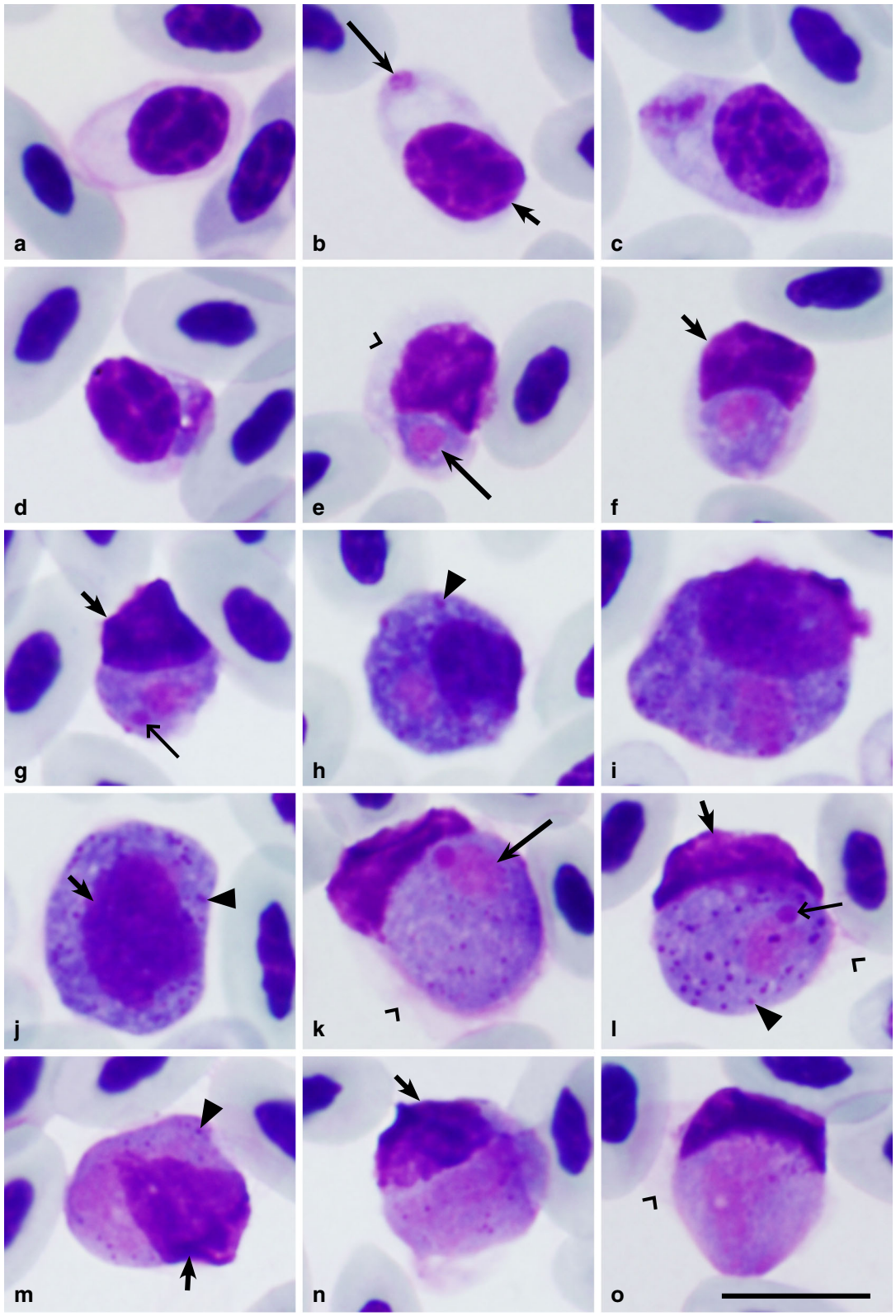
and positive controls (samples from infected birds previously confirmed by sequencing and microscopy) conducted in 25 μl reactions. Visualization of the resulting PCR product used a 1.8% agarose gel to check for positive infections. Bi-directional sequencing of positive PCR products was done by Elim Biopharmaceuticals Inc. (Hayward, CA).

Phylogenetic analysis

Alignment and editing of sequences used Geneious software (v.11.0.4) (http://www.genious.com, Kearse et al., 2012). Trimming primers produced a sequence length of 476 base pairs. Unique sequences differed from other sequences by one or more nucleotides (Martinsen et al., 2006; Hellgren et al., 2007). Identification of three distinct *Leucocytozoon* lineages was done by comparing samples to other haemosporidian sequences using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (NCBI Resource Coordinators, 2016) and the MalAvi Database (Bensch et al., 2009). These distinct lineages were deposited in GenBank (MW626892, MW626893, MW626894). A Maximum-likelihood (ML) tree was generated using PAUP* (v.4.0a.b161) (Swofford, 2018) incorporating sequences from this study and 12 reference sequences from GenBank. The analyses only included reference sequences that have well-established morphological species identifications, which were based on microscopic examination of blood films. The outgroup used a partial cyt *b* sequence from *Haemoproteus velans* (MH311672), a woodpecker parasite infecting white-headed woodpeckers and northern flickers (Groff et al., 2019). The ML phylogenetic construction used a GTR+Γ model based on an AICc analysis of models and 1,000 bootstrap replicates. Calculation of a molecular distance matrix used to determine genetic differences between lineages used Geneious software (Table 1).

Morphological analysis

Examination of blood films, preparation of illustrations, and measurements used an Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE. Examination of blood films included approximately 100-150 fields at low magnification (400×), and then at least 100 fields were studied at high magnification (1,000×). The morphometric features studied (Table 2) are those defined by Valkiūnas (2005) and Lotta et al. (2019). Estimation of the intensity of parasitemia as a percentage included actual counting of the number of parasites per 10,000 randomly observed red blood cells. The morphometric analysis used the 'Statistica 7' package.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◀**Fig. 2** *Leucocytozoon polynuclearis* **n. sp.** (lineage NOFL1) from the blood of a northern flicker (*Colaptes auratus*): a – uninfected thrombocyte, b-f –young gametocytes, g-l – macrogametocytes, m-o – microgametocytes. Note that distinct thrombocyte morphological characters (thick envelope and large avoid nuclei) were readily visible at an early stage of infection (compare figures a with b, c), but the host cells were markedly deformed by the growing parasites (f-o) making it impossible to identify the origin of host cells using morphological characters at these stages of gametocyte development. Long simple arrows – parasite nuclei. Short simple arrows – host cell nuclei. Triangle arrowheads – volutin granules. Long simple wide arrows – nucleolus. Simple wide arrowheads – remnants of the host-cell cytoplasm. Giemsa-stained thin blood films. Scale bar = 10 μm

Results

Phylogenetic analysis

Based on the phylogenetic analysis (Figure 1), all lineages detected in woodpeckers appear in a well-supported clade and genetic differences among these lineages were low (Table 1), indicating they all should belong to one species. This is in accordance with morphological similarity of gametocytes of all these lineages. An unidentified lineage found in New Mexico from northern flickers clusters in this clade with no genetic differences and likely is the same species.

Parasite description

***Leucocytozoon polynuclearis* n. sp.** (Fig. 1a-o, Table 2)

Young gametocytes (Fig. 2b-f) were seen in numerous thrombocytes, which are likely the main host cells (Fig. 2a). The early-stage gametocytes lie free in the cytoplasm and usually do not touch the nuclei of thrombocytes (Fig.2b, c). The parasites look like roundish or oval bodies with prominent nuclei and a poorly visible cytoplasm (Fig. 2b, c). The smallest visible gametocytes do not change the shape of infected cells nuclei at the earliest stages of their development, and the shape of non-infected thrombocytes can be readily recognized in the parasitized cells (compare Fig. 2a with Fig. 2b-d); this is a characteristic feature of this species' development. Advanced young gametocytes (Fig. 2e, f) deform host cells, which become rounded; the parasites are closely appressed to the thrombocyte nuclei and lie in the indentation of the nuclei (Fig. 2e, f). At this stage of development, the host cell nuclei assume various cap-like shapes (Fig. 2e, f). The host-cell cytoplasm usually is visible (Fig. 2e, f), but not in all cells containing advanced young gametocytes. Vacuolization of the cytoplasm is light and volutin granules were not yet seen. Parasite nuclei are prominent, usually approximately centrally located. Nucleolus is invisible in advanced young gametocytes.

Macrogametocytes (Fig. 2g-l) are roundish to slightly oval in form and develop in roundish host cells; fusiform cytoplasmic processes do not develop. The cytoplasm is heterogeneous in appearance and contains numerous prominent, roundish, randomly scattered volutin granules, which are markedly variable in size (Fig. 2h-l). Vacuolization of the cytoplasm is low. The parasite nucleus is prominent and is variable both in form and in position. Nucleoli are readily visible with distinct outlines, located close to the periphery of nuclei (Fig. 2g, k, l). In cells with growing parasites, the host cell nuclei are closely appressed to gametocytes. The parasite pushes aside the nuclei, which looks deformed and lies peripherally, often assuming broadly triangular (Fig. 2g, h) or roundish (Fig. 2i, j) forms, which are characteristic features of this species development. Host cell nuclei often assume markedly various positions above gametocytes (Fig. 2h, j), which is an additional characteristic feature of this species development. In cells with fully-grown gametocytes, the host cell nuclei appeared as more or less evident caps (Fig. 2k) or, sometimes, bands (Fig. 2l). They extend less than half the circumference of the gametocytes (Fig. 2k, l). Mature gametocytes usually largely replace the cytoplasm of host cells. It is often is invisible (Fig. 2g), but more frequently present around maturing gametocytes as a minute, very pale, margin of variable form (Fig. 2k, l).

Microgametocytes (Fig. 2m-o) have the same general configuration and other main features as macrogametocytes with the usual haemosporidian sexual dimorphic characters, such as the paler staining of the cytoplasm (compare Fig. 2h-j with Fig. 2n, o) and the large diffuse nuclei of parasites (Fig. 2m-o). The nucleolus was invisible. The amount of volutin in microgametocytes is less than in macrogametocytes,

and volutin granules are smaller in size and more difficult to recognize than in macrogametocytes (compare in Fig. 2l with Fig. 2m, n).

Taxonomic summary

Type host: northern flicker (*Colaptes auratus*) (Piciformes, Picidae)

Additional host: white-headed woodpecker (*Dryobates albolarvatus*) (Piciformes, Picidae). No reports of *L. polynuclearis* **n. sp.** lineages in other bird species, indicating restriction of the parasite to woodpeckers. There are reports of similar lineages (2 nucleotide differences) in two passerine species, the gray catbird (*Dumetella carolinensis* Linnaeus, Mimidae) and the common yellowthroat (*Geothlypis trichas* Linnaeus, Palulidae) in the USA. Morphological description of leucocytozoids in these birds is absent and the relationships with *L. polynuclearis* remain unclear.

Vectors: Unknown.

DNA sequence: Lineage NOFL1, GenBank accession MW626894.

Type locality: Eastern Cascade Mountains (46.694731, -121.080742), Yakima County, Washington, USA.

Site of infection: Thrombocytes; no other data

Prevalence: PCR and microscopic examination found infections of *L. polynuclearis* **n. sp.** in 27 of 44 (38.6%) sampled northern flickers. This parasite infected both in adult and nestlings, indicating local transmission. Two of 39 (5.1%) sampled white headed woodpeckers were found to be infected as well. However, the lower prevalence in white-headed woodpeckers may be biased low, as there were an unusual number of nestling mortalities from unknown causes in 2017 (data not shown).

Type specimens: Hapantotype (accession numbers 49027, 49028 NS, intensity of parasitemia is approximately 0.6%, *C. auratus*, Yakima County, Washington, USA, June 2017, coll. Tierra Claire Groff) was deposited in the Nature Research Centre (NRC), Vilnius, Lithuania. Parahapantotypes (accession nos. No 49029-49031 NS and G466227, intensity of parasitemia ranging between approximately 0.1% and 1%, other data as for the hapantotype) were deposited in the Nature Research Centre (NRC), Vilnius, Lithuania and in the Queensland Museum, Queensland, Australia, respectively.

Additional material: Voucher blood films with gametocytes of *L. polynuclearis* **n. sp.** from the type vertebrate host were deposited in Nature Research Centre, Vilnius, Lithuania (accession nos. 49035 and 49036 NS).

Distribution: Samples from woodpeckers both in Washington and New Mexico were infected with this parasite, indicating a broad distribution in woodpeckers across the western United States.

Etymology: The specific name (*Leucocytozoon polynuclearis*) reflects the marked morphological variability of gametocyte host cell nuclei (see Fig. 1f-o).

ZooBank registration: The life science identifier (LSID) for *L. polynuclearis* **n. sp.** is urn:lsid:zoobank.org:pub:283F445B-DA23-467F-A080-EB18EBD87A30

Remarks

This is the second species of *Leucocytozoon* described from birds belonging to the woodpecker order Piciformes. Distinguished from *L. squamatus*, described from the Asian scaly-bellied woodpecker in India (Nandi, 1986), there are reports of similar gametocytes and their host cells from numerous species of Piciformes in North America and the Old World (Bennett et al., 1982; Bishop and Bennett, 1992; Valkiūnas, 2005; Atkinson et al., 2008). Fully-grown gametocytes of both species occur in roundish host cells, which nuclei assume various cap-like or band-like shapes (Fig. 1k, l, o). Due to these characters both parasite species are similar. However, there are no reports of triangular-shaped host cell nuclei (Fig. 1g, h, m) nor the positioning of host cell nuclei above gametocytes (Fig. 1i, j) in *L. squamatus*, which is common during *L. polynuclearis* **n. sp**. infection.

Based on these characters, *L. squamatus* and *L. polynuclearis* **n. sp.** can be readily distinguished.

Host cells of *L. squamatus* gametocytes remain non-identified, and this parasite lacks molecular characterization. *Leucocytozoon polynuclearis* **n. sp.** parasitizes thrombocytes, which can be readily identified due to the presence of a colourless cytoplasm and the readily distinguishable outline of the host cell envelope (Fig. 1a, b), which is not the case in other avian blood cells (Clark et al., 2009). Comparison of phylogenetic relationships between *L. polynuclearis* **n. sp.** and *L. squamatus* currently is premature. The detection of DNA sequence information of *L. squamatus* from its type vertebrate hosts from the type locality (India) would be ideal for such a comparison.

The morphology of some fully-grown gametocytes and their host cells observed in *L. polynuclearis* **n. sp.** is similar to many other species of leucocytozoids that develop in roundish host cells possessing cap-like or band-like nuclei (see for example, Fig.1k, l, o) and parasitizing birds in other avian orders (Valkiūnas, 2005). Sequence information is important for *L. polynuclearis* **n. sp.** identification, particularly because parasitemias are often light and few morphological features are available for taxonomic work, as is the case with the majority of other avian *Leucocytozoon* parasites. Nuclei of host cells show several rare features, primarily the broadly triangular shapes (Fig. 1g, m) and various positions above the gametocytes (Fig.1h, j). That provides opportunities to distinguish this parasite from other avian leucocytozoids belonging to *L. fringillinarum* Woodcock, 1910 and *L. majoris* morphological groups even by observation of single gametocytes.

Discussion

The key result of this study is the description of a morphologically unique *Leucocytozoon* parasite of woodpeckers, whose gametocytes inhabit thrombocytes (i.e., blood clotting platelets). A study by Zhao et al. (2015) applied cell-type specific antibodies to determine the identity of the host cells infected by the gametocytes of *L. sabrazesi* Mathis and Léger, 1910 (possible synonym of *L. macleani* Sambon, 1908) and concluded that thrombocytes were the main host cells. This pathogen often parasitizes domestic chickens in the tropics of the Old World. Our microscopic observations support their conclusion regarding thrombocytes being the host cells of leucocytozoids morphologically. The colorless cytoplasm and readily distinguishable outline of the host cell envelope – the distinct characteristics of thrombocytes (Clark et al., 2009) – were observable during early-stage infections (Fig. 1a-d). This study shows that the origin of some host cells can be determined at early stages of *Leucocytozoon* infection with microscopic examination. Available data indicate that species of *Leucocytozoon* inhabit several blood cell types, including erythrocytes and different types of leukocytes, including thrombocytes (Wingstrand, 1947; Ramisz, 1962; Khan and Fallis, 1971; Valkiūnas, 2005; Atkinson et al., 2008) however, the precise range of host cells and the sequence of occurrence of the parasites in different blood cells during the course of parasitemia remain unidentified in the majority of the described avian leucocytozoids.

White-headed woodpeckers are year-long residents at the study site in the eastern Cascade Mountains indicating there is active local transmission of these parasites. Many infected birds were nestlings 18-25 days old, which also points to local transmission and infection in the nests; this also would suggest a prepatent period of infection in nestlings of less than 25 days. The vectors of *L. polynuclearis* **n. sp.** remain unknown, but likely are species of blackflies belonging to Simuliidae as is the case in other species of leucocytozoids, which are phylogenetically related (i.e., *L. schoutedeni* Rodhain, Pons, Vandenbranden and Bequaert, 1913, *L. dubreuili* Mathis and Léger, 1911, *L. fringillinarum*, *L. sakharoffi* Sambon, 1908, *L. danilewskyi* Ziemann, 1898, *L simondi*) (see Fig. 1 and Atkinson et al., 2008; Valkiūnas 2005).

The primers described by Hellgren et al. (2004) readily amplify *L. polynuclearis* **n. sp.** DNA using PCR. As these primers successfully screen for avian leucocytozoids worldwide, the available information regarding the distribution of this parasite likely corresponds to its true distribution in wildlife. This is not a case with some South American *Leucocytozoon* species, as well as the parasites of *L. toddi* Sambon, 1908 species group, which might be undetectable using the same primers (Himmel et al., 2019; Lotta et al., 2019). While woodpeckers are an under sampled group, the *L. polynuclearis* **n. sp.** distribution is likely restricted to North America as lineages have only been identified in this region.

It is interesting that *L. polynuclearis* **n. sp.** is closely related (appeared in the same clade) to *L. californicus* Walther et al., 2016, a common parasite of the American kestrel (*Falco sparverius sparverius*), which is also a cavity nester like woodpeckers. These two parasites are similar because their gametocytes develop only in roundish host cells. *L. californicus* differs by 18 nucleotides from the new species (Table 1). These parasites might have evolved during a recent host switch and adaptation to develop in birds of different orders (Piciformes and Falconiformes). Gametocytes of both parasites are similar and develop only in roundish host cells (Walther et al., 2016). Thus, although the avian hosts are vastly divergent, the morphologies of *L. polynuclearis* **n. sp.** and *L. californicus* are similar and congruent with their phylogeny.

Leucocytozoon squamatus was originally described in India (Nandi, 1986) and then found at many sites in the Old World (Atkinson et al., 2008; Valkiūnas, 2005). Although North American Picidae species have been identified as infected with this species using microscopic examination of blood films, these detections were not genetically characterized and may be misidentifications. Gametocytes of *L. squamatus* and *L. polynuclearis* **n. sp.** have some similar morphological characteristics, particularly the inhabitation of only roundish host cells. Available molecular data indicate that is it is likely that *L. polynuclearis* **n. sp.** is restricted to North American Picidae species. *L. squamatus* is probably restricted to Old World Piciformes birds.

It is important to both morphologically and genetically describe parasites from rare, declining, or otherwise species of conservation concern. One model projects an approximately 70% loss of the threatened white-headed woodpecker's range by 2100 due to impacts of climate change (Bateman et al., 2020). The risk and severity of infectious diseases are projected to increase among wildlife (Patz et al., 2000; Garamszegi, 2011) and may impact species in unexpected ways. This is particularly true in case of leucocytozoonosis, which often is lethal in non-accustomed avian hosts due to damage of organs by exo-erythrocytic stages and heavy parasitemia of large-size gametocytes (Atkinson et al., 2008; Valkiūnas, 2005). It has previously been reported that haemosporidian infections can be lethal to white-headed woodpecker nestlings already impacted by other environmental conditions such as reduced foraging opportunities and increased spring temperatures (Groff et al., 2019). It will be important to study other woodpecker species to determine the prevalence and distribution of these parasites, and the potential impact of these avian diseases on temperate forest ecology.

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Author's contributions TCG did the bulk of the field and molecular work, TJL organized the field work and initiated the project. TAI and GV did the microscopy and described the parasite. RNMS coordinated the work and conceived of the project. TCG, GV and RNMS wrote the paper.

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Availability of data and material All data not included in manuscript will be made available upon request.

Code availability Not applicable

Declarations

Conflicts of interest The authors have no conflicts of interest to declare that are relevant to the content of this article

Ethics approval All bird handling was conducted in compliance with the Ornithological Council Guidelines for the Use of Wild Birds in Research (Fair et al., 2010) and U.S. Department of Agriculture, Forest Service, Institutional Animal Care and Use Committee (Proposal number 2016-007).

Consent to participate Not applicable.

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