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Research Article

Molecular characterization of Haemoparasites, genus *Lankestrella* (Apicomplexa: Coccidia) in two lizard species from Iran

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Abstract

Reptiles are known hosts of a wide variety of parasites, including haemoparasites. Species of *Lankestrella* (Apicomplexa; Haemococcidia) have been described from the blood of lizards distributed in American and western European areas. In the present study, we characterized morphologically and molecularly the haemococcidian parasites (sporozoites) that infect the blood cells of *Eremias persica* and *Ophisops elegans*, two species of lacertid lizards from central Iran. By the microscopic examination of the smears, we identified parasites as the genus *Lankestrella*. In total, two positive samples of each species have investigated the basis of the partial 18S rRNA gene analyses. The result of similarity analysis with our sequences using the Basic Local Alignment Search Tool (BLAST) indicated a11 four parasites belonging to one haplotype of *Lankestrella* sp. The phylogenetic analysis revealed that the new *Lankestrella* sp is related to *Lankestrella* sp identified from two lizards belonging to Spain and Socotra Archipelago. This is the first report of *Lankestrella* parasites in the new lizard's host. The present study provided additional information about the new host of *Lankestrella* species and added new knowledge to clarify the future phylogenetic relationship between these parasites. Our results emphasize the importance of screening haemococcidian parasites in Iran.

Keywords: Apicomplexan, blood parasite, haemogregarine, reptiles

Introduction

The phylum Apicomplexa contains a large diversity of inter and intracellular parasites that are highly diverse and known to parasitize vertebrates and invertebrates' hosts. Within the more than 300 recognized genera, the best-studied group is the suborder Haemosporina (e.g., genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*), which is common in birds and mammals (Bench et

al., 2000; Chagas et al., 2021). However, much less is known of the suborder Eimeriorina, especially in reptiles (Quillfeldt et al., 2018).

Species of *Lankestrella* belonging to the family Lankesterellidae (order Eucoccidiorida, subclass Coccidia, class Conoidasida, Apicomplexa) are parasites with heteroxenous life cycles that parasitize saurian hosts which are highly host-specific (Megía-Palma et al., 2018). Lankesterellids are considered closely related to the Eimeriidae family (Telford, 2008). This genus is described primarily in amphibians around the world. However, diverse groups of lankesterellids parasites described from American lizards (Megia Palma et al., 2017) and recently by the molecular study are known from birds (Biedrzycka et al., 2013; Chagas et al., 2021). The life cycle of *Lankesterella* spp. is only starting to be understood. They are transmitted by dipteran and acarine arthropods (Megía-Palma et al., 2017). In the peripheral blood of vertebrate hosts, the intraerythrocytic stages of these parasites are found as sporozoites (Zechmeisterova et al., 2019). It is understood the transmission is finally accomplished by ingestion of the infected invertebrate (Telford, 2008).

The parasites undergo several replication cycles of sexual and asexual reproduction in vertebrate's host's gut. Merogony, gametogony, sporogony, and then invasive sporozoites in lizards, whereas in the gut of haematophagous blood-sucking invertebrates (i.e., mites, dipterans, or leeches) can serve as the paratenic vectors without undergoing any development or modification (Poinar, 2009). Hematic stages (i.e., sporozoites) of *Lankesterella* spp. are morphologically distinguished by the two differential numbers of refractile bodies in the cytoplasm (Megía-Palma et al., 2013; Megía-Palma et al., 2018).

In the past, these parasites were described using morphological features, life cycles, and on their occurrence in certain host species. However, morphology of blood stages of several genera are often similar and led to debates about the validity of many species names and their generic position. This is particularly true for *Hepatozoon*, and *Lankesterella* parasites in their various vertebrate hosts (O'Donoghue, 2017). Due to this uncertainty, further investigation of the life cycle remains important in order to better understand the biology of *Lankesterella* spp. and to distinguish them from *Hepatozoon* parasites. For example, in birds, several species of *Hepatozoon* were described based exclusively on morphological and morphometric parasite features, later based on molecular diagnostics understood they are closely related to the amphibian parasites, *Lankesterella* sp, but not *Hepatozoon* species (Biedrzycka et al., 2013; Merino et al., 2006; Chagas et al., 2021).

The taxonomic relationship among haemococcidian parasites in a controversial issue are unresolved (Ghimire, 2010; Chagas et al., 2021). During of scarcity of differential phenotypic

traits, the use of more molecular data is needed to shed light on the relationships within haemococcidian parasites (Megía-Palma et al., 2013). On the other hand, little is known about the molecular diversity and the phylogenetic relationship of the *Lankestrella* that infect lizard hosts (Megia Palma et al., 2017). Iran is a rich country in terms of reptile species. More than 171 lizard species belonged to 10 families, and 47 genera were described (Kafash et al., 2020). However, the blood parasites of reptiles are one of the most poorly studied organisms in Iran. In the current study, we investigated blood samples from two species of lizards in Iran in order to add new knowledge about saurian haemoparasites, namely haemogregarines and eimeriids, which are frequently, reported infecting reptiles (Maia et al., 2016).

Materials and methods

During the field season of 2019, we captured 40 individual lizards of two species (*Eremias persica* and *Ophisops elegans*) from Markazi province in Iran (33° 58′ N, 49° 52′ E). The blood samples were taken from the ventral vein at the base of the tail. All the lizards were released after manipulation in the original sampling site. Two samples were obtained from each lizard: blood smears were prepared from one drop of the sample, while the remaining blood was preserved in absolute ethanol for later DNA extraction. All blood smears were immediately air-dried and later, fixed with absolute methanol (Svahn, 1975). All blood smears were stained with Giemsa stain (1/10 v/v) for 15 min. Slides were examined for haemoparasites by light microscopy. The intensity of infection in the blood smears was determined by counting the total number of cells infected per 10.000 erythrocytes or leukocytes. Pictures of parasites were taken with camera for microscopic camera incorporated digital camera (UCMOS10000KPA; China) to a microscope Nikon (Alphaphoto YS, Japan).

DNA was extracted from blood using the tissue DNA extraction kit. Conventional PCR amplification was made using the primers Hep300 (5′-GTTTCTGACCTATCAGCTTTCGACG-3′) and Hep900 (5′-CAAATCTAAGAATTTC ACCTCTGAC-3′) (Ujvari et al., 2004), targeting part of the 18S rRNA gene region. PCR reactions were run in a 25 μl reaction mixture using 12.5 μl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, 0.4 mM of each dNTP, and 4 mM MgCl2), 1.25 μl of each primer, and at least 25 ng DNA (Cook et al., 2016). PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles, entailing a 95 °C denaturation for 30 s, annealing at 60 °C for 30 s with an end extension at 72 °C for 1 min, and following the cycles a final extension of 72 °C for 10 min as detailed according to previous methods (Netherlands, 2019) Purification and sequencing of PCR products were

commercially performed by Macrogen Inc. (Seoul, South Korea) using both primers mentioned above. The size of the PCR products was about 600 base pairs (bp).

Sequence alignment and phylogenetic analysis

The obtained sequences were edited using the BioEdit software version 7.0.5.3 (Hall, 2005).We performed a similarity analysis with our sequences using the Basic Local Alignment Search Tool (BLAST) to find the best match against published sequences in GenBank (blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences with 512 bp length, were aligned with published GenBank sequences using Clustal W in BioEdit v 7.0.5.3 (Hall, 2005). Details of sequence data set including locality, origins, and accession numbers for the *18S* rRNA gene are presented in table 1.

Table 1. The data set of *Lankesterella*, *Isospora*, *Caryospora*, *Eimeria* and *Goussia* species used in this study. The information includes the accession numbers, locality and origin for the 18S rRNA gene.

N0	species	Accession numbers	Locality	Origin
1	*Lankesterella* sp.	xxxx	Iran	This study
2	*Lankesterella* sp.	MF167549.1	USA (South California)	Megía -Palma et al. (2017)
3	*Lankesterella* sp.	MF167546.1	USA	Martinez et al. (2017)
4	*Lankesterella* sp.	MF167545.1	USA	Megía -Palma et al. (2017)
5	*Lankesterella* sp.	MF167554.1	Argentina	Megía -Palma et al. (2017)
6	*Lankesterella* sp.	MF167550.1	USA	Megía -Palma et al. (2017)
7	*Lankesterella* sp.	MF167552.1	USA	Megía -Palma et al. (2017)
8	*Lankesterella* sp.	MF167555.1	Chile	Martinez et al. (2017)
9	*Lankesterella* sp.	MF167544.1	Chile	Martinez et al. (2017)
10	*Lankesterella* sp.	MH459286.1	Mexico: San Benito Oeste Island	Quillfeldt et al. (2018)
11	*Lankesterella* sp.	MH459288.1	Mexico: San Benito Oeste Island	Quillfeldt et al. (2018)
12	*Lankesterella* sp.	MH459292.1	Mexico: San Benito Oeste Island	Quillfeldt et al. (2018)
13	*Lankesterella* sp.	KX453658.1	Oman	Maia et al. (2016)
14	*Lankesterella* sp.	KX453654.1	Oman: Jabel Samha	Maia et al. (2016)
15	*Lankesterella* sp.	KX453652.1	Oman: Wadi Bani Khalid	Maia et al. (2016)
16	*Lankesterella* sp.	KX453655.1	Oman	Maia et al. (2016)
17	*Lankesterella* sp.	KX453653.1	Oman	Maia et al. (2016)
18	*Lankesterella* sp.	KJ131417.2	Spain	Martinez et al. (2017)
19	*Lankesterella* sp.	MW076442.1	Yemen: Socotra	Tome et al. (2020)
20	*Lankesterella* sp.	KU180248.1	Spain: pet trade	Megía -Palma et al. (2016)
21	*Isospora albogularis*	KU180243.1	Spain	Martinez et al. (2016)
22	*Isospora tarentolae*	KU180245.1	Spain: Tenerife Island	Martinez et al. (2016)
23	*Caryospora ernsti*	KU180247.1	Spain	Martinez et al. (2016)
24	*Eimeria uptoni*	KU192953.1	Germany: Sollichau	Macova et al. (2018)
25	*Eimeriorina* sp	KM234611.1	South America	Harris et al. (2015)
26	*Eimeria rioarribaensis*	AF307877.1	-	Zhao and Duszynski (2001)
27	*Goussia neglecta*	FJ009242.1	-	Jirku et al. (2009)
28	*Goussia noelleri*	FJ009241.1	-	Jirku et al. (2009)

The appropriate model for Maximum Likelihood (ML) and Bayesian Inference (BI) analysis was selected using the jModelTest v 0.1.1 program (Posada, 2008) with the Akaike Information

Criterion (AIC). Bayesian inference (BI) phylogeny was carried out using the MrBayes, v 3.2.2 program (Ronquist et al., 2012) with 10^{*6} generations. Maximum likelihood (ML) was carried out using the PhyML, v 3.0 program (Guindon et al., 2010) with 1500 bootstrap replicates. The FigTree v1.4.0 program (Rambaut, 2016) was used to visualized the phylogenetic tree. The sequence obtained in the present study was uploaded to the GenBank database under the accession number XXX.

Results

Haemoparasites detected by microscopy in blood smears based on their morphological descriptions and having refractile bodies were identified to the genus level as *Lankestrella* (Fig.1). The common shape of the sporozoites presented by these parasites goes from somewhat ellipsoidal to elongate. A roundish to elongated (Fig.1) vacuole-like structure is present in each sporozoite. We observed sporozoites infecting erythrocytes or leucocytes in 10 of the 27 (10/27) thin blood smears of *Ophisops elegans* and 7 of the 16 (7/16) *Eremias persica***.** The mean intensity per 10000 erythrocytes in the positive smears were 0.85± 0.058 and 0.052± 0.042, respectively. The higher intensity belonged to one sample of *O. elegans* that 36/10000 erythrocytes were infected. Only the juveniles of *E. persic*a species were available, and the intensity of infection was 0.05 ± 0.016.

For the molecular analysis, using the HEP300/HEP900 primers yielded usable DNA sequences of approximately 512 bps. The BLAST analysis revealed a 100% identity with published sequences from the genus *Lankestrella*. The Bayesian (BI) and maximum-likelihood (ML) inferences conducted using the phylogenetic relationships of the obtained *Lankestrella*, and the sequences with published data for available in GenBank. The best fit model identified by AIC was TIM3+I. The results of the analysis gave the same overall estimate of phylogenetic patterns where the species of *Lankestrella* sp detected in the present study form a well-supported group on its own, closely related to *Lankestrella* sp. obtained from *Acanthodactylus erythrurus* (genebank: KJ131417.2) from Spain and *Mesalina balfuri* (genebank: mw076442.1) from Socotra Archipelago (Fig. 2). The clade formed by *Lankestrella* sp isolated from *Phymaturus payuniae* was a sister group to this group (support > 70%). These two clades formed a third monophyletic clade within *Lankestrella* sp from *Uta stansburiana* and *Liolaemus pictus* in the family Lankesterellidae. It was the first report of *Lankesterella* parasites in these two species of lizards in the world.

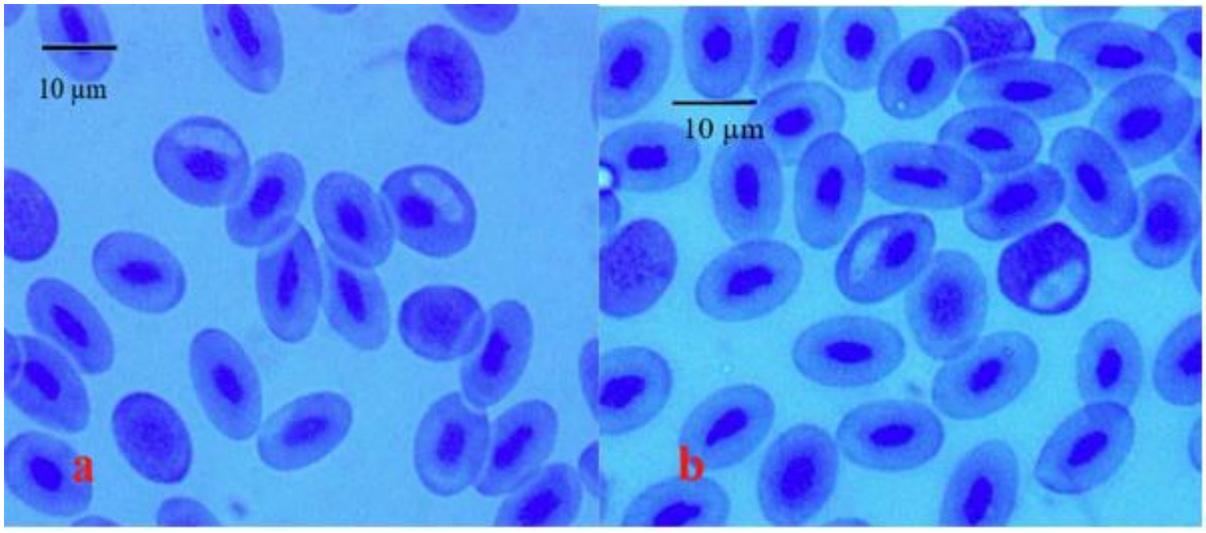

Figure 1. Photographs of the erythrocytes (a) and leucocyte (b) of the lizards infected with a *Lankestrella* blood parasites from Iran.

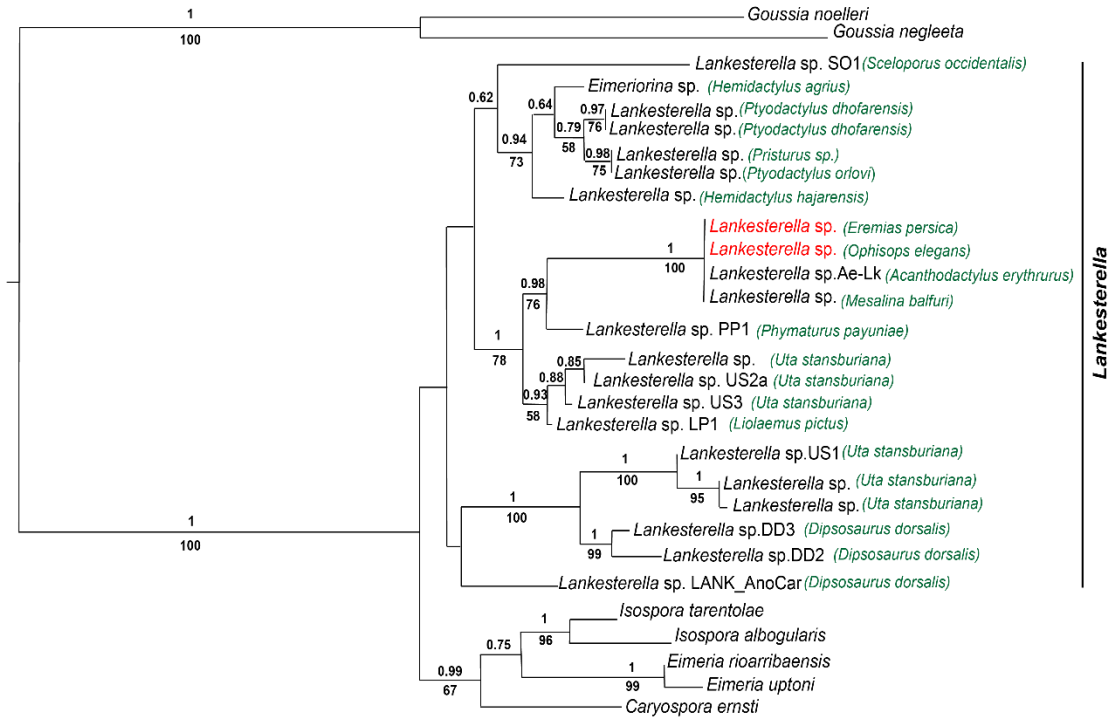

Figure 2. The phylogenetic tree of *Lankestrella* species implemented in PhyML and MrBayes based on partial 18S rRNA gene. PhyML and MrBayes trees showed the same topology. therefore, only the ML tree was presented. Bayesian posterior probability values are at the top of the branch/Maximum likelihood bootstrap values are at the bottom. Details of accession numbers are presented in Table 1.

Discussion

Previously species of *Lankestrella* were known to infect American and Iberian lizards. We found these parasites in blood of two different new hosts that are morphologically and genetically compatible with other described *Lankestrella* sp. Besides, the sporozoites were found infecting erythrocytes and leukocytes (Fig. 1) In both types of host cells, intracellular parasites assumed elongate to bean-like shapes. The parasites do not occupy the entire cytoplasm of host cells. On the other hand, parasites found in the blood samples of the lizard hosts *Ophisops elegans* and *Eremias persica* are likely the same parasite formerly described from Spain and Socotra Archipelago lizards. This parasite is phylogenetically related to other *Lankesterella* parasites of birds, lizards, and amphibians.

Results of the phylogenetic tree of the sporozoites revealed two same haplotypes in *Eremias persica* and *Ophisops elegans*, whose genetic identity was 100% with the *Lankestrella* sp. obtained from *Acanthodactylus erythrurus* from Spain (Megía-Palma et al., 2013) and *Mesalina balfuri* genebank from Socotra Archipelago (Tomé et al., 2020). This is the first report of *Lankestrella* parasites in the new lizard host. Our results might be indicated these haplotypes were more common and widely distributed between host species in different areas. Prevalence can be determined by the distribution of same invertebrate host encounter with suitable hosts (Simberloff, 2010), environmental factors (Wood, 2007). Our result might show low host-specificity in this species. Host-specificity between haemoparasites groups could be due to many factors, including differences in life-cycle strategies and/or transmission pathways (Maia et al., 2016). For example, within haemogregarines, some haplotypes are more common and widely distributed than others, according to their host-specificity and the biogeography of their hosts (Maia et al., 2016). A lower host-specificity could be benefit to parasite geographical distribution (Tome et al., 2020). However, further data are necessary to investigate the effect of host- specificity on distribution.

Historically, the morphology of sporozoites in Lankestrellids parasites was known with having two refractile bodies in sprozoites. However, Fayer (2011) demonstrated that refractile bodies are dynamic structures within the Eimeriorina that can fluctuate in number even in the sporozoites infecting an individual host. Thus, the number of refractile bodies are not valid diagnostic characteristics to differentiate the parasites between the genera *Schellackia* and *Lankesterella* (Megía-Palma et al., 2017).

The molecular and morphological study by Megía-Palma et al. (2016) demonstrated that all haemococcidan parasites detected in American lizard hosts that were previously identified as *Schellackia*, are closely related to the genus *Lankesterella*, and distant to the genus *Schellackia*.

They resulted that at least in the Americas, lankesterellids are a molecularly diverse group of parasites in lizard hosts and the genus *Schellackia* might be restricted to old world lizards. This suggests that Lankestrellidae parasites should not be recognized exclusively based on morphological and morphometric parasite features, and phylogenetic molecular analysis is a good alternative tool particularly helpful when the parasite life cycle proves elusive (Biedrzycka et al., 2013). The diversity of hemococcidians haplotypes in the new world lizards might be related to the high diversity of their vectors, indicating high vector-parasite specificity (Megía-Palma et al., 2016).

In this study, we reported lankesterellid (one haplotype) parasites in a screening of 43 host species (10/27 of *O. elegans* and (7/16) *E. persica*). Our sampling is Comparable with the results of the Oman lizards, where only 5% of the analyzed gekonids and 28% of amphibian *Sclerophrys arabica* were infected (Maia et al., 2016). It is particularly interesting to compare these results with the prevalence of *Lankestrella* sp in *Mesalina balfuri* from Socotra Archipelago, where only 3.9% of samples were infected. The high host-specificity and the lower prevalence of parasites on Socotra compared to our study is in line with apicomplexan studies from other island systems (Tome et al., 2018; Tome et al., 2020). Study on blood parasites in eastern Spain during an entire annual activity revealed 16.9% -29.4% of the analyzed *Acanthodactylus erythrurus* and 6.9% of *Psammodromus algirus* were infected by *Lankestrella* spp. (Drechsler et al., 2021). The differences can be related to differences between the method of qPCR and light microscopy. It also might be due to various biotic and abiotic factors influencing variation in the distribution, specificity, and abundance of competent vectors, as well as host behavior (Chagas et al., 2021). In our study haplotypes were widely distributed between two species of lizards, suggesting low host-specificity and the possible occurrence of host-shift (Bensch et al., 2000).

Previous studies have demonstrated that vector diversity and vector-protozoan specificity may be high in avian and American lizard's models (Martínez-de la Puente. et al 2011; Megia Palma et al., 2017). In the present study, same haplotype detected in two different hosts might be related to the low diversity of their vectors, indicating low vector-parasite specificity.

The life cycle of *Lankesterella* sp obtained in the present study is unknown, no invertebrate host was found during samples collection. According to Megía-Palma et al. (2017), dipteran and acarine arthropods (Ball & Oda, 1971) are known vectors of American lankesterellids. In the Mediterranean lizards, sand flies and other haematophagous dipterans feeding on lizards, are known to potential vectors of haemococcidians (Drechsler et al., 2021). However, the life cycle of *Lankesterella* spp. in other distribution areas is unknown (Megía-Palma et al., 2017).

Conclusion

we present new molecular data on a haemococcidian species of the genus *Lankesterella* isolated from two species of lizard of Markazi province in Iran for the first time, carry out a molecular phylogenetic analysis. Additionally, we present data on a new host for *Lankestrella* sp in the different geographical areas. To date, there was no information available on the lankestrelids parasites in Iran. It is necessary to use more molecular data to solve the molecular phylogeny of this group. Results of our study could shed further light on the phylogenetic relationships and host specificity of the haemococcidian parasites lineages. As a whole, our results emphasize the importance of screening parasites in Iran.

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