VILNIAUS UNIVERSITY

Life Sciences Center

Institute of Biosciences

VYTAUTAS PREIKŠA

PREVALENCE AND GENETIC DIVERSITY OF *LANKESTERELLA* SPECIES (APICOMPLEXA, COCCIDIDA) IN JUVENILES OF *ACROCEPHALUS* BIRDS

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LANKESTERELLA RŪŠIŲ (APICOMPLEXA, COCCIDIDA) PARAZITŲ PAPLITIMAS IR GENETINĖ ĮVAIROVĖ _ACROCEPHALUS_ PAUKŠČIŲ GENTIES JAUNIKLIUOSE

Genetikos magistrinių studijų baigiamasis darbas

SANTRAUKA

Lankesterella (Lankesterellidae) yra kraujo parazitai, priklausantys Apicomplexa tipui. Jie parazituoja varliagyvius, driežus ir visai neseniai, molekulinių metodų pagalba, buvo aptikti parazituojantys paukščius, bet jie nebuvo pakankamai tiriami. Paukščiuose šie parazitai dažnai buvo priskiriami *Hepatozoon* rūšims, tačiau naudojant molekulės filogenijos metodus buvo nustatyta, kad jie priklauso *Lankesterella* genčiai. Kadangi apie šių parazitų, įskaitant, jų paplitimą, gyvenimo ciklą, šeimininkus, pernešėjus ir molekulinę charakteristiką, yra mažai žinių, šio tyrimo tikslas buvo ištirti *Lankesterella* haplotipų paplitimą *Acrocephalu*s paukščių genties jaunikliuose, derinant molekulinius ir mikroskopinius diagnostikos metodus. *Acrocephalus* gentis yra migruojantys paukščiai, pasižymintys plačia parazitų įvairovę. Paukščių jauniklių užsikrėtimas parazitais atspindi infekcijos lokalią transmisiją. Iš viso buvo ištirti 264 jaunikliai, priklausantys keturioms *Acrocepalus* rūšims. Bendras *Lankesterella* sp. paplitimas *Acrocephalus* paukščiuose buvo 6,4% (17 iš 264). Visi *Acrocephalus arundinaceus* kraujo mėginiai buvo neigiami, infekcijos ekstensyvumas *Acrocephalus schoenobaenus* paukščiuose buvo - 8%, *Acrocephalus palustris* - 5% ir *Acrocephalus scirpaceus* - 6%. Palyginus 17 sėkmingai amplifikuotų mėginių, buvo nustatyti 5 nauji haplotipai (1-5). *Acrocephalus schoenobaenus* paukščių rūšį parazitavo 1 haplotipas ir 2 haloptipas, *Acrocephalus palustris* - 3 haplotipas ir 5 haplotipas, *Acrocephalus scirpaceus* - 4 haplotipas ir 5 halotipas. Šiuo tyrimu galima patvirtinti, kad *Lankesterella* parazitų transmisija vyksta Europoje. Pirmą kartą nustatytos ir aprašytos šių

haplotipų kraujo stadijos. Tačiau neaišku, ar gyvenimo ciklas paukščiuose yra toks pats kaip ir parazitų, kurie parazituoja driežus ir varliagyvius. Norint išsiaiškinti šį klausimą, reikia atlikti papildomus tyrimus.

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Master Thesis

SUMMARY

Lankesterella (Lankesterellidae) parasites belong to the Apicomplexa phylum. They infect amphibians, lizards, and have been reported in birds. In birds, these parasites have been usually referred as belonging to *Hepatozoon* species however, recent molecular genetic data showed that they are more closely related to *Lankesterella* species. Because little is known about these pathogens, including their distribution, life cycle, hosts, vectors, and molecular characterization, **the objective** of the present study was to investigate the prevalence and genetic diversity of *Lankesterella* sp. in *Acrocephalus* juveniles birds combining molecular genetics and microscopic diagnostic tools. Birds of the genus *Acrocephalus* are migratory and are infected with a wide variety of parasites. Infections in juvenile birds can be considered as an indicator of local parasite transmission. In total, 264 juveniles belonging to four *Acrocephalus* species were examined. The overall prevalence of *Lankesterella* infection in *Acrocephalus* birds was 6.4% (17 of 264). All blood samples of *Acrocephalus arundinaceus* were negative, the prevalence of infection was 8% in *Acrocephalus schoenobaenus*, 5% in *Acrocephalus palustris* and 6% in *Acrocephalus scirpaceus*. When the sequences of the 17 positive samples were compared, five new closely related *Lankesterella* sp. haplotypes (1-5) were identified. *Acrocephalus schoenobaenus* was infected by haplotypes 1 and 2, *Acrocephalus palustris* by haplotype 3 and 5, and *Acrocephalus scirpaceus* by haplotypes 4 and 5. This study proved that the transmission of all these haplotypes takes place in Europe. Blood stages of these parasite haplotypes were determined and described for the first time. However, it is still unclear if the life cycle of found

parasites is the same as proposed for these parasites in lizards and amphibians. Further investigation is needed to clarify this subject.

INTRODUCTION

Parasites belonging to Apicomplexa phylum are relatively well-studied due to their medical, veterinary, and livestock importance. Some of the most well-known organisms belong to this parasite group, including *Plasmodium* spp., responsible for human and animal malaria, and *Toxoplasma* sp., which causes human and animal toxoplasmosis. However, some Apicomplexa species remain insufficiently studied, as is the case of *Lankesterella* species. This blood parasite belongs to Coccidia sub-class and has been reported infecting amphibians, lizards and, more recently, birds. In general, parasites can have a major impact on their hosts (Cox, 1993). In birds, blood parasites were reported to affect their physiology, ecology, health, population dynamics, sexual selection, and reproduction (Hamilton, Zuk, 1982; Marzal et al., 2005; Shurulinkov, Chakarov, 2006; Nourani et al., 2017). That calls for detail investigation of poorly studies parasitic infections.

The application of molecular diagnostic tools has opened a new opportunity to study parasitic infections, particularly in terms of genetic characteristics, epidemiology, populational biology and their taxonomic relationships. Most parasites belonging to the Coccidia, including the *Lankesterella* genus, are morphologically similar at many stages of their life cycle and have not been genetically characterized, resulting in many inaccuracies and disagreements in the systematics (Remple, 2004). In parasitology, rRNA (ribosomal RNA) gene have been used to assess the interspecific and intermediate genetic diversity of an organism (Johnston et al., 1993). Several studies that genetically reported infections by *Lankesterella* have used this gene in their parasite studies (Megía-Palma et al., 2015; Quillfeldt et al., 2018).

Even though that molecular diagnostic tools have been extensively used nowadays, it is important to correlate the molecular genetic findings with parasite morphological data (Nadler, León, 2011). This is particularly important in epidemiology research. These approaches complement each other: molecular tools are sensitive in diagnostics and are essential for identification of haplotypes, but often do not distinguish parasite life stages, but microscopic examination can be used for the latter purpose. This is especially true for *Lankesterella* species. There are several publications reporting these parasites as belonging to *Hepatozoon* genus. Only

recently, some molecular studies have pointed out the possibility that these parasites in birds actually belongs to *Lankesterella* genus (Merino et al., 2006). One of the bird species that have been reported to be infected with this parasite was *Acrocephalus schoenobaenus* (Biedrzycka et al., 2013). Because avian *Lankesterella* blood parasites remain insufficiently studies and have never been researched in Lithuania before, the present study focused on the investigation of this parasite in juveniles of *Acrocephalus* species.

Objective of this study: to evaluate the prevalence and genetic diversity of *Lankesterella* sp. (Apicomplexa, Coccidia) in juveniles of *Acrocephalus* species.

The following tasks were aimed to achieve:

1. To determine the prevalence of *Lankesterella* infections in *Acrocephalus* species;
2. To evaluate the genetic diversity of *Lankesterella* haplotypes in *Acrocephalus* birds;
3. To compare obtained haplotypes with the other genetic sequences that are deposited in GenBank public database;
4. To develop a phylogenetic inference of possible evolutionary relationship of *Lankesterella* parasites using detected haplotypes;
5. To determine blood stages of reported parasite haplotypes and illustrate their morphology.

1. LITERATURE REVIEW

1.1. Parasitism and the parasites of *Acrocephalus* birds

The term parasitism defines the relationship between two different organisms, where one (the parasite) uses the other (the host) as its environment from where it can obtain the nutrients necessary for its survivor. Parasites are a diverse group of organisms that have developed different strategies to infect their hosts and to obtain all the nutrients that they need to survive. Some parasites, such as lice and ticks, are found on the outer parts of the body (ectoparasites), but most of them are found inside (endoparasites) (Edosomwan, Igetei, 2018). Migratory birds can harbour a high diversity of pathogens. In Europe, different species of birds migrate to warmer regions of the globe, such as Africa, during winter. This increases the potential for parasite spreading, including zoonotic pathogens (Fuller et al., 2012).

Birds can have a high diversity of ectoparasites, such as feather mites (Acari: Astigmata, and lices (Phthiraptera: Ischnocera and Amblycera). They live on the surface of the body, on the feathers or in the host skin, feeding on skin, fat secretions, feathers, or blood (Proctor, 2003; Clayton et al., 2010). Gruianu et al. (2017) investigated the prevalence of parasites in *Acrocephalus* birds and reported infections in about 22% of studied animals. Also Holmstad et al. (2008) observed that the amount of endoparasites is directly correlated to the ectoparasites.

Endoparasites are more diverse and represented by several taxonomic groups of organisms, such as protists (Apicomplexa), nematodes (Nematoda), tapeworms (Cestoda), acantocephalans (Acantocephala), trematodes (Trematoda), and some others (Cox, 1993). They can infect various organs and tissues of bird's body and cause disease and even mortality in wild birds (Kruszewicz, 2000; Atkinson et al., 2008; Wojczulanis-Jakubas et al., 2012). However, the virulence of different parasites is markedly different, and not all parasites are pathogenic and able to cause disease and kill their hosts (Atkinson et al., 2008; Best et al., 2010).

The transmission of parasites can happen directly, when the host ingests the infective stage of the parasite (oocysts, eggs or larval stages) or indirectly, through vectors (ectoparasites and dipteran insects such as mosquitoes) or even by ingestion of other vertebrates or invertebrates (Kopečná et al., 2006; Atkinson et al., 2008). *Acrocephalus* birds were reported to harbour infections by different groups of organisms, such as Coccidia, Ascaridia, *Ornisthostrongylus* sp.,

Hepatozoon kabeeni, *Haemoproteus* spp. and *Lankesterella* sp. (Kruszewicz, 2000; Shurulinkov, Chakarov, 2006; Chagas et al., 2020).

1.2. Apicomplexa pathogens

Apicomplexa phylum is known as a large group of unicellular eukaryotic organisms that parasitize vertebrate and invertebrate hosts. With rare exceptions, apicomplexans are obligate parasites. It is estimated that there are between 1.2 to 10 million of species, and only about 0.1% of them have been detected, identified, and described to date (Adl et al., 2007). The majority of known and identified Apicomplexa parasites are found in vertebrates, including humans, but these parasites are equally diverse in marine, terrestrial, and invertebrates (Mihalca et al., 2008; Gupta et al., 2011; Renoux et al., 2017; Greay et al., 2018; Xavier et al., 2018). This is a large group of organisms, with a big number of morphological forms that vary according to the genus and life cycle (O'Donoghue, 2017).

Plasmodium spp. are the most well-known Apicomplexa parasites that cause human malaria, which kills about 1 million people per year (Mackintosh et al., 2004; Manguin et al., 2010). Another common parasite is *Toxoplasma gondii*, which is found in about 30% of human population. Even though this parasite often does not cause serious diseases in adults, it can cause severe disease in pregnant women and harm their foetus (McAuley, 2014). Parasitic apicomplexan infections of domestic animals are mainly associated with livestock animals and are known to cause significant economic losses. Of these, it is possible to highlight *Eimeria* spp., protozoans that cause about US$ 1.5 billion in losses to the poultry broiler industry worldwide each year (Sharman et al., 2010), and *Neospora caninum* that is associated with losses in the dairy and cattle industries (Trees et al., 1999).

Although Apicomplexa organisms are widely studied all over the world, there are many species in which life cycles are not completely understood. One of these species is the *Lankesterella* blood parasite. Until these days, scientists disagree about its taxonomic position inside of the Apicomplexa (Nocciolini et al., 2018). Phylogenetic information on avian *Lankesterella* parasites is limited (Sebaio et al., 2012). Most of the life cycle stages of these parasites, including the blood stage that allows microscopic diagnosis, remain insufficiently

investigated and are morphologically similar to *Hepatozoon* spp., the other Coccidia species that is commonly found in reptiles, and most commonly in several species of canids and felids (Merino et al., 2006). In fact, there are several species of *Hepatozoon* species that were described in birds using only morphological characteristics of blood stages in the past (Bennet et al., 1992).

Recently, Merino et al. (2006) suggested that the parasites morphologically identified as *Hepatozoon* in birds might, in fact, belong to *Lankesterella* genus. Blood stages were found in Blue tit (*Cyanistes caeruleus*) and microscopic analysis showed that they were similar to *Hepatozoon parus*. However, molecular analysis revealed that the sequence obtained from infected birds was more closely related to *Lankesterella minima*, a parasite of amphibians, than to other *Hepatozoon* species. That raised a question about the taxonomic status of *Hepatozoon*-like infections in birds.

A similar study was performed by Biedrzycka et al. (2013), that examined blood samples from Sedge warbler (*Acrocephalus schoenobaenus*). This bird species is known for harbouring infections by *Hepatozoon kabeeni*. In that study, authors managed to amplify and identify three different haplotypes, and phylogenetic analysis showed that two of them were closely related to *Lankesterella minima* and the *Lankesterella* sequence found by Merino et al. (2006). The third haplotype was grouped with *Caryospora* and *Eimeria*. None of the haplotypes were similar to other *Hepatozoon* sequences. Martínez et al. (2018) found similar genetic results when juveniles Snow bunting (*Plectrophenax nivalis*) were investigated for the presence of haemoparasites. The results of all these three studies confirmed the need for further investigation and elucidation on taxonomy of this group.

1.2.1. Origin of Apicomplexa

According to the literature, Apicomplexa organisms evolved from the ancestor of dinoflagellates, which are free-living unicellular organisms that use photosynthesis to obtain energy for their metabolism. However, a large proportion of them are mixotrophs, receiving nutrients through photosynthesis and parasitizing the host (Stoecker, 1999). Phylogenetic analysis shows that Apicomplexa forms a sister group with members of the genus *Colpodella*, that is an alga from Alveolata superphylum (Kuvardina et al., 2002). This genus unites unicellular

organisms that mature into unicellular algae by means of myzocytosis. Myzocytosis is a mode of feeding during which a parasite attaches to a prey (cell) and sucks out the cell's cytoplasm through specialized structures. The cell-parasite interaction is mediated by specific organelles (apical complex) that are similar to those used by Apicomplexa species to attach and to invade the host cells. Thus, Apicomplexa most likely evolved from these organisms or their relatives (Arisue, Hashimoto, 2015).

Another link between apicomplexan and algae is an organelle that is chloroplast residue called apicoplast. This organelle is found in almost all Apicomplexa parasites, with exception of *Cryptosporidium* spp. and gregarines (Liu et al., 2016). Apicoplasts are involved in important metabolic processes, such as fatty acid synthesis and isoprenoid precursor synthesis. Furthermore, it plays an important role in the process of host cell invasion by the parasite. According to former studies, this plastid is the secondary endosymbiotic origin and is derived from red algae (Lim et al., 2010).

1.2.2. Important ultrastructural characteristics of Apicomplexa parasites

Most apicomplexans have a sporozoite stage (Figure 1.1), which is the infectious stage and is characterized by a group of distinctive morphological features. Sporozoites are covered by pellicle, which consists of outer membrane and a complex of alveoles. Inside the sporozoite a nucleus, mitochondria, dense granules and other cellular structures are present. The most distinctive sporozoite feature is the apical complex (polar ring, conoide and other structures), which is located at the anterior cell pole. This complex is connected with numerous microtubules, which locate below pellicle. Micronemes and rhoptries ducts open on apical sporozoite end. These structures are located throughout the sporozoite but are more dense on apical end; they produce various enzymes being involved in parasite penetration into host cells. Sporozoites also possess micropores, which are responsible for absorbing nutrients (Morrissette, Sibley, 2002).

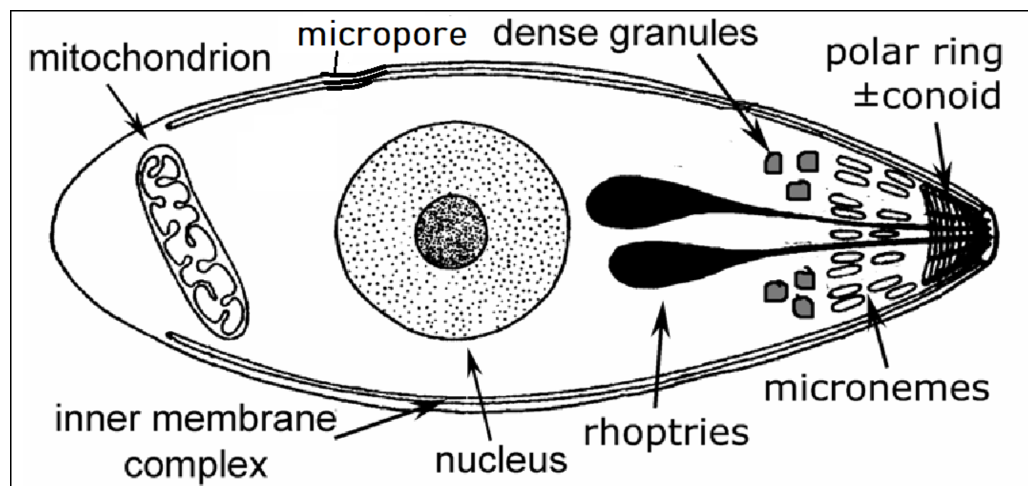


Figure 1.1. Schematic representation of ultrastructure of sporozoites of Apicomplexa parasites (according to Ghimire, 2010).

1.3. Classification of *Lankesterella* parasites

Lankesterella genus was described by Labbé in 1899, that first observed this parasite in frogs (Labbé, 1899). Avian *Lankesterella* taxonomy has been debated for many years being considered as belonging to different groups of organisms such as *Haemogregarina*, *Toxoplasma*, *Atoxoplasma* (Box, 1971) and *Isospora* (Desser, 1980). Levine (1982) addressed this taxonomic problem and proposed that different protozoan genera that have similar blood cells stages cannot be assigned to certain genera until their life cycle is clear.

Currently, the most broadly accepted classification is the one provided by Levine (1980). Eimeriina is a suborder of Apicomplexa that includes many genera and species (Duszynski, Upton, 2009). This suborder includes the family Lankesterellidae, which includes two genera, *Lankesterella* (Labbé, 1899) and *Schellackia* (Reichenow, 1919). This family is more commonly called hemococcidia because it can be found in the blood of vertebrates. Classification according to Levine (1980):

Phylum: Apicomplexa

Class: Sporozoea

Sub-class: Coccidia

Order: Eucoccidiorida

Sub-order: Eimeriina

family: Lankesterellidae

Genus: *Lankesterella*

The *Schellackia* genus was originally found in European lizards (Reichenow, 1919). *Schellackia* species infects frogs and lizards in Europe, America, Asia, Africa and Oceania have been described to date (Bonorris, Ball, 1955; Rogier, Landau, 1975; Lainson et al., 1976; Godfrey et al., 2006; Telford, 2009). The second genus of hemococcidia is *Lankesterella*, which species were first reported parasitizing frogs (Labbé, 1899). Later species of this genus were found in lizards (Desser et al., 1990) and in birds (Mansour, Mohammed, 1962; Lainson, Paperna, 1995; Paperna, Ogara, 1996; Merino et al., 2006; Biedrzycka et al., 2013; Martínez et al., 2018). It is believed that the *Lankesterella* and *Schellackia* form a monophyletic clade in phylogeny of the Lankesterellidae family. However, recent phylogenetic analyses have shown that they are evolutionarily independent groups (Megía-Palma et al., 2017).

The main morphological differences used to identify hemococcidia parasites, which are present in lizard blood cells and classified as *Lankesterella* or *Schellackia*, are the characteristics of oocysts during endogenous parasite development. Usually, *Schellackia* sp. oocyst consists of eight naked sporozoites surrounded by a soft-walled of the oocyst in *lamina propria* of the gastrointestinal tract. In contrast to *Schellackia*, *Lankesterella* consists of 32 or more sporozoites (Telford, 2009). However, the taxonomic value of these features requires further testing.

1.3.1. Life cycle

Parasites of the Lankesterellidae family have and obligate-heteroxenous life cycle, with sexual and asexual replication stages (merogony, gamontogony and sporogony) occurring in the intestinal tissues of vertebrates (Megía-Palma et al., 2014). However, differently from what happens with other coccidians, the oocysts are not expelled to the environment, and sporozoites are released in the host. They reach the bloodstream, inhabit mononuclear white cells, and maintained in the circulation. The parasites are ingested by hematophagous invertebrate hosts, where they became dormant stages, and a new vertebrate host is infected when it ingests the invertebrate host (Upton, 2000). In *Hepatozoon* spp., the gamontogony and sporogony takes place in invertebrates (Ewing, Panciera, 2003; Megía-Palma et al., 2014). Although it has been reported that *Lankesterella* sp. protozoans parasitize amphibians, lizards, and birds, the life cycle has been elucidated only for *Lankesterella minima*, which parasitizes the Edible frog (*Rana esculenta*) (Merino et al., 2006; Biedrzycka et al., 2013; Martínez et al., 2018).

Briefly (Figure 1.2), transmission occurs when a vertebrate host ingests an invertebrate that is infected with the parasite, and it can also occur through direct ingestion of infected blood or liver (experimental infections) (Figure 1.2, A) (Telford, 2009). Sporozoites enter the intestinal epithelial cells and form meronts (Figure 1.2, B), from which merozoites develop (Figure 1.2, C), which become macro- and microgametes (Figure 1.2, D). Sexual reproduction will happen, and zygote will be formed (Figure 1.2, E). The zygote will develop and form oocysts (Figure 1.2, F) where sporogony will occur and form sporozoites. Between 32 and 50 sporozoites are produced in three days which then travel to the circulatory blood system and infect white or red blood cells (Kreier, 1993; Telford, 2009).

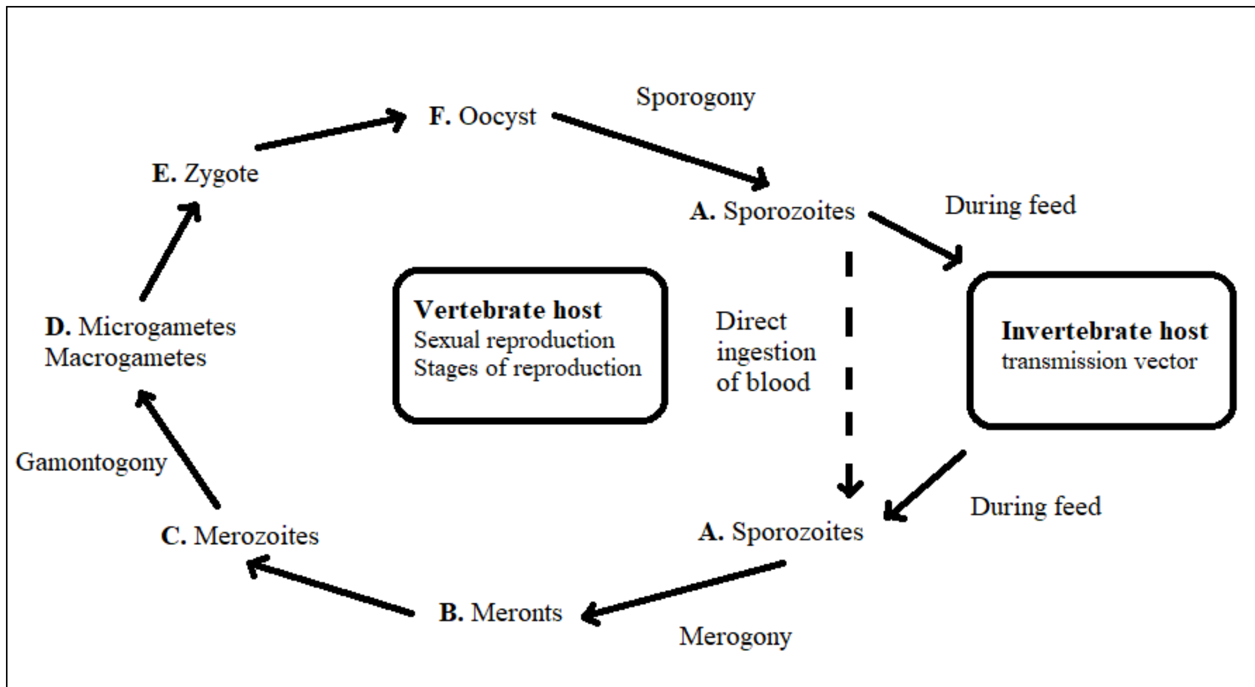


Figure 1.2. Schematic representation of *Lankesterella minima* life cycle (according to Telford, 2009).

1.4. Parasite detection methods

Study of parasite taxonomy, prevalence and diversity in vertebrates is the first step to better understand ecological interactions between parasites and hosts (Poulin, 2005). This can be done in different forms and using different methodologies, each one with different requirements and indications.

Microscopy is the traditional and gold standard method for the detection and identification of blood parasites. This analysis can be done using blood smears prepared in glass slides followed by its fixation and staining with Giemsa (Valkiūnas et al., 2008; Telford, 2009). Microscopic observation of parasite's morphological features and stages of development can provide a better understanding of the infection and parasite's life cycle. Apicomplexans parasites are able to infect different types of cells, so when performing microscopically analysis, it is possible to see the parasite inside of the cell. In the case of *Lankesterella*, they can infect mononuclear leukocytes and thrombocytes (Baker et al., 1972). The identification and characterization of blood parasites depends on the characterization of the stages of development

of the parasite in the host, as well as in which cells or tissues reproduction takes place. Thus, microscopy is a valuable tool because it allows to monitor the morphological features of the parasite, to assess the prevalence of the infections, as well as the intensity of the infection, to identify co-infections, the presence of more than one parasite in the same samples, and to identify in which cells or tissues the parasites live (Moody, 2002; Jovani et al., 2004; Valkiūnas et al., 2008). Importantly, this method can show if parasite complete life cycle and produce invasive stages in a host, but molecular diagnostic tolls often cannot answer this question.

PCR (polymer chain reaction)-based methods have been extensively used in the diagnosis of parasitic infections (Singh, 1997). They consist of targeting and amplifying a fragment of the DNA (deoxyribonucleic acid) or RNA (ribonucleic acid) of the parasite in order to determine if the parasite is present or not in the sample. PCR-based methods are particularly attractive because they provide information on the phylogenetic relationships of parasites and the diagnosis of parasitic diseases (Kimura et al., 2006; Križanauskienė et al., 2006; Palinauskas et al., 2007; Perkin et al., 2007). Thus, molecular diagnostics is a valuable tool because it allows the detection of a parasite at low intensity of infection, comparison and phylogenetic relationships between organisms, elucidation of the parasite life cycle by determining the DNA sequence of the parasite in the vector, paratenic (reservoir) organism and final host and showing directions for most productive microscopic examinations (Valkiūnas et al., 2008).

However, a disadvantage PCR-based protocols using general primers in pathogen diagnostics is that they often are insufficiently sensitivity to detect co-infections, which are common in wild animals, especially in the diagnostic of parasites, which belong to different closely related genera (Bernotienė et al., 2016). To determine the prevalence of parasite species, it is necessary to improve PCR diagnostics, for example, to use specific primers, which is not always available, even for parasites that are well-known and studied worldwide (Valkiūnas et al., 2008)

When microscopy and molecular techniques are compared, microscopy usually is referred to as being significantly less sensitive than molecular methods and is less accurate in determining the prevalence and intensity of parasitic infections (Mangold et al., 2005). However, microscopy often is more sensitive to detect co-infections, which are common in the wild. It is important to highlight that depending on the protocol used, DNA extraction requires several days to finish.

Sequencing can also take several days if sequencing equipment is not available on site. These details are very important if the diagnostic should be performed quickly. Valkiūnas et al. (2008) compared microscopy and molecular diagnostic methods and advised to combine both methods.

1.4.1. DNA molecular marker for the Apicomplexa parasite detection

To study the phylogenetic relationships of the protists of Apicomplexa, the most widely targeted gene is the nuclear ribosomal DNA (rDNA). The rRNA gene sequences are convenient in such research because this gene is relatively conserved, allowing the use of universal PCR primers to amplify sequences and also because there have many copies of this gene in the same organism, which makes amplification easier. The disadvantage of using these genes is that they are not very informative when relatively recent evolutionary changes are studied (Abouheif et al., 1998).

The structure of rDNA is composed of repetitive clusters of rRNA genes, which length is about 8-14 kb. The rRNA cluster consists of the 18S rRNA gene (small ribosomal RNA subunit), the 5.8S rRNA gene sequence, which is separated by internal transcribed spacers ITS1 and ITS2, and the 28S rRNA (large ribosomal subunit) gene sequence. The whole complex is surrounded by external transcribed spacers 5' ETS and 3' ETS, which do not transcribe regions or IGS (intergenic spacers) connect adjacent copies of rRNA gene clusters (Figure 1.3). Both internal and external transcribed spacers have high structural variability, so these regions are used less frequently or not at all in phylogenetic studies. Also, the 5.8S rRNA gene is not often used due to low informativeness of phylogenetic relationships (Dyomin et al., 2016).

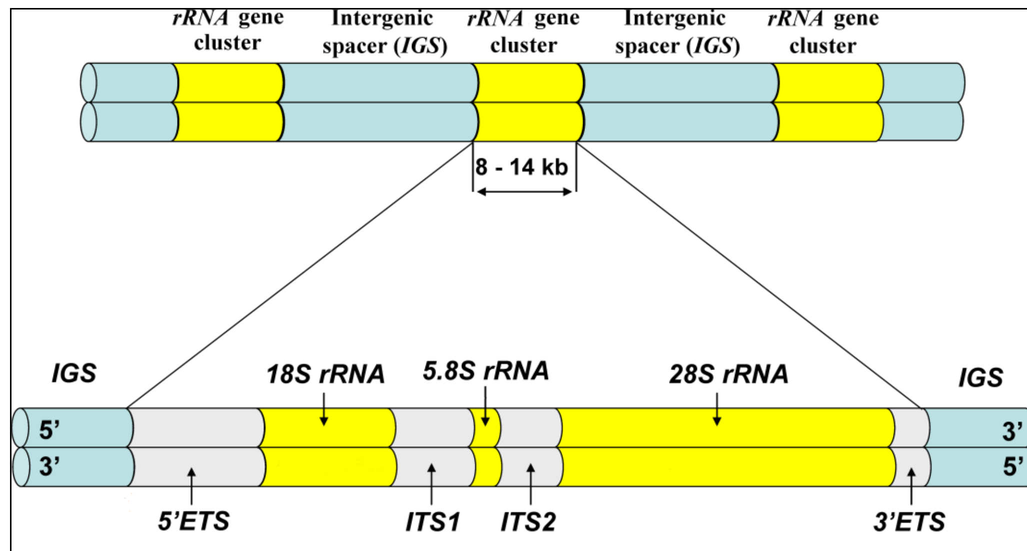


Figure 1.3. Schematic representation of rDNA sequence structure (according to Srivastava, Schlessinger, 1991).

1.5. *Acrocephalus* genus

The reed warblers (*Acrocephalus*) is a genus of birds that belong to the Acrocephalidae family and Passeriformes order. Representatives of this genus are small insectivores. It is now recognized that most of these birds are found in moist habitats such as wetlands, in reeds as well as in shrubs and bushes. Many species have fairly simple colouration of feathers. According to the International Ornithological Committee World Bird List database, 43 species are registered in the world and seven of them reported in Lithuania (Karalius et al., 2019; Gill, Donsker, 2020), being: Aquatic warbler (*Acrocephalus paludicola*), Paddyfield warbler (*Acrocephalus agricola*), Blyth's reed warbler (*Acrocephalus dumetorum*), Sedge warbler (*Acrocephalus schoenobaenus*), Marsh warbler (*Acrocephalus palustris*), Eurasian reed warbler *(Acrocephalus scirpaceus*), Great reed warbler (*Acrocephalus arundinaceus*). The last four species of the list were used in the present study, and they are going to be described in more details below.

1.5.1. *Acrocephalus schoenobaenus*

The appearance of Sedge warbler is dominated by yellowish-brown feathers. The most important features by which this bird can be identified are from the beak, a bright white band

above the eye, a brown tail, brown sides, and a whitish belly. The juveniles are similar to adults but with a darker chest. This bird is often found in dense grass, as well as in shrub vegetation along water bodies. It feeds mainly on flies (order Diptera). In Lithuania, the population of Sedge warblers varies between 300,000 – 400,000 (Karalius et al., 2019).

Monogamy predominates among Sedge warblers, but there are exceptions among males (Leisler, Wink, 2000). Sedge warbler begins to migrate from mid-March to mid-June in Europe and South Asia, wintering in central and southern Africa. The female lays and hatches three to five eggs during the breeding season. The incubation period is of 16 days. After hatching, both female and male feed the juveniles until they become independent, this takes about 11 to 12 days (Alker, Redfern, 1996).

1.5.2. *Acrocephalus palustris*

The upper part of the body of the Marsh warbler is one-coloured, greenish-brown, the middle part is slightly browner, and the lower part is whitish with a yellowish-brown tinge. It has an "eyebrow" that is blurred compared to a Sedge warbler. It has a bright white ring around the eyes. Juveniles differ from adults in a browner shade. These birds are found in various bushes, mowed meadows, and river valleys. A water body is not a necessary element for their hatcheries. In recent years, the population of Marsh warblers in Lithuania has decreased, currently, the population ranges from just 100,000 – 150,000 (Karalius et al., 2019).

The Marsh warbler is a monogamous species. They breed in Europe, to where they migrate in mid-April and stay until mid-June in Europe and then, for the winter, fly to Africa. During the season, three to six eggs are laid, the offspring hatch within ten to eleven days. Only the females hatch the eggs during the night, but both males and females hatch equally during the day. Juveniles are fed for 15 to 20 days by both parents until they become independent (Dowsett-Lemaire, Collette, 1980).

1.5.3. *Acrocephalus scirpaceus*

The Eurasian reed warbler is slightly larger than the Great tit, the top of the bird is greenish brown, the equator and tail are brown, the bottom whitish with a yellowish-brown tinge.

The eyebrows are short compared to the birds described as enemies, extending from the beak to the eyes. Juveniles are slightly browner than mature birds, but in nature it is difficult to distinguish them. These birds place high demands on their habitats, usually dense and large reedbeds. They mainly feed on small invertebrates. According to 2019 data, in Lithuania the population size is 300,000 – 500,000 (Karalius et al., 2019).

Monogamy prevails among these birds. Breeding season in Europe lasts from mid-March to mid-June, they are wintering in central Africa. Females lay three to five eggs per season. Both females and males breed juvenile. The juvenile hatch in nine to 12 days, before they become independent, they are fed for ten to 14 days by both partners. Nests are often parasitized by cuckoo (Holden, 2018).

1.5.4. *Acrocephalus arundinaceus*

The Great reed warbler is much larger than other birds of this genus. The appearance is very similar to the Eurasian reed warbler, however, they are different in size and songs. They defend their territory. Juveniles are similar to the adults, only slightly browner. Great reed warbler habitats are most commonly found on the shores, as well as on high, and dense reedbeds of water bodies. Usually they feed on insects, but rarely they also feed on small vertebrates. In Lithuania, the population of these birds is from 40,000 – 80,000 (Karalius et al., 2019).

Monogamous and polygamous relationships prevail among males of the Great reed warbler. The female chooses the male according to the quality of the area, the abundance of food, and the protection of the nest from predators. Males with an attractive nest are polygamous, while males with lower quality nests are monogamous or do not mate at all. Females choose a second partner only if they have a larger repertoire of songs, which is associated with higher offspring survival (Hansson et al., 2000; Nowicki et al., 2000). The Great reed warbler breed from early April to August in northern Europe, wintering in Africa. Females lay an average of three to six eggs per season. The juvenile hatch within 14 days and become independent after two weeks (Hansson et al., 2004).

1.6. Influence of parasites on bird population dynamics

Ectoparasites such as lice (Phthiraptera: Ischnocera, Amblycera), which feed on feathers and skin, can damage the feathers, which would decrease its quality, puncturing small holes, reducing feather aerodynamic and causing chronic dermatosis. These factors affect bird flying, consequently migration distance and survival (Vas et al., 2008; Gruianu et al., 2017).

Based on the hypothesis of Hamilton and Zuk (1982), female birds evaluate the genetic quality of males based on their resistance to endoparasites. Resistance is correlated with certain bird traits, such as bird mass, feather size, and colour brightness, which should be less developed in parasitized birds compared to healthy ones. The authors suggested that these traits help inform females about the genetic resistance of the male to endoparasites, so they reflect the current physical condition of the individual. The degree of decorativeness of all species should be positively related to the prevalence of parasites (Hamilton, Zuk, 1982). There are many studies, which partly supported or do not supported at all this hypothesis, which still remains attractive to evolutionary biology and need further testing using sensitive genetic methods.

For pale-coloured species such as those of *Acrocephalus*, a trait that could correlate with the intensity of parasitism may be song complexity, which should correlate with body weight and fat deposits. Buchanan et al. (1999) reported that *Acrocephalus schoenobaenus* individuals who were infected with blood parasites had a significantly smaller song repertoire compared to non-parasitized males. Body weight is also directly correlated with the intensity of parasites in the blood (Buchanan et al., 1999).

2. MATERIAL AND METHODS

2.1. Study site

The study was conducted in late July, in the years of 2016 to 2018. Samples were collected in Ventės Ragas Ornithological Station (VROS) (55°20′28.1″N, 21°11′25.3″E), Lithuania (Figure 2.1). Samples of this study were collected by the staff of P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, and provided for the thesis author for investigation.

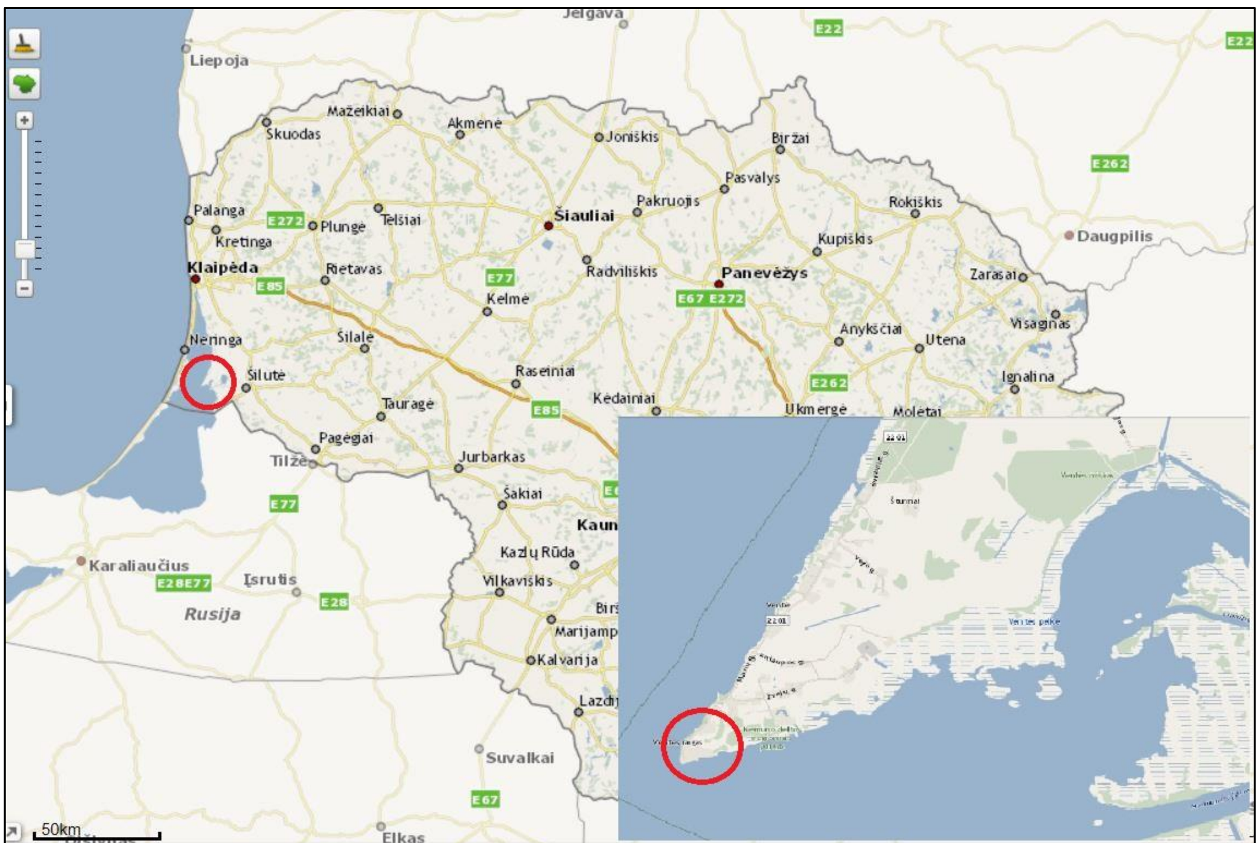


Figure 2.1. Location of the study site. The Ventės Ragas Ornithological Station, Ventė, Lithuania. Source: http://maps.lt/map/.

2.2. Sample collection and blood smears preparation

Acrocephalus juveniles were caught using zigzag nets and big funnel traps, identified and ringed by the ornithologists of VROS. About 30 µl of blood was taken from the brachial vein using heparinized microcapillaries. Few drops were used to prepare blood smears, that were air-dried, fixed in absolute methanol, and stained with Giemsa (Valkiūnas et al., 2008). Remaining blood was transferred to an Eppendorf containing 500 µl of SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for further DNA extraction.

Blood smears were examined using an Olympus BX-43 light microscope equipped with an Olympus SZX2-FOF digital camera and QCapture Pro 6.0, Image Pro Plus (Olympus, Tokyo, Japan) imaging software. The preparations were analysed with 1000x magnification. Parasites were morphologically identified according to Paperna and Martin (2001).

All procedures described here were performed by experienced researchers and comply with the current laws of Lithuania and were approved by the Environmental Protection Agency, Vilnius, under permits 2016-05-05 Nr. 23, 2017-04-26 Nr. 23 and 2018-04-13 Nr. 24.

2.3. DNA extraction

The DNA extraction of blood samples was conducted in P. B. Šivickis Laboratory of Parasitology, at Nature Research Centre, Vilnius. An ammonium acetate protocol was used (Dolnik et al., 2009). Briefly, 250 µl of the mixture of blood and SET buffer was transferred to an eppendorf tube, adding 7 µl of 20% SDS and 5 µl of 20 mg / ml Proteinase K (Thermo Fisher Scientific, Lithuania). Then, the samples were vortexed and incubated at 56 °C in a water bath overnight. Next, 250 µl of 4M ammonium acetate solution was added to the samples and incubated for 40 – 60 min at 700 rpm on an automatic shaker. Next, samples were centrifuged for 10 minutes at 13,000 rpm. After that, the supernatant was transferred to a new tube and about 800 µl of 96% ethanol was added, followed by centrifugation for 15 minutes at 13,000 rpm. Finally, the supernatant was carefully discarded, and the pellet was washed with 70% ethanol. Extracted DNA was left to dry overnight and then resuspended with 1x TE buffer.

2.4. DNA amplification

A nested PCR protocol was used to amplify DNA fragments of the 18S rRNA. PCR was performed using a MiniAmp™ Plus Thermal Cycler - Thermo Fisher Scientific amplifier. The primers used in this protocol were developed by Dr. Josef Harl, from the University of Veterinary Medicine, Vienna, Austria (Table 2.1). The primers were generated based on the 18S rDNA sequences of parasites belonging to various genera of Apicomplexa (*Hepatozoon*, *Eimeria*, *Isospora* and *Lankesterella*). The primary target was 1105 bp fragment located in the middle part of 18S rDNA.

Table 2.1. The primers used to amply a fragment of 18S rDNA of *Lankesterella* parasites.

Primers	Nucleotide sequence	Fragment length
Cocc18S_n1F:	5'- CAGCTTTCGACGGTATGGTATTGG - 3'	1135 bp
Cocc18S_n1R:	5'- CAGACCTGTTATTGCCTCAAACTTCCT - 3'	
Cocc18S_n2F:	5'- GTATTGGCTTACCGTGGCAGTGAC - 3'	1105 bp
Cocc18S_n2R:	5'- GCCTCAAACTTCCTTGCGTTAGACA - 3'	

All reactions were performed in a 25 µl total volume, including 2 µl of genomic DNA, 12.5 µl of Dream Taq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µl of nuclease-free water and 1 µl of each primer. In the first reaction primers Cocc18S_n1F / Cocc18S_n1R were used, and in the second reaction primers Cocc18S_n2F / Cocc18S_n2R were used. In each PCR, one negative control (nuclease-free water) and one positive control (an infected sample, which was positive by microscopy examination of blood smears) were used. The temperature profile is shown in table 2.2.

After the PCR was conducted, the product was evaluated in 2% agarose gels electrophoresis (Magdeldin, 2012). Briefly, 2 g of agarose powder (Thermo Fisher Scientific, Lithuania) was added in prepared 1x TAE buffer (100 ml of 10x TAE buffer mixed with 900 ml of distilled water). Using a microwave, the solution was heated and stir until agarose was completely dissolved. Next, 0.5 µl of Green Dye was added to the solution and mixed well. Then,

the solution was poured into a gel mold with a comb holder. Once the gel had solidified, the comb was removed, and the gel was immersed in an electrophoresis vessel with 1x TAE buffer.

Table 2.2. The temperature profile of nested PCR amplification.

Steps	Temperature	Time	Cycle
Initial denaturation	95 °C	2 min	1x
Denaturation	95 °C	30 sec	35x
Primers hybridization	58 °C	30 sec	
DNA synthesis	72 °C	1 min	
Final DNA synthesis	72 °C	5 min	1x
Hold	4 °C	∞	-

The protocol for loading PCR products into electrophoresis gel consisted of first, adding 1 µl of Loading dye (Thermo Fisher Scientific, Lithuania) on parafilm for each sample. Next, 2 µl of PCR product was taken and mixed with loading dye, then poured into a gel well (repeat with all samples). Gene Ruler 100bp Plus DNA Ladder (Thermo Fisher Scientific, Lithuania) was used as a molecular marker which was added to the edge of the gel. Electrophoresis was conducted at 90 V for 30 minutes. Finally, the results of electrophoresis gel were analysed under ultraviolet light. Samples were considered positive when they amplified a fragment of about 1100 bp.

The samples that were considered as positive were submitted to DNA precipitation and then were sent for sequencing. An ammonium acetate protocol was used for precipitation and purification of positive samples. For precipitation, 21 µl of PCR product were transferred to a new 0.5 ml tube and mixed with 11 µl of 8M ammonium acetate and 37 µl of 96% ethanol. The solution was incubated for 15 minutes at room temperature. Next, the samples were centrifuged at 10,000 rpm for 10 minutes. Then, the supernatant was discarded and 150 µl of 70% ice-cold ethanol was added. After that, samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded, and the tubes were left to dry overnight. Finally, 17 µl of distilled water were added to the tubes and the samples were vortexed and centrifuged twice. Prepared samples were stored in the freezer. The purified samples were sequenced from both ends 5'- and

3'- with primers applied in the nested reaction. The sequencing was carried out using an ABI PRISM TM 3100 capillary sequencing robot (Applied Biosystems, Foster City, California, USA).

After all samples were processed, the prevalence of infections was calculated. This was done dividing the number of infected animals by the total number of studied birds, the result was divided by 100, and represented by percentage. Yates's correction was used for correcting for approximation error, it was considered as being statistically significant $p \leq 0.05$ (Maxwell, 1976).

2.5. Phylogenetic inference, genetic distance and haplotype polymorphism

Both forward and reverse sequences were visualized and edited with BioEdit software (Hall, 1999) in order to create a consensus sequence for each positive sample. The best-fitting model of evolution was the general time return model (GTR + G + I) selected by MrModeltest2 (Nylander, 2018).

Phylogenetic inference was constructed using a partial sequence (983 bp) of 18S rRNA. In all, 63 sequences were used, and four sequences were used as outgroup. The Bayesian phylogenetic tree was constructed using MrBayes v.3.2.0 (Ronquist, Heulsenbeck, 2003). Analysis was conducted using the Markov Chain Monte Carlo (MCMC), searches were done in 3,000,000 generations, with a sample frequency of every 100th generation. Before the constructions of consensus tree, 25% of the initial tree were discarded as a "burn in". Phylogeny was visualized using FigTree v.1.4 software. (Rambaut, 2006-2012).

The genetic distance of the sequences was determined by MEGA X software, analysis was run using the Compute pairwise method and q-distance model included transitions and transversions (Kumar et al., 2018).

To determine haplotype sequence polymorphisms, DnaSP v6 software (Rozas, 2018) was used. Method to detect polymorphisms were "DNA polymorphisms", analysed sites was 983 bp. GeneDoc software (Nicholas, Nicholas, 1997) was used to visualize nucleotide differences.

3. RESULTS

3.1. Prevalence of *Lankesterella* infections

Samples from four species of *Acrocephalus* juveniles were analysed in the present study. Overall prevalence of infection was of 6.4% (Table 3.1 and Figure 3.1). The highest prevalence was found in *Acrocephalus schoenobaenus*, and the lowest prevalence was found in *Acrocephalus palustris*. All samples from *Acrocephalus arundinaceus* were negative. The results were not statistically significant ($x^2 = 0.6771$ and p = 0.71282).

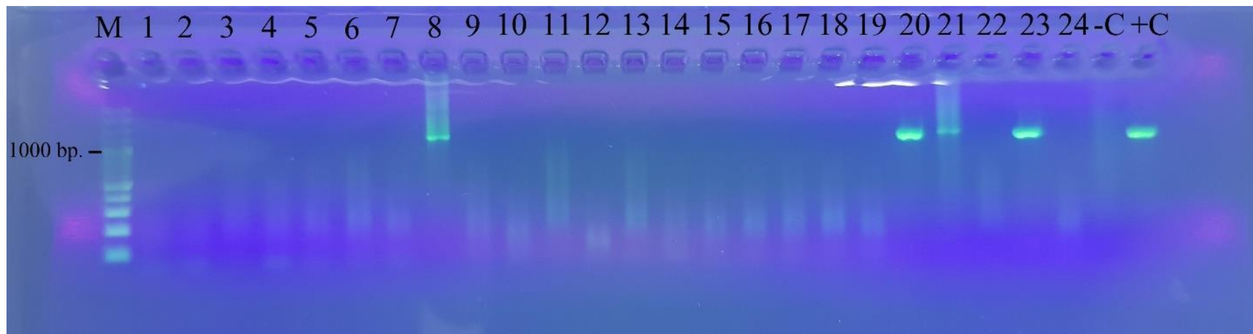


Figure 3.1. The photo of electrophoresis gel. M: Gene Ruler 100 bp. Plus DNA Ladder; number 1 to 24 represent PCR products; -C: negative control (ultra-pure water); +C: positive control (an infected sample, which was positive by microscopy examination of blood smears).

Table 3.1. The number of studied birds, prevalence of *Lankesterella* infections and haplotypes found in different host species.

Bird species	Samples	Prevalence (%)	*Lankesterella* sp. haplotypes[a]
Acrocephalus arundinaceus	20	0 (0)	-
A. schoenobaenus	150	12 (8.0)	Haplotype 1 (7) Haplotype 2 (5)
A. palustris	42	2 (4.8)	Haplotype 3 (1) Haplotype 5 (1)
A. scirpaceus	52	3 (5.8)	Haplotype 4 (1) Haplotype 5 (2)
Overall	**264**	**17 (6.4)**	**5 haplotypes**

[a] Number in parentheses represents the number of positive samples for each haplotype.

3.2. Molecular and morphological results

3.2.1. Molecular characterization

Five different haplotypes of *Lankesterella* sp. were detected. Each positive bird species was found to have infection by two different haplotypes (Table 3.1). Both haplotypes found in *A. schoenobaenus* were exclusively from this species, while *A. palustris* and *A. scirpaceus* had one unique and one shared haplotype each.

3.2.2. Morphological characterization

Morphological analysis was conducted in order to determine the presence of morphological differences between blood stages of found haplotypes (Figure 3.2). Some characteristics were common to all haplotypes, such as the presence of vacuoles, which is usually closely appressed to parasite nucleus, and the presence of a clear edge around the parasite (Figure 3.2, C, E, J). However, it was possible to notice some differences between them. Blood stages of *Lankesterella* haplotype 1 not always have a clearly visible nucleus (Figure 3.2, A-D). *Lankesterella* haplotype 2 has a more basophilic cytoplasm than the other ones and the parasite nucleus is not clearly visible (Figure 3.2, E-H). *Lankesterella* haplotype 4 and 5 have similar characteristics, with a lightly stained cytoplasm, which makes the nucleus readily distinguishable (Figure 3.2, I-P). These morphological features might have taxonomic value for the *Lankesterella* parasite species description and identification. This needs further testing.

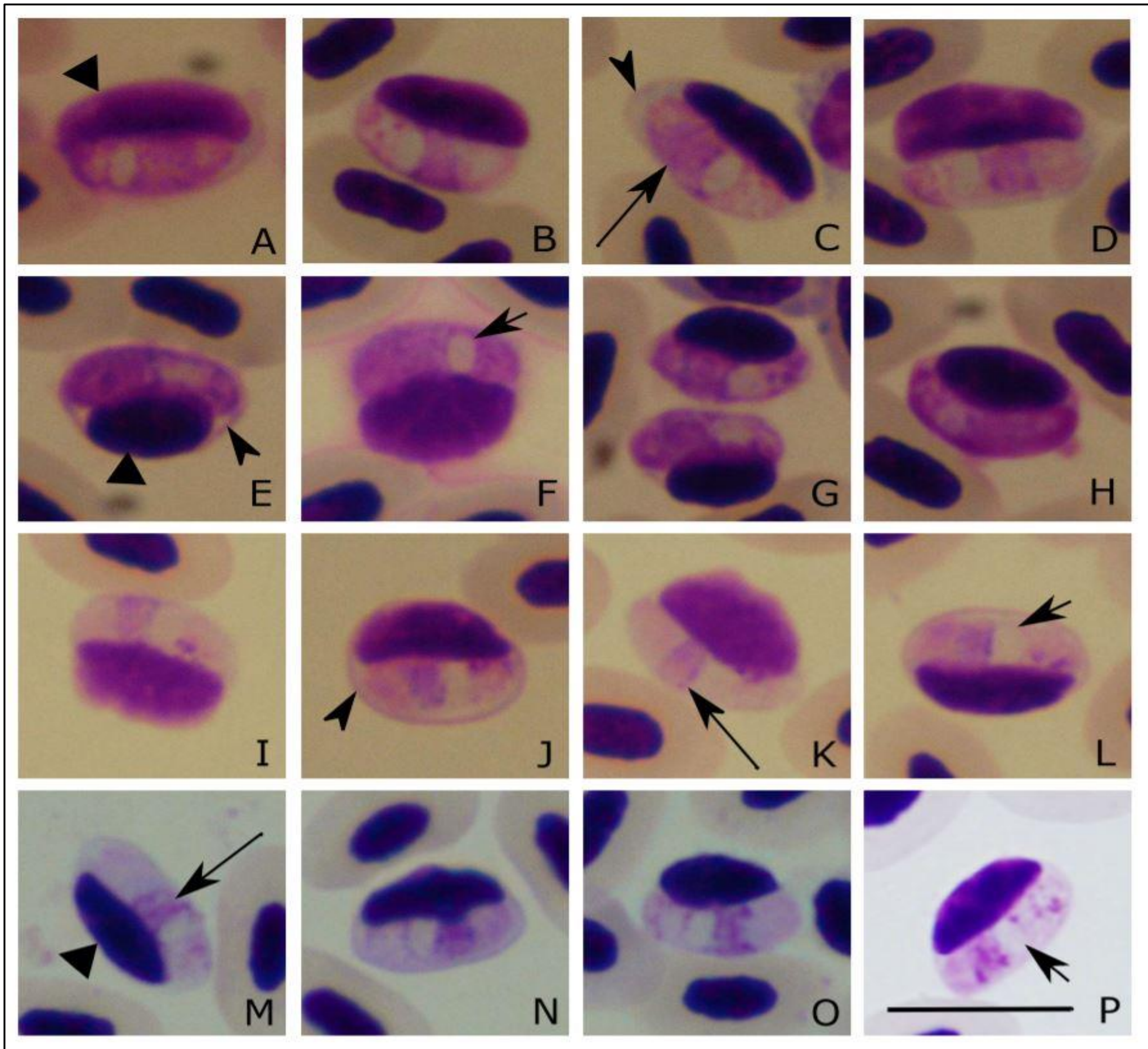


Figure 3.2. The microscopic image of *Lankesterella* parasite in leucocytes. Morphological characteristics of *Lankesterella* parasites encountered in *Acrocephalus schoenobaenus* belonging to haplotype 1 (A-D) and haplotype 2 (E-H); and of *Acrocephalus scirpaceus* belonging to haplotype 4 (I-L) and haplotype 5 (M-P). *Lankesterella* parasites are found inside mononuclear leucocytes (triangle). *Lankesterella* sp. nucleus (long simple arrow). Vacuoles (short simple arrow). A visible edge around the parasite (arrowhead). Methanol-fixed and Giemsa-stained thin films. Scale-bar: 10µm.

3.2.3. *Lankesterella* haplotypes characterization

The nucleotide frequency was calculated and compared between the five different haplotypes found. Guanine (G) and adenine (A) had the highest nucleotide frequencies in all fragments of the haplotype 18S rDNA sequences (Table 3.2).

Table 3.2. The nucleotide frequency in a fragment of 983 bp of the18S rRNA gene sequence.

Sequence name	T	C	A	G
Lankesterella sp. haplotype 1	26.04	19.23	26.65	28.08
Lankesterella sp. haplotype 2	26.25	19.02	26.65	28.08
Lankesterella sp. haplotype 3	26.35	19.02	26.75	27.87
Lankesterella sp. haplotype 4	26.25	19.02	26.75	27.98
Lankesterella sp. haplotype 5	26.35	18.92	26.65	28.08
Average	**26.25**	**19.04**	**26.69**	**28.02**

In 18S rRNA gene sequence fragments eleven polymorphic regions were detected (129, 273, 283, 293, 296, 331, 338, 353, 364, 431 and 454) at the studied fragment (Figure 3.3), of which nine regions are variable and two regions are parsimony active. The diversity of haplotypes was equal to Hd = 0.750, the diversity of nucleotides was equal to π = 0.00323.

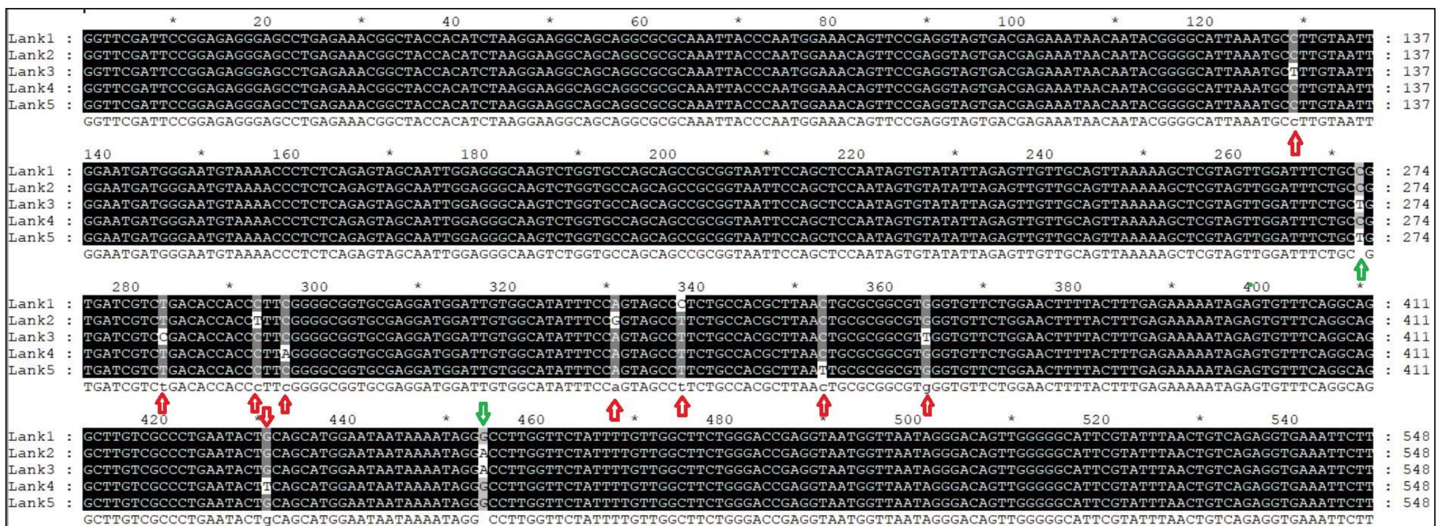


Figure 3.3. *Lankesterella* sp. comparison of differences between five different haplotypes found in the present study (DNA fragment 1-548). Red arrows represent variable sites; green arrows represent parsimony informative sites.

3.2.4. Assessment of genetic distance

The genetic distance between five *Lankesterella* haplotypes detected in the juvenile *Acrocephalus* birds during this study was calculated between each other and to the other nine *Lankesterella* sp. recorded in different hosts (birds, amphibians, lizards). A *Hepatozoon* sp. sequence reported in the bird was also included in the analysis. All sequences are available on GenBank and accession numbers of used sequences are given in table 3.3.

The biggest genetic distance is among *Lankesterella* haplotype 3 and haplotype 4 (0.7% or 7 bp). The smallest distance was recorded between haplotype 1 and haplotypes 4 and 5 (0.3% or 4 bp) (Table 3.4.). When haplotypes from this study were compared to other *Lankesterella* sequences, the biggest difference was between *Lankesterella* sp. (GenBank accession number MF167555.1) and *Lankesterella* haplotype 1 (5.5%) and the lowest was between *Lankesterella valsainensis* (DQ390207.1) and *Lankesterella* haplotype 3 (1.4%). Genetic distances of *Lankesterella* haplotypes from this study compared to *Hepatozoon* sp. (KF022102.1) found in a bird was of 15% for haplotypes 1, 2, and 4, 14.7% for haplotype 3, and for 5 haplotypes was of 14,9%.

Table3.3. 18S rRNA gene sequences of *Lankesterella* parasites from the GenBank database.

Parasite	**Host**	**GenBank accession**	**Reference**
Lankesterella valsainensis	*Parus caeruleus*	DQ390207	Martinez et al., 2006
Lankesterella sp.	*Plectrophenax nivalis*	MG808272	Martinez et al., 2018
Lankesterella sp.	*Plectrophenax nivalis*	MG808273	Martinez et al., 2018
Lankesterella sp.	*Plectrophenax nivalis*	MG808274	Martinez et al., 2018
L. minima	-	AF080611	Barta et al., 2001
Lankesterella sp.	*Liolaemus pictus*	MF167555	Megía-Palma et al., 2017
Lankesterella sp.	*Uta stansburiana*	MF167544	Megía-Palma et al., 2017
Lankesterella sp.	*Sceloporus occidentalis*	MF167551	Megía-Palma et al., 2017
Lankesterella sp.	*Dipsosaurus dorsalis*	MF167547	Megía-Palma et al., 2017
Hepatozoon sp.	*Oceanodroma melania*	KF022102	Merino et al., 2014

Table 3.4. The differences in genetic distance between partial DNA sequences of *Lankesterella* sp. and *Hepatozoon* sp.

	Lankesterella sp. haplotype 1	*Lankesterella* sp. haplotype 2	*Lankesterella* sp. haplotype 3	*Lankesterella* sp. haplotype 4	*Lankesterella* sp. haplotype 5	DQ390207.1 *L. valsainensis*	MG808272.1 *Lankesterella* sp.	MG808273 *Lankesterella* sp.	MG808274 *Lankesterella* sp.	AF080611 *L. minima*	KF022102.1 *Hepatozoon* sp.	MF167555.1 *Lankesterella* sp.	MF167544.1 *Lankesterella* sp.	MF167551.1 *Lankesterella* sp.	MF167547.1 *Lankesterella* sp.
Lankesterella sp. haplotype 1															
Lankesterella sp. haplotype 2	0.004														
Lankesterella sp. haplotype 3	0.006	0.006													
Lankesterella sp. haplotype 4	**0.003**	0.005	**0.007**												
Lankesterella sp. haplotype 5	**0.003**	0.005	0.005	0.004											
DQ390207.1 *L. valsainensis*	0.015	0.015	**0.014**	0.016	0.015										
MG808272.1 *Lankesterella* sp.	0.016	0.018	0.017	0.017	0.015	0.016									
MG808273 *Lankesterella* sp.	0.017	0.019	0.018	0.018	0.016	0.017	0.001								
MG808274 *Lankesterella* sp.	0.016	0.018	0.017	0.017	0.015	0.016	0.000	0.001							
AF080611 *L. minima*	0.051	0.053	0.051	0.052	0.052	0.052	0.051	0.052	0.051						
KF022102.1 *Hepatozoon* sp.	**0.150**	**0.150**	**0.147**	**0.150**	0.149	0.151	0.145	0.146	0.145	0.173					
MF167555.1 *Lankesterella* sp.	**0.055**	0.051	0.054	0.054	0.054	0.055	0.049	0.050	0.049	0.067	0.147				
MF167544.1 *Lankesterella* sp.	0.054	0.050	0.053	0.053	0.053	0.054	0.048	0.049	0.048	0.066	0.144	0.003			
MF167551.1 *Lankesterella* sp.	0.053	0.051	0.051	0.052	0.051	0.056	0.048	0.049	0.048	0.072	0.146	0.039	0.038		
MF167547.1 *Lankesterella* sp.	0.046	0.045	0.043	0.046	0.048	0.052	0.044	0.045	0.044	0.065	0.154	0.035	0.036	0.041	

Numbers marked in blue represent the biggest distances and in red the lowest distances between *Lankesterella* sp. and *Hepatozoon* sp.

3.2.5. *Lankesterella* sp. polymorphism

Comparison of all five *Lankesterella* sp. haplotypes reported in this study with parasite haplotypes, which were detected in birds and are available in the databases, revealed 30 polymorphic nucleotides. Of the 983 nucleotides, 953 were conserved (Figure 3.4).

Figure 3.4. The sequence comparison of cluster C 18S RNA gene (sequence fragment 1-528). Lank1: *Lankesterella* haplotype 1; Lank2: *Lankesterella* haplotype 2; Lank3: *Lankesterella* haplotype 3; Lank4: *Lankesterella* haplotype 4; Lank5: *Lankesterella* haplotype 5; DQ390207.1, MG808272.1, MG808273, MG808274: GenBank accession numbers of *Lankesterella* sp. Red arrows mark variable sites; green arrows are marking parsimony informative sites (two variants) and blue arrows, parsimony informative sites (three variants).

Of the 30 polymorphic regions, 13 (121, 125, 128, 129, 287, 293, 296, 331, 337, 338, 340, 431, 462) nucleotide positions are variable, 15 (87, 171, 273, 280, 283, 297, 306, 313, 339, 344,

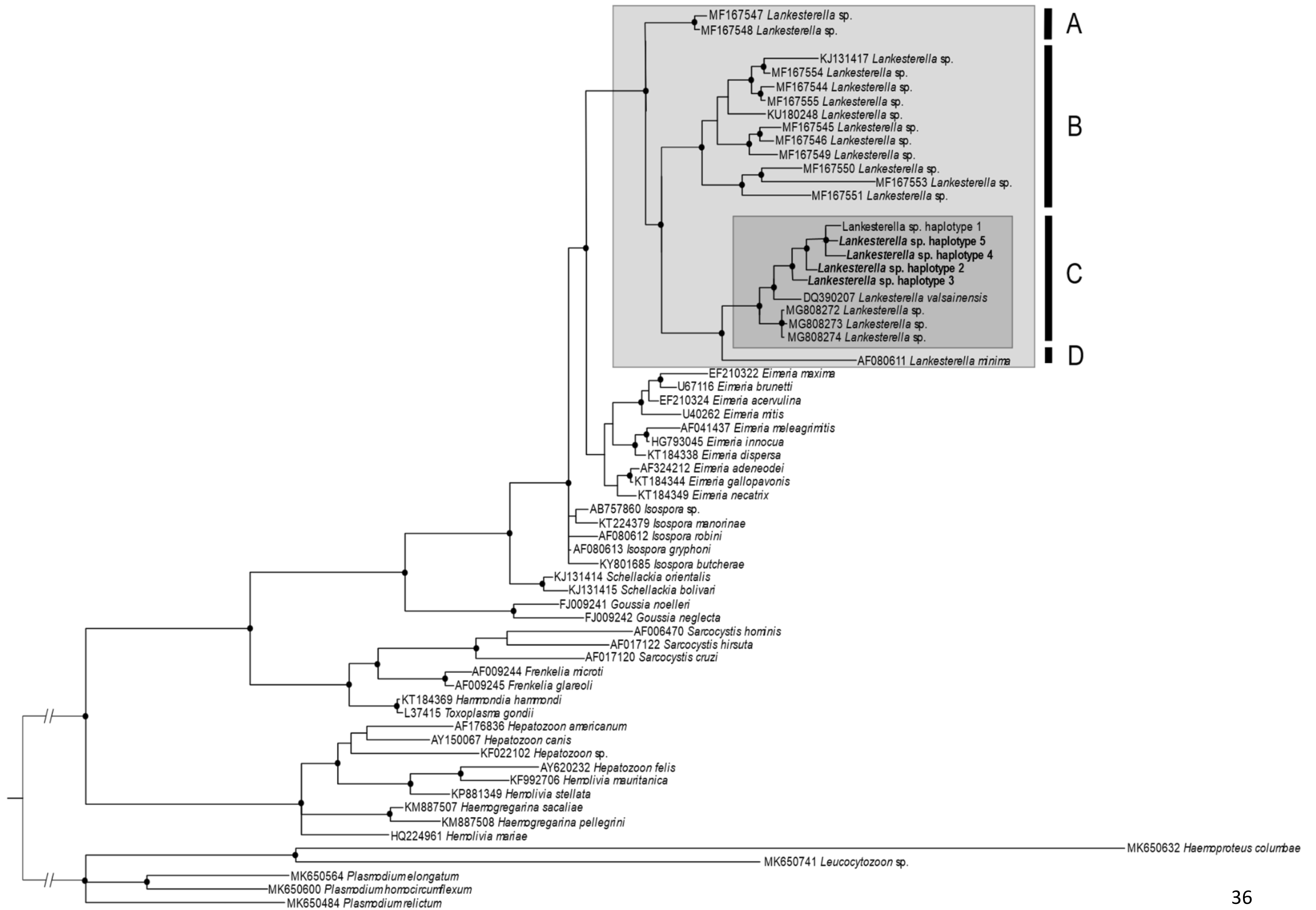
363, 407, 454, 485, 495) nucleotide positions are parsimony informative sites (two variants) and two (353, 364) are parsimony informative sites (three variants) (Figure 3.4).

3.2.6. Phylogenetic tree

The phylogenetic analysis showed that *Lankesterella* haplotypes form a separate clade in comparison to other coccidian parasites. In total, four *Lankesterella* sp. distinct clades were observed. *Lankesterella* sequences of clades A and B were reported in lizards, clade C sequences were reported in birds, including the sequences found in the present study, and clade D contains a sequence that was reported in amphibians (Figure 3.5).

It was also possible to notice that *Lankesterella* sequences obtained from birds clustered in a different and distant clade from the *Hepatozoon* sequences, including the sequence obtained from birds (Figure 3.5).

Figure 3.5 (Next page). Apicomplexa parasites' Bayesian inference using sequences of 18S rRNA gene. GTR + G + I model was used. The genetic distances are shown (scale bar). Black dots indicate posterior probability ≥ 85. Clade A and B indicate *Lankesterella* from lizards; clade C shows *Lankesterella* sequences reported in birds, including the sequences from the present study and clade D a *Lankesterella* sequence reported in amphibians.


MF167547 Lankesterella sp.
MF167548 Lankesterella sp.
KJ131417 Lankesterella sp.
MF167554 Lankesterella sp.
MF167544 Lankesterella sp.
MF167555 Lankesterella sp.
KU180248 Lankesterella sp.
MF167545 Lankesterella sp.
MF167546 Lankesterella sp.
MF167549 Lankesterella sp.
MF167550 Lankesterella sp.
MF167553 Lankesterella sp.
MF167551 Lankesterella sp.
Lankesterella sp. haplotype 1
Lankesterella sp. haplotype 5
Lankesterella sp. haplotype 4
Lankesterella sp. haplotype 2
Lankesterella sp. haplotype 3
DQ390207 Lankesterella valsainensis
MG808272 Lankesterella sp.
MG808273 Lankesterella sp.
MG808274 Lankesterella sp.
AF080611 Lankesterella minima
A
B
C
D
EF210322 Eimeria maxima
U67116 Eimeria brunetti
EF210324 Eimeria acervulina
U40262 Eimeria mitis
AF041437 Eimeria meleagrimitis
HG793045 Eimeria innocua
KT184338 Eimeria dispersa
AF324212 Eimeria adeneodei
KT184344 Eimeria gallopavonis
KT184349 Eimeria necatrix
AB757860 Isospora sp.
KT224379 Isospora manorinae
AF080612 Isospora robini
AF080613 Isospora gryphoni
KY801685 Isospora butcherae
KJ131414 Schellackia orientalis
KJ131415 Schellackia bolivari
FJ009241 Goussia noelleri
FJ009242 Goussia neglecta
AF006470 Sarcocystis hominis
AF017122 Sarcocystis hirsuta
AF017120 Sarcocystis cruzi
AF009244 Frenkelia microti
AF009245 Frenkelia glareoli
KT184369 Hammondia hammondi
L37415 Toxoplasma gondii
AF176836 Hepatozoon americanum
AY150067 Hepatozoon canis
KF022102 Hepatozoon sp.
AY620232 Hepatozoon felis
KF992706 Hemolivia mauritanica
KP881349 Hemolivia stellata
KM887507 Haemogregarina sacaliae
KM887508 Haemogregarina pellegrini
HQ224961 Hemolivia mariae
MK650632 Haemoproteus columbae
MK650741 Leucocytozoon sp.
MK650564 Plasmodium elongatum
MK650600 Plasmodium homocircumflexum
MK650484 Plasmodium relictum
0.2

DISCUSSION

This is the first report of *Lankesterella* parasites in juveniles of *Acrocephalus* birds in Lithuania, consequently, it was possible to confirm that the transmission of this parasite takes place in the country and in Europe. Other studies have reported the presence of *Lankesterella* infections in adult migrant birds in close study sites, such as in Poland (Biedrzycka et al., 2013) and in a Mediterranean country, in Spain (Merino et al., 2006). Interestingly, the presence of *Lankesterella* was also reported in juveniles of Snow bunting in a remote island in Norway (Martínez et al., 2018). Based on these data, it is possible to conclude that transmission of *Lankesterella* spp. might occur in a broad range of countries in Europe and as far north as Norway.

The prevalence of infections was similar in all avian hosts screened in this study, with the highest prevalence found in *Acrocephalus schoenobaenus*, followed by *A. scirpaceus* and *A. palustris*. These differences were not statistically significant. The overall prevalence of *Lankesterella* infections in juveniles of *Acrocephalus* in the present study (6.4%) was smaller than the prevalence found in Snow bunting juveniles (20%) (Martínez et al., 2018). Since these studies were conducted with different hosts and in different geographical regions, this might be the reason of such differences. However, little is known about *Lankesterella* parasites in birds, so more studies are necessary to better understand the real situation of this parasite in the European migrant birds.

Additionally, it is important to mention that the prevalence of infections in adults of *Acrocephalus* reported by Biedrzycka et al. (2013) was of 32.7%, much higher than the 6.4% found in juveniles. However, it was similar to the infections reported in adults of *C. caeruleus* (31.2%). This might be mainly due to the fact that transmission also occurs in wintering grounds, increasing the number of infected birds when they are in warmer regions. Yet, more studies addressing how transmission occurs and which are the vectors are still required to better understand these parasites.

We found five new haplotypes of *Lankesterella* sp. in *Acrocephalus* bird species. The nucleotide diversity was relatively low $\pi = 0.00323$ (Table 3.2). The nucleotide distribution in *Lankesterella* parasite was very similar as reported by Martínez et al. (2018). In the present study,

the obtained 18s rDNA sequences were compared with other *Lankesterella* sequences reported in birds. It was possible to notice the presence of 30 variables nucleotides in amplicon while Gericota et al. (2010) reported 28 variable nucleotides in *Lankesterella* sequences obtained from amphibians. This study shows that bird *Lankesterella* parasites have more variable 18s rRNR gene than the parasites of amphibians. This finding is important for evolutionary research and better understanding of phylogenetic relationships between these parasitic organisms.

This is the first study, which addressed haplotype diversity in avian *Lankesterella* parasites. We found five unique *Lankesterella* sp. haplotypes which diversity based on our studies was Hd = 0.75. It is interesting to note that Perles et al. (2019) investigate *Hepatozoon* sp. haplotype prevalence in rodents, they used two different sets of primers that were able to amplify different regions of the 18S rRNA gene, according to his findings, depending on the protocol used, haplotype diversity was Hd = 0.154 and Hd = 0.426. These data suggest that avian *Lankesterella* spp. sequences are more variable than in parasites inhabiting other vertebrate hosts. However, it is recommended to conducted studies of more parasites inhabiting other bird species to reach the final conclusion on this issue.

The sequences obtained in this study were more closely related to other *Lankesterella* sequences reported in birds (Figure 3.5, clade C). This phylogenetic analysis showed that *Lankesterella* parasites from different avian hosts forms a separate well supported clade inside of the apicomplexans. *Lankesterella* species from birds, amphibians, and reptiles were placed in different clades. In total, five different haplotypes were identified, four of them were exclusively found in separate bird species, while the other one was shared between species (Table 3.1), indicating their broader host-specificity. This finding is important epidemiologically. It would be interesting to conduct studies with other avian host species for better understand the diversity of haplotypes that have been found, and also to include other genes in the analysis in the future.

This study calls for a revision in the taxonomy of Lankesterellidae parasites at species level. Many species descriptions of these parasites were done using only morphological features of blood stages and limited information about differences reported on other stages of their life cycle. However, sporozoites, the most commonly found stage in *Lankesterella* and other related coccidian parasites the blood, is similar in several species. Limited number of taxonomic characters visible at the blood stage may lead to misidentification of the parasite even on a

generic level (Merino et al., 2006; Biedrzycka et al., 2013; Megía-Palma et al., 2017). Merino et al. (2006) addressed these taxonomic problems for the first time, after analysing the genetic data obtained, raising two main issues. First, because the same parasites were attributed to different species and even genera by different authors, their true prevalence remains unclear in wildlife. Second, the prevalence of *Lankesterella* spp. may be significantly higher in birds, and this infection may have a greater impact on avian hosts than previously thought. It is worth mentioning that two studies conducted in Bulgaria using only microscopic identification of parasites, have identified infections by *Hepatozoon sylvae* in *Acrocephalus* birds (Shurulinkov, 2005; Shurulinkov, Chakarov, 2006). It is likely that instead of dealing with *Hepatozoon* infections the authors were dealing with *Lankesterella* infections, which were reported in the same avian hosts during this study.

Morphological analysis of blood stages showed that different *Lankesterella* sp. haplotypes might have distinctive characteristics (Figure 3.2). This would allow morphological identification of the parasites in blood films. However, more detailed studies are needed to draw a more precise conclusion about the taxonomic value of certain morphological characters. It is essential to combine molecular genetic data and morphological examination in taxonomy and identification of *Lankesterella* species. It is important to highlight that the morphology of blood stages of *Lankesterella* parasites is similar to the formally described *Hepatozoon* species in birds, as was reported by Bennett et al. (1992). This reinforces the need for taxonomic revision of the described *Hepatozoon* species in birds as well.

CONCLUSIONS

1. The overall prevalence of *Lankesterella* parasites was 6.4% in juveniles of *Acrocephalus* birds. All blood samples of *Acrocephalus arundinaceus* were negative. The prevalence of infection in *Acrocephalus schoenobaenus, Acrocephalus palustris* and *Acrocephalus scirpaceus* was 8%, 4.8% and 5.8%, respectively.
2. Five new haplotypes (1-5) were identified. *Acrocephalus schoenobaenus* was infected by parasites of haplotypes 1 and 2, *Acrocephalus palustris* by haplotypes 3 and 5, and *Acrocephalus scirpaceus* by haplotypes 4 and 5. The diversity of these haplotypes was equal to Hd = 0.750, and the diversity of nucleotides was equal to $\pi = 0.00323$. The biggest genetic distance was between *Lankesterella* haplotype 3 and haplotype 4 (0.7% or 7 bp). The smallest distance was between haplotype 1 and haplotypes 4, and between haplotypes 1 and 5. (0.3% or 4 bp).
3. Transmission of all reported *Lankesterella* parasite haplotypes occurs in Lithuania.
4. Biggest genetic distance was found between *Lankesterella* sp. (GenBank accession number MF167555.1) and *Lankesterella* haplotype 1 (5.5%), and the smallest was between *Lankesterella valsainensis* (DQ390207.1) and *Lankesterella* haplotype 3 (1.4%).
5. All available haplotypes of avian *Lankesterella* parasites are phylogenetically closely related. The sequences obtained in this study are closely related to *Lankesterella valsainensis*.
6. Phylogenetic clades of *Lankesterella* sp. parasites correspond well to groups of their vertebrate hosts, indicating the high specificity of these pathogens.
7. As indicated by molecular genetic differences, the morphological characters of blood stages of different haplotypes have taxonomic value and can be used in the taxonomy of *Lankesterella* parasites on species level.

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ANNEX

Annex 1. Sequences of 18S rDNA fragments of five *Lankesterella* sp. haplotype reported in the present study (fasta format).

>*Lankesterella* haplotype 1

GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATGGAAACAGTTCCGAGGTAGTGACGAGAAATAACAAT
ACGGGGCATTAAATGCCTTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT
AGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTG
TATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGCCGTGATCGTCTG
ACACCACCCTTCGGGGCGGTGCGAGGATGGATTGTGGCATATTTCCAGTAGCCCTCT
GCCACGCTTAACTGCGCGGCGTGGGTGTTCTGGAACTTTTACTTTGAGAAAAATAGA
GTGTTTCAGGCAGGCTTGTCGCCCTGAATACTGCAGCATGGAATAATAAAATAGGGC
CTTGGTTCTATTTTGTTGGCTTCTGGGACCGAGGTAATGGTTAATAGGGACAGTTGGG
GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTAC
TGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTG
AAGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGG
AAACGCCTACCTTGGCTTCTCCTGCATCTCATGAGAAATCAAAGTCTCTGGGTTCTGG
GGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACA
TGGGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGTGATCTGTCTGGTTAATTTCGATAACGAACGAGACCTTA
GCCTGCTAAATAG

> *Lankesterella* haplotype 2

GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATGGAAACAGTTCCGAGGTAGTGACGAGAAATAACAAT
ACGGGGCATTAAATGCCTTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT
AGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTG
TATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGCCGTGATCGTCTG
ACACCACCTTTCGGGGCGGTGCGAGGATGGATTGTGGCATATTTCCGGTAGCCTTCT
GCCACGCTTAACTGCGCGGCGTGGGTGTTCTGGAACTTTTACTTTGAGAAAAATAGA
GTGTTTCAGGCAGGCTTGTCGCCCTGAATACTGCAGCATGGAATAATAAAATAGGAC
CTTGGTTCTATTTTGTTGGCTTCTGGGACCGAGGTAATGGTTAATAGGGACAGTTGGG
GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTAC
TGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTG
AAGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGG
AAACGCCTACCTTGGCTTCTCCTGCATCTCATGAGAAATCAAAGTCTCTGGGTTCTGG
GGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACA
TGGGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGG

CCGTTCTTAGTTGGTGGAGTGATCTGTCTGGTTAATTTCGATAACGAACGAGACCTTA
GCCTGCTAAATAG

> *Lankesterella* haplotype 3

GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATGGAAACAGTTCCGAGGTAGTGACGAGAAATAACAAT
ACGGGGCATTAAATGCTTTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT
AGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTG
TATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGCTGTGATCGTCCG
ACACCACCCTTCGGGGCGGTGCGAGGATGGATTGTGGCATATTTCCAGTAGCCTTCT
GCCACGCTTAACTGCGCGGCGTTGGTGTTCTGGAACTTTTACTTTGAGAAAAATAGA
GTGTTTCAGGCAGGCTTGTCGCCCTGAATACTGCAGCATGGAATAATAAAATAGGAC
CTTGGTTCTATTTTGTTGGCTTCTGGGACCGAGGTAATGGTTAATAGGGACAGTTGGG
GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTAC
TGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTG
AAGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGG
AAACGCCTACCTTGGCTTCTCCTGCATCTCATGAGAAATCAAAGTCTCTGGGTTCTGG
GGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACA
TGGGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGTGATCTGTCTGGTTAATTTCGATAACGAACGAGACCTTA
GCCTGCTAAATAG

> *Lankesterella* haplotype 4

GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATGGAAACAGTTCCGAGGTAGTGACGAGAAATAACAAT
ACGGGGCATTAAATGCCTTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT
AGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTG
TATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGCCGTGATCGTCTG
ACACCACCCTTAGGGGCGGTGCGAGGATGGATTGTGGCATATTTCCAGTAGCCTTCT
GCCACGCTTAACTGCGCGGCGTGGGTGTTCTGGAACTTTTACTTTGAGAAAAATAGA
GTGTTTCAGGCAGGCTTGTCGCCCTGAATACTTCAGCATGGAATAATAAAATAGGGC
CTTGGTTCTATTTTGTTGGCTTCTGGGACCGAGGTAATGGTTAATAGGGACAGTTGGG
GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTAC
TGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTG
AAGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGG
AAACGCCTACCTTGGCTTCTCCTGCATCTCATGAGAAATCAAAGTCTCTGGGTTCTGG
GGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACA
TGGGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGTGATCTGTCTGGTTAATTTCGATAACGAACGAGACCTTA
GCCTGCTAAATAG

> *Lankesterella* haplotype 5

GGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGCAAATTACCCAATGGAAACAGTTCCGAGGTAGTGACGAGAAATAACAAT
ACGGGGCATTAAATGCCTTGTAATTGGAATGATGGGAATGTAAAACCCTCTCAGAGT
AGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGTG
TATATTAGAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCTGCTGTGATCGTCTG
ACACCACCCTTCGGGGCGGTGCGAGGATGGATTGTGGCATATTTCCAGTAGCCTTCT
GCCACGCTTAATTGCGCGGCGTGGGTGTTCTGGAACTTTTACTTTGAGAAAAAATAGA
GTGTTTCAGGCAGGCTTGTCGCCCTGAATACTGCAGCATGGAATAATAAAATAGGGC
CTTGGTTCTATTTTGTTGGCTTCTGGGACCGAGGTAATGGTTAATAGGGACAGTTGGG
GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTAC
TGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGACAGTAGGGGGTTTG
AAGACGATTAGATACCGTCGTAATCTCTACCATAAACTATGCCGACTAGAGATAGGG
AAACGCCTACCTTGGCTTCTCCTGCATCTCATGAGAAATCAAAGTCTCTGGGTTCTGG
GGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAG
GCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACA
TGGGAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGG
CCGTTCTTAGTTGGTGGAGTGATCTGTCTGGTTAATTTCGATAACGAACGAGACCTTA
GCCTGCTAAATAG