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Host specificity of passerine *Lankesterella* (Apicomplexa: Coccidia)

Ashwin Kumar Saravana Bhavan Venkatachalam [a,*], Ivan Čepička [b], Kristýna Hrazdilová [c], Milena Svobodová [a,*]

[a] *Department of Parasitology, Faculty of Science, Charles University, Czech Republic*
[b] *Department of Zoology, Faculty of Science, Charles University, Czech Republic*
[c] *Department of Chemistry and Biochemistry, Mendel University, Czech Republic*

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Abstract

Lankesterella parasites are blood coccidians that have recently gained attention as their records in common passerine species emerge. To date, their occurrence has been molecularly confirmed in several passerine genera, mainly among members of the families Paridae and Acrocephalidae. Despite their relatively high prevalence in some host populations, their life cycles remain unclear, mosquitoes or mites being the proposed vectors. The aim of this study was to reveal *Lankesterella* host specificity, focusing mainly on parasites of tit and warbler species (families Paridae and Acrocephalidae). We have determined the 18S rRNA gene sequences of *Lankesterella* from 35 individuals belonging to eight different host species. Phylogenetic analysis revealed that passerine *Lankesterella* are host-specific, with specificity at the host genus or species level. Besides *Lankesterella, Isospora* sequences were obtained from avian blood as well, pointing out the need for barcoding.
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Keywords: Avian parasites; *Acrocephalus*; *Parus*; *Cyanistes*; *Poecile*; *Sturnus*

Introduction

Avian blood protists are well known yet unevenly studied parasites. Among the apicomplexan blood parasites, the genus *Lankesterella* Labbé, 1899 is perhaps the most neglected, but it is recently gaining attention (Biedrzycka et al. 2013; Chagas et al. 2021a; Merino et al. 2006). *Lankesterella* belongs to true coccidians (Lankesterellidae, Eucoccidiorida, Apicomplexa; Adl et al. 2019) and is traditionally placed to the family Lankesterellidae, together with the genus *Schellackia* Reichenow, 1919. However, phylogenetic studies revealed that the two genera are not closely related to each other (Megía-Palma et al. 2017).

Members of *Lankesterella* are known to infect reptiles and amphibians (Desser 1993), but recent findings suggest that avian infections are not an exception (Chagas et al. 2021a). Interestingly, the blood stages of avian extraintestinal coccidians, previously assigned to *Hepatozoon* genus (Bennett and Peirce, 1989; Biedrzycka et al. 2013; Kruszewicz and Dyrcz, 2000) were shown to be closely related to a frog species, *Lankesterella minima* based on molecular barcoding of 18S rDNA gene (Merino et al. 2006). Passerines known to host *Lankesterella*, as confirmed by molecular barcoding, now include two species of tits – Blue Tit and Great Tit (*Cyanistes caeruleus* and *Parus major*; Bennett and Peirce 1989; Chagas et al.

*Corresponding authors at: Department of Parasitology, Faculty of Science, Charles University, Czech Republic.
E-mail addresses: saravana@natur.cuni.cz (A.K. Saravana Bhavan Venkatachalam), ivan.cepicka@natur.cuni.cz (I. Čepička), kristyna@hrazdilova.cz (K. Hrazdilová), milena@natur.cuni.cz (M. Svobodová).

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2021a; Merino et al. 2006), five warbler species – Sedge, Marsh, Reed, Great Reed and Icterine Warblers (*Acrocephalus* spp. and *Hippolais icterina*; Biedrzycka et al. 2013; Chagas et al. 2021a; Kruszewicz and Dyrcz 2000), Snow Bunting (*Plectrophenax nivalis*; Martínez et al. 2018), Common House Martin (*Delichon urbicum*; Bennett and Peirce 1989; Chagas et al. 2021a), and Eurasian Blackbird (*Turdus merula*, Chagas et al. 2021b; Kučera 1982). The number of hosts is probably underestimated since other records of morphologically similar parasites can be found in literature, and at least some of them probably represent *Lankesterella* as well (Bennett and Peirce, 1989; Bennett et al. 1992; Desser 1993; Kučera 1982).

The life cycle of avian *Lankesterella* is poorly known; most of the information about their life cycle was obtained while studying amphibians and reptiles (Desser 1993). These parasites are heteroxenous and their infective sporozoites, circulating in blood cells, are taken up by bloodsucking invertebrates (mites, ticks, leeches), but no multiplication in the vector was observed. Merogony, gametogony, and sporogony take place in the liver and gut of the vertebrate, which is unique among coccidians (Desser 1993; Levine 1982a, b). Transmission to the vertebrate host occurs by ingestion of the vector (Desser 1993). Due to the lack of replication of the parasites, the vectors are sometimes called paratenic hosts (Tse et al. 1986), which is perhaps not appropriate since they are necessary for transmission. Vectors responsible for transmission of avian species remain unknown, although Lainson (1959) suggested mites as vectors of a putative *Lankesterella* from sparrows. However, the identity of the parasite remains controversial (Box 1970). Mosquitoes are another putative vectors, and *Lankesterella* life stages survived in mosquitoes fed on naturally infected passerines; although transmission to experimental birds was unsuccessful (Chagas et al. 2021a).

Species of *Lankesterella* described from birds that have been sequenced and whose SSU rRNA gene sequences are available in GenBank include *Lankesterella macrovacuolata* from the Great Tit (Chagas et al. 2021a), *Lankesterella vacuolata* from the Common House Martin (Chagas et al. 2021a), *Lankesterella kabeeni* from Sedge Warbler (Chagas et al. 2021a; Kruszewicz and Dyrcz, 2000), *Lankesterella bivacuolata* from Eurasian Blackbird (Chagas et al. 2021b); *Lankesterella valsainensis*, occasionally used for lineages from Blue Tit, is probably an invalid name (Chagas et al. 2021a).

To shed light on the diversity and, potentially, the transmission of *Lankesterella* species, knowledge of their host specificity is needed. To achieve this, we focused on three tit species, Blue Tit (*C. caeruleus*), Great Tit (*P. major*), and Marsh Tit (*Poecile palustris*), and three warbler species: Marsh Warbler (*A. palustris*), Reed Warbler (*A. scirpaceus*), and Sedge Warbler (*A. schoenobaenus*) that occur sympatrically. We also included available occasional findings from other passerine hosts such as Eurasian Jay (*Garrulus glandarius*) and Starling (*Sturnus vulgaris*) with the aim to reveal host-parasite relationships and host specificity of avian *Lankesterella*.

Material and methods

Bird trapping and sampling

Common passerine species were trapped during the breeding season (May-July) from 2014 to 2021, at several localities in Czechia, namely, Zeměchy (50.230346, 14.267905), Choteč (49.989565, 14.282534), Tisý (49.056739, 14.724126), and Milovický forest (48.808441, 16.648047). Adults and yearlings were caught using mist nets and were sampled using a tuberculine syringe. Blood was drawn from the metatarsus vein articulation (*vena metatarsalis plantaris superficialis media*); a drop of blood was used to prepare a blood smear, and 10–20 µL of blood was stored in 96% ethanol for further use.

Animal experimentation guidelines

All experiments were performed by licensed workers. Birds were trapped and identified by licensed ringers; blood was sampled by people certified for experimentation with animals by the Ministry of Agriculture of the Czech Republic. Blood sampling was approved by the Committee on the Ethics of Laboratory Experiments of Charles University and performed under the permissions 50982/ENV/14-2961/630/14 and MZP/2019/630/1081 of the Ministry of the Environment of the Czech Republic.

Microscopy

Blood smears were fixed with methanol usually the day of sampling and stained with Giemsa (Sigma) according to the manufacturer's instructions for 30 min. Smears were microscopically checked with light microscope at 1000x magnification for 10 min by a single person (MS). Blood stages of *Lankesterella* were photographed at 1000x magnification with a CDC camera (DP70) using an Olympus BX51 microscope and light microscope.

DNA extraction, amplification, and sequencing

DNA was isolated using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Manheim, Germany) according to the manufacturer´s protocol. A specific nested PCR protocol was developed to amplify an approximately 1300 bp-long fragment of the 18S ribosomal RNA gene of *Lankesterella*. The first PCR step was performed in the final volume of 16 µL: 7 µL of PCR H_2O, 7 µL of PCR mix (PrimeSTAR Max DNA Polymerase Master Mix, TaKaRa, Shiga, Japan), 0.5 µL of primers EF (5′-GAAAC TGCGAATGGCTCATT-3′) and ER (5′-CTTGCGCCTAC

TAGGCATTC-3′) (10 μM concentration) (Kvičerová et al. 2008), and 1 μL of DNA. The PCR conditions were as follows: initial denaturation temperature at 98 °C for 3 min, denaturation at 98 °C for 10 sec, annealing at 55 °C for 20 sec, extension at 72 °C for 30 sec for 35 cycles, and final extension at 72 °C for 5 min. The second PCR step was performed in the final volume of 18 μL; 8 μL of PCR H_2O, 8 μL of PCR mix, 0.5 μL primers Hep153F (5′-GTAATTC TATGGCTAATACATGCGC-3′) and Hep1496R (5′-TTAT TGCCTCAAACTTCCTTGCG-3′) (10 μM concentration), which were newly designed using available sequences of *Lankesterella*, and 1 μL of the initial PCR product. The PCR conditions for the nested step was as follows; initial denaturation temperature at 98 °C for 3 min followed by the denaturation at 98 °C for 20 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 30 sec for 35 cycles, and final extension at 72 °C for 5 min. PCR products were analysed on 1% agarose gels, stained with SybrSafe, visualized under UV light, purified using the ExoSAP-IT™ PCR Product Clean up Reagent (ThermoScientific, Waltham, MA, USA), and sequenced with the primer Hep153F using Applied Biosystems® 3500 Genetic Analyzer at the core facility of the Faculty of Science, Charles University. Avian host DNA in the blood samples was barcoded as described in Valinsky et al. (2014) to confirm the species identity of Reed and Marsh Warblers.

Phylogenetic analysis

A data set containing 118 18S rRNA gene sequences was used for the phylogenetic analysis, out of which 35 were newly determined avian *Lankesterella* sequences. Four to seven *Lankesterella* sequences from each model species (three species of tits and three of warblers), occasional findings from other hosts and all available avian *Lankesterella* sequences from GenBank were used in the analysis. The rest were *Lankesterella* sequences from non-avian hosts or other closely related parasites such as *Eimeria, Isospora, Caryospora*, and *Schellackia.* The sequences were aligned using MAFFT with the G-INS-i algorithm (Katoh et al. 2002) on the server https://mafft.cbrc.jp/alignment/software/. The sequences were masked, and the alignment was slightly trimmed using BioEdit 7.2.5. The final data set used for the phylogenetic analysis consisted of 1864 positions. A maximum likelihood tree was constructed under GTRGAMMAI model with 10 starting trees in RAxML v8.2.10 (Stamatakis 2014). Statistical support was assessed by 1000 bootstrap pseudoreplicates in RAxML. Sequences from *Eimeria* and related genera were used as the outgroup (Fig. 1).

Results and discussion

We determined 44 new 18S rRNA gene sequences of which 35 belonged to *Lankesterella* while nine belonged to *Isospora* (Fig. 1). Seven of the new sequences that clustered with *Isospora* were from Sedge, Marsh, and Reed warblers and two sequences originated from Hawfinch (*Coccothraustes coccothraustes*) and Chaffinch (*Fringilla coelebs*), respectively (Fig. 1). The genus *Lankesterella* appears to be monophyletic since sequences from avian, amphibian, and reptile hosts clusters together although without sufficient support. All *Lankesterella* sequences from avian hosts cluster together forming an avian-specific clade. Interestingly, a lineage from Bocage's wall lizard (*Podarcis bocagei*) groups with the avian clade while all other *Lankesterella* sequences from reptiles are separated from the avian clade as shown previously (Chagas et al. 2021b). On the other hand, sequences originating from the genus *Schellackia* form a separate clade together with *Eimeria* sequences, thus supporting the non-monophyly of the family Lankesterellidae (Megía-Palma et al. 2014). The genus *Caryospora* seems to be non-monophyletic, which is consistent with Chapman et al. (2016). The reduction of sporocyst number occurred probably several times in coccidian evolution resulting thus in paraphyly of the genus *Caryospora* and hence the inconsistencies in the phylogenetic tree (Megía-Palma et al. 2015).

Most of the avian *Lankesterella* sequences formed host-specific clades. Interestingly, sequences obtained from the three tit species, each belonging to a different genus of the Paridae family, did not cluster together although they formed genus-specific clades. On the other hand, all sequences from *Acrocephalus* warblers clustered together. Species identification of Reed and Marsh Warblers done by ringers was confirmed by molecular barcoding; nevertheless, *Lankesterella* sequences from Reed and Marsh Warblers do not show any host-specific pattern while sequences from Sedge Warbler form a host-specific clade within the warbler clade, however with low support. As for the hosts, Common Reed and Marsh Warblers are more closely related to each other than each to the Sedge Warbler (Fregin et al. 2009); however, *Lankesterella* sequence from the Great Reed Warbler clusters together with Reed and Marsh Warbler sequences although the hosts are not so closely related. The sequence from Icterine Warbler (*Hippolais icterina*) clustered with *Acrocephalus* lineages as well, representing the only exception to the host specificity at the species/genus level.

Previous studies on avian *Lankesterella* always worked with a single avian host species (Sedge Warbler, Biedrzycka et al. 2013; Snow Bunting, Martínez et al. 2018; Blue Tit, Merino et al. 2006). Recently, a wider host range has been sampled, and several sequences from the Acrocephalidae family (*Acrocephalus scirpaceus*, *A. palustris*, *A.schoenobaenus* and *A. arundinaceus*) were compared (Chagas et al. 2021a). In our study, we extensively sampled not only warblers but also tits belonging to different genera. *Lankesterella* parasites were suggested to be host-specific on the family level (Chagas et al. 2021a); our data suggest that members of *Lankesterella* are probably specific on the

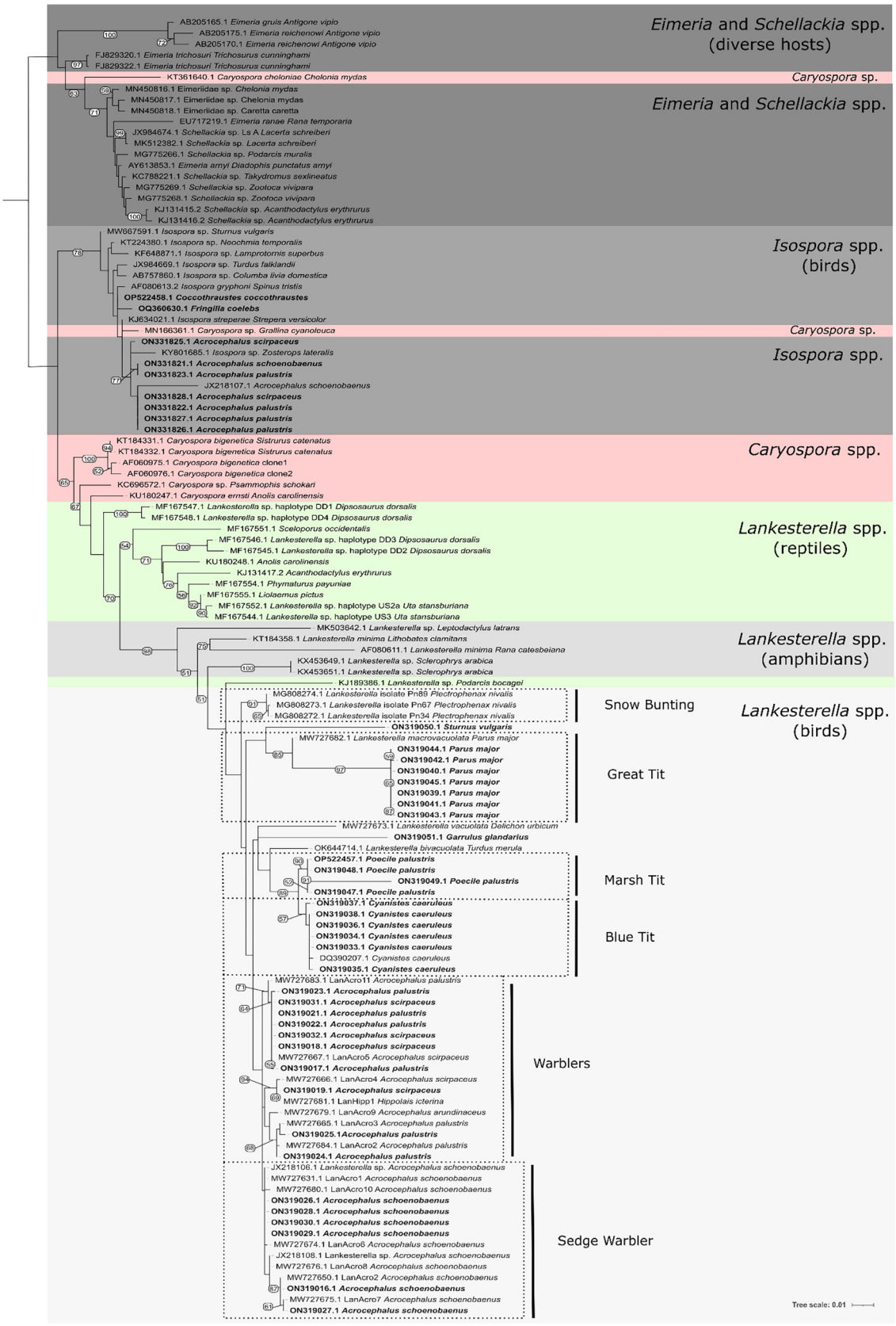

Fig. 1. Phylogenetic tree of avian *Lankesterella* spp. based on the 18S rRNA gene sequences. The tree was constructed by maximum likelihood in RAxML 8.0.0 (GTRGAMMAI model). Bootstrap values are shown at the branches (only support values > 50 are indicated). Newly determined sequences are highlighted in bold.

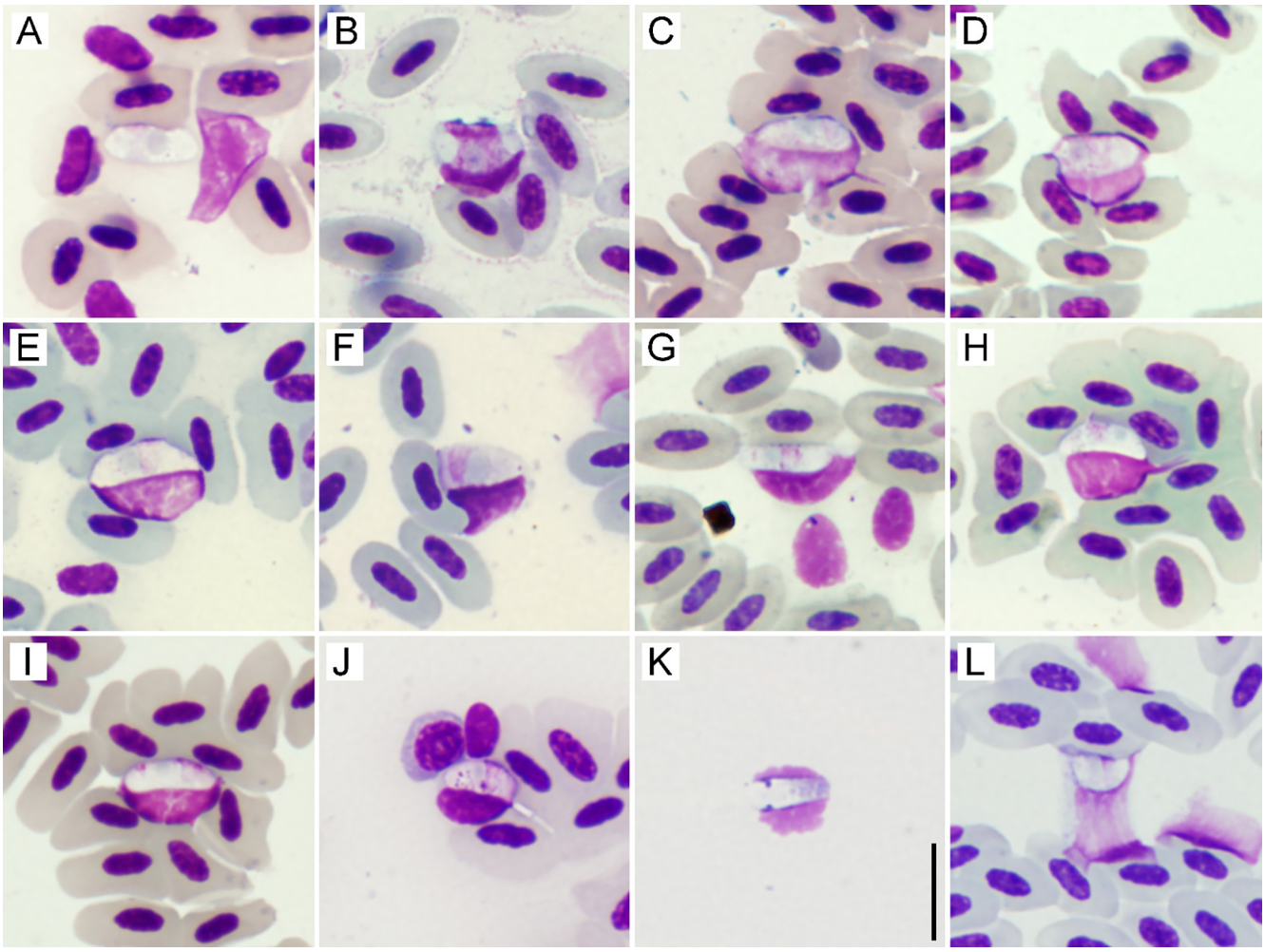

Fig. 2. *Lankesterella* sporozoites in blood of different passerine hosts. The parasites were found in thrombocytes and leukocytes. **(A-B)** Great Tit (*Parus major*), **(C-D)** Blue Tit (*Cyanistes caeruleus*), **(E-F)** Marsh Tit (*Poecile palustris*), **(G-H)** Sedge Warbler (*Acrocephalus schoenobaenus*), **(I)** Reed Warbler (*Acrocephalus scirpaceus*), **(J)** Marsh Warbler (*Acrocephalus palustris*) **(K)** Eurasian Jay (*Garrulus glandarius*), **(L)** Starling (*Sturnus vulgaris*). Bar: 10 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genus level or are even species-specific in some cases (Fig. 1). More sampling of different avian species belonging to the same genus and sampling of genera belonging to the same family would shed light on this topic. As concerns the host specificity of amphibian and reptile *Lankesterella*, the information is scarce, but it seems that they have a considerable level of host specificity as well (Maia et al. 2016). In this respect, the single lizard lineage clustering within the avian/amphibian clade is surprising and might result from an opportunistic interaction (Maia et al. 2014).

Host specificity of coccidians closely related to *Lankesterella* is usually narrow as well. *Schellackia* parasites are known to be highly host specific, especially among lizards (Megía-Palma et al. 2018; Zechmeisterová 2021). Members of the family Eimeriidae are thought to have a considerable degree of host specificity as well (Joyner, 1982; Knight et al. 2018; Kubiski et al. 2022; Schrenzel et al. 2005); but exceptions including spillover or host switching occur (Kvičerová and Hypša 2013). Some phylogenetic analyses suggest that the genus *Lankesterella* is closely related to the genus *Caryospora* (Barta 2001, Megía-Palma et al. 2015), which tends to heteroxenous development represented by the existence of so-called primary and secondary hosts, for which the host specificity is not so strict. Some *Caryospora* sequences including those of *C. bigenetica* cluster with the *Lankesterella* clade in our analysis. It could be possible that this flexibility in life cycles/transmission occurs in *Lankesterella* as well and that *Lankesterella* might be able to use multiple modes of transmission or several invertebrate vectors. In fact, a strictly host-specific parasite probably cannot use a vector with opportunistic feeding preferences, since its transmission to the next susceptible individual would be unlikely. Therefore, transmission by permanent ectoparasites (e. g., ticks, fleas, lice or mites) or direct life cycles seem to be more probable for *Lankesterella*.

The avian isolates of the genus *Isospora* Schneider, 1881 form its own clade in the phylogenetic tree; nine newly determined sequences from blood of several Warbler species, a Chaffinch, and a Hawfinch are closely related to *Isospora* lineages obtained from different species of passerines. The grouping of the *Caryospora*-like isolate from Magpie-lark (*Grallina cyanoleuca*) with *Isospora* suggests that the genus may be paraphyletic (Liu et al. 2020). *Isospora* parasites can be found as faecal oocysts or as blood merozoites in the avian host (Schrenzel et al. 2005). Extraintestinal stages of *Isospora* were not detected in the blood of tits. It is probable that, besides hosting *Lankesterella*, warblers of the studied populations are infected with *Isospora* coccidians with extraintestinal (blood) stages; this type of life cycle has been demonstrated in canaries (Box 1967) and was suspected previously in Polish warbler populations (Biedrzycka et al. 2013). Interestingly, all the warblers that we found positive for *Isospora* by PCR were negative by microscopy, thus preventing morphological comparison with *Lankesterella* blood stages (Fig. 2). Hence, unless a PCR protocol specific for the genus *Lankesterella* is developed, samples positive by the available PCR protocols should be sequenced to avoid confusion with extraintestinal *Isospora* species.

Conclusion

This study shows that avian *Lankesterella* parasites are host-specific at the genus or even the species level. The life cycle of avian *Lankesterella* still remains unresolved, and consistent data concerning prevalence and factors influencing *Lankesterella* distribution in host populations are lacking. Being widespread blood parasites, avian *Lankesterella* thus deserves further attention.

CRediT authorship contribution statement

Ashwin Kumar Saravana Bhavan Venkatachalam: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Ivan Čepička:** Writing – review & editing, Formal analysis, Software. **Kristýna Hrazdilová:** Writing – review & editing, Methodology. **Milena Svobodová:** Supervision, Writing – review & editing, Investigation, Conceptualization.

Data availability

Data available in GenBank.

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