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First Report on Karyolysus sp. (Apicomplexa: Adeleorina) from Green Bellied Lizard Darevskia chlorogaster in the North of Iran

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ABSTRACT

Parasites play a crucial role in ecosystems by interacting in population processes, shaping entire community structures and significantly reducing host fitness in the wild. The phylum Apicomplexa is a diverse group of obligate unicellular blood parasites with a vast distribution. Species of reptiles are exposed to apicomplexan blood parasites, mainly haemogregarines and haemosporidians. Haemogregarine parasites belonging to the suborder Adeleorina are common and widely distributed in lizards. The genus Karyolysus Labbe, 1894 (Apicomplexa: Adeleorina: Karyolysidae), is composed of intracellular haemogregarine parasites which can be found in various genera of Palearctic lizards. The vectors of the parasites are gamasid mites from the genus Ophionyssus. In the present study, we characterised molecularly the haemogregarine parasites from green bellied lizards, Darevskia chlorogaster in the north of Iran. The fragments of the 18S rRNA gene of reptile haemogregarines were amplified using the primer set Hep300 and Hep900. DNA sequences of 493 bp length were aligned with DNA sequences obtained from GenBank through blasting. The BLAST analysis revealed a 100% identity with published sequences of the genus Karyolysus. Phylogenetic analyses indicated that the obtained haplotypes were identical to the Karyolysus sp. (KJ461944) sequence from Ophionyssus mites isolated from Lacerta viridis from Hungary. In addition to being the first molecular characterisation of a Karvolvsus within the Iranian lizards, it was also the first report of a species of Karyolysus infecting the Darevskia genus. The present study provided additional information about the new host of Karyolysus species, its distribution and host specificity and provided further hints to clarify future phylogenetic relations between these parasites.

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Introduction

Most groups of parasites have received little attention from biologists and, as a result, much of their biodiversity remains to be described or even detected (Morrison, 2009). The phylum Apicomplexa Levine, 1970, is a large unicellular clade, composed of a diverse array of obligate intracellular parasitic organisms (Morrison, 2009). Haemogregarines (Apicomplexa: Coccidia: Adeleorina) have been known as an important group of parasites mainly from the genera Hemolivia (Petit, Landau,

Baccam & Lainson, 1990; Hepatozoon Miller, 1908; and Karvolysus Labbé, 1894; Haklová-Kočíková et al., 2014). They have heteroxenous life cycles that include merogony and gamonts formation in the visceral tissues and erythrocytes of the vertebrate host, as well as sporogony within the gut of haematophagous invertebrates (Telford et al., 2008). Even though three haemogregarine genera may be transmitted to the saurian host through the ingestion of the infected invertebrate vector, Hepatozoon spp. may be transmitted by a variety range of arthropod vectors (such as mosquitoes and ticks).

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However, *Hemolivia* spp. and *Karyolysus* spp. have been recorded only to be transmitted by the ingestion of ticks and mites' vectors, respectively.

Karyolysus parasite is mainly known for lacertid lizards of Europe and Asia (Beyer and Sidorenko, 1984; Mihalca et al., 2008). It is different from the closely related genera Hepatozoon and Hemolivia in terms of the life cycle (Haklová-Kočíková et al., However, due to its morphological similarity, species identification based on morphological characteristics of gamonts in erythrocytes is very difficult and ambiguous in Karyolysus (Haklová-Kočíková et al., 2014). Therefore, many researchers have identified these parasites only as "haemogregarines" (Amo et al., 2004; Mihalca et al., 2008; Garrido and Pérez-Mellado, 2013). However, phylogenetic analysis based on parasite gamonts data found in erythrocytes were nested within the Karyolysus group in comparison to other parasites for more precise identification of these parasites. Moreover, a growing number of studies have uncovered high levels of undescribed diversity within this group and information on their taxonomy is uncertain (Maia et al., 2016).

There are rare studies about the haemoparasites of lizards in Iran. Only a few small records on reptile blood parasites have been reported (Javanbakht *et al.*, 2015a, b). Therefore, it is necessary to obtain molecular data on blood parasites from lizards. *Darevskia chlorogaster* (Boulenger, 1908) is a common lizard in Hyrcanian forests (Tohidifar *et al.*, 2016). This study aims to detect *Darevskia chlorogaster* blood parasites genetically and compare them to the available data.

Materials and Methods

The lizard specimens were captured by hand net during the spring and summer of 2019 from Rasht in the North of Iran (37°19′N, 49°64′W). The blood samples were taken via a ventral puncture in the caudal vein of 40 green bellied lizards, *D. chlorogaster*. A drop of blood was preserved for molecular analysis (stored in 96% ethanol) (Siroky *et al.*, 2009).

DNA was extracted from the blood samples using buffer-detergent (Triton X100). Based on the previous studies, we amplified fragments of

18S rRNA the gene from reptile haemogregarines using the primer set Hep300 (5'-GTT TCT GAC CTA TCA GCT TTC GACG-3') and Hep900 (5'-CAA ATC TAA GAA TTT CACCTC TGA C-3'). The PCR reactions amplified have a fragment (approximately 600 bp) of the 18S rRNA gene (Ujvari et al., 2004). PCR reactions were run in a 25 ul reaction mixture using 12.5 ul 2X. Thermo Scientific DreamTag PCR master mix (Contain: 2X, DreamTag buffer; 0.4 mM, dNTP; 4 mM, MgCl₂), 1.25 µl of each primer, and at least 25 ng DNA (Cook et al., 2015). 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 et al., 2014). Purification and sequencing of PCR products of four positive samples were commercially performed by Macrogen Inc. (Seoul, South Korea) using both primers mentioned above. The size of the PCR products was about 610 base pairs (bp).

Sequence alignment and phylogenetic analysis

In addition to one haplotype identified in this study (from three sequenced samples), 41 DNA sequences were obtained from **NCBI** (http://www.ncbi.nlm.nih.gov/BLAST/) GenBank (Table 1). Details of the sequence data set, including locality, origins, and numbers for the 18S rRNA gene are presented in Table 1. DNA sequences of 493 bp length were aligned using Clustal W in BioEdit v 7.0.5.3 (Hall, 2005) and Muscle in MEGA X (Kumar et al., 2018). Bayesian Inference (BI) phylogeny was carried out using the MrBayes, v 3.2.2 program (Ronquist et al., 2012) with 10⁶ generations. Maximum Likelihood (ML) was carried out using the PhyML, v 3.0 Program (Guindon et al., 2010) with 1500 bootstrap replicates. The appropriate model for BI and ML analysis was selected using the iModelTest v 0.1.1 Program (Posada, 2008) with the Akaike Information Criterion (AIC). The best fit model identified by AIC was TIM3+I. The FigTree v1.4.0 Program (Rambaut, 2016) was used to visualize the phylogenetic tree. The uncorrected pairwise distances (p-distances) between the haplotypes were computed using the MEGA7. The new sequence in the present study was uploaded to the GenBank database under the accession number OK638134.

Results

Four of 40 samples of *D. chlorogaster* (10% prevalence) were PCR positive. Results of DNA sequences of approximately 493 bps indicated that all sequences identified in this study were the same and belonged to one haplotype. The BLAST analysis revealed a 100% identity with published sequences from the genus *Karyolysus*.

The *p*-distance values between them were 0 %. Both BI and ML trees showed the same estimated relationships among the sequences of the *Karyolysus* lineages with high bootstrap values (Fig. 1).

According to tree topology, the obtained haplotype was identical to the sequence of *Karyolysus* sp. (KJ461944) from *Ophionyssus* mites isolated from *Lacerta viridis* from Hungary (Fig. 1, Table 1). This is the first *Karyolysus* species reported from *D. chlorogaster*.

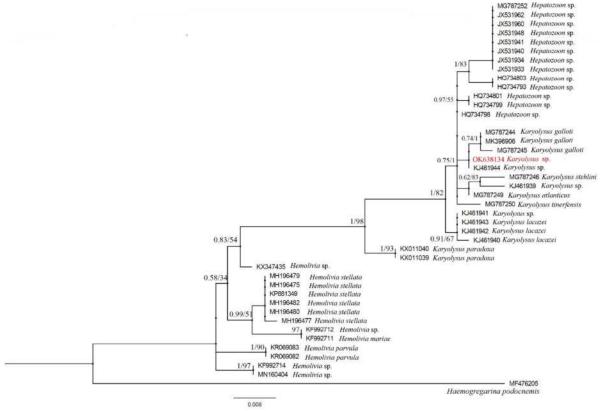


Fig. 1. The phylogenetic tree of *Karyolysus*, *Hepatozoon*, and *Hemolivia* species implemented in PhyML and MrBayes based on the 18S rRNA gene. PhyML and MrBayes trees showed the same topology; therefore, only the PhyML tree was presented. Bayesian posterior probability values are on the left of the slash/Maximum likelihood bootstrap values are on the right. Details of accession numbers are presented in Table 1.

Taxonomic summary

Phylum: Apicomplexa Levine 1970; Order: Eucoccidiorida Léger & Duboscq 1910; Suborder: Adeleorina Léger 1911; Family: Karyolysidae Wenyon 1926; Genus: *Karyolysus* Labbé 1894; Species: *Karyolysus* sp. (Haklová-Kočíková *et al.*, 2014).

Type-host: *Darevskia chlorogaster*; Other hosts: Unknown; Vector: Unknown, probably mites of

the genus *Ophionyssus* Haklová-Kočíková *et al.* 2014.

Type-locality: Rasht, Iran (37°19'N, 49°64'W). Other localities: Unknown.

Representative DNA sequences: The nucleotide sequence data reported here were deposited in the gene bank database under accession number OK638134.

Table 1. The data set of *Karyolysus*, *Hepatozoon*, and *Hemolivia* species were used in this study. The information includes the accession numbers, locality, and origin of the 18S rRNA gene.

No	Species Species	AC No*	Locality	Origin
1	Karyolysus sp.	OK638134	Rasht, Iran	This study
2	Karyolysus galloti	MG787244	Canary Islands	Tomé et al. (2019)
3	Karyolysus galloti	MG787245	Canary Islands	Tomé et al. (2019)
4	Karyolysus galloti	MK396906	Canary Islands	Tomé et al. (2019)
5	Karyolysus stehlini	MG787246	Canary Islands	Tomé et al. (2019)
6	Karyolysus atlanticus	MG787249	Canary Islands	Tomé et al. (2019)
7	Karyolysus tinerfensis	MG787250	Canary Islands	Tomé et al. (2019)
8	Karyolysus paradoxa	KX011040	South Africa	Cook et al. (2016)
9	Karyolysus paradoxa	KX011039	South Africa	Cook et al. (2016)
10	Karyolysus sp.	KJ461939	Slovakia	Haklová-Kočíková et al. (2014)
11	Karyolysus lacazei	KJ461940	Poland	Haklová-Kočíková et al. (2014)
12	Karyolysus sp.	KJ461941	Hungary	Haklová-Kočíková et al. (2014)
13	Karyolysus lacazei	KJ461942	Romania	Haklová-Kočíková et al. (2014)
14	Karyolysus lacazei	KJ461943	Hungary	Haklová-Kočíková et al. (2014)
15	Karyolysus sp.	KJ461944	Hungary	Haklová-Kočíková et al. (2014)
16	Hepatozoon sp.	HQ734801	Northern Portugal	Maia et al. (2014)
17	Hepatozoon sp.	HQ734799	Northern Portugal	Maia et al. (2014)
18	Hepatozoon sp.	MG787252	Canary Islands	Maia et al. (2014)
19	Hepatozoon sp.	JX531962	Western Mediterranean	Maia et al. (2014)
20	Hepatozoon sp.	JX531960	Western Mediterranean	Maia et al. (2014)
21	Hepatozoon sp.	JX531948	Western Mediterranean	Maia et al. (2014)
22	Hepatozoon sp.	JX531941	Western Mediterranean	Maia et al. (2014)
23	Hepatozoon sp.	JX531940	Western Mediterranean	Maia et al. (2014)
24	Hepatozoon sp.	JX531934	Western Mediterranean	Maia et al. (2014)
25	Hepatozoon sp.	JX531933	Western Mediterranean	Maia et al. (2014)
26	Hepatozoon sp.	HQ734803	North Africa	Maia <i>et al.</i> (2014)
27	Hepatozoon sp.	HQ734793	North Africa	Maia et al. (2014)
28	Hepatozoon sp.	HQ734798	North Africa	Maia et al. (2014)
29	Hemolivia sp.	KX347435	Thailand	Ahantarig et al. (2016)
30	Hemolivia stellata	MH196480	Northeastern Colombia	Cotes-Perdomo et al. (2018)
31	Hemolivia stellata	MH196479	Northeastern Colombia	Cotes-Perdomo et al. (2018)
32	Hemolivia stellata	KP881349	Brazil	Karadjian et al. (2015)
33	Hemolivia stellata	MH196475	Northeastern Colombia	Cotes-Perdomo et al. (2018)
34	Hemolivia stellata	MH196482	Northeastern Colombia	Cotes-Perdomo et al. (2018)
35	Hemolivia stellata	MH196477	Northeastern Colombia	Cotes-Perdomo et al. (2018)
36	Hemolivia sp.	KF992714	Nicaragua	Kvičerová et al. (2014)
37	Hemolivia sp.	KF992712	Australia	Kvičerová et al. (2014)
38	Hemolivia mariae	KF992711	Australia	Kvičerová et al. (2014)
39	Hemolivia sp.	MN160404	-	Nordmeyer <i>et al.</i> (2020)
40	Hemolivia parvula	KR069083	Southern Africa	Cook et al. (2015)
41	Hemolivia parvula	KR069082	Southern Africa	Cook et al. (2015)
42	Haemogregarina podocnemis	MF476205	Brazil	Úngari <i>et al.</i> (2018)

^{*} AC No= Accession numbers

Discussion

Species of the genus *Karyolysus* have been considered the most frequent haemogregarines, infecting Palearctic lizards, mainly from European countries (Haklová-Kočíková *et al.*, 2014). Recently seven new species of *Karyolysus* were described in the Canary Islands (Tomé *et al.*, 2019). Moreover, a report of *Karyolysus* species from South Africa (Cook *et al.*, 2016) indicated the wide distribution of this genus (Zechmeisterová *et al.*, 2019). Nevertheless, information about the presence of these blood parasites in Iranian lizards is uncertain. The identified vertebrate host of *Karyolysus* are lacertid lizards of the genus *Gallotia*, *Lacerta*, *Podarcis* and *Zootoca*, and

newly varanid, scincid and geckonid lizards (Zechmeisterová et al., 2019). In this study, we identified a haplotype of Karyolysus blood parasites in the new lizard host, D. chlorogaster from Iran. The taxonomy of haemogregarines is complicated and the identification of parasite species is limited only to morphological attributes, which are not consistent (Barta et al., 2012). However, it is possible molecular data without morphology becomes insufficient to distinguish (O'Donoghue, 2017). Therefore, in recent years, the identification of Karyolysus species has been based on both morphological and molecular data of gamonts in infected lizard hosts (Haklová-Kočíková et al., 2014; Cook et al., 2016; Tomé et al., 2019;

Zechmeisterová et al., 2019). Haklová-Kočíková et al. (2014) investigated the common blood parasites in European lizards with 30.5% prevalence based on both molecular and morphological characterization. They reported two species of Karyolysus and concluded mites are known as the main hosts of the Karyolysus species. They also expressed that Karyolysus haplotypes identified in their study were similar to *Hepatozoon* haplotypes from lizards in the Iberian Peninsula. The prevalence of Karvolvsus among the three host genera in Canarian lizards was 69.7% in Gallotia, 4.9 % in Tarentola, and 3.3 % in Chalcides. (Tomé et al., 2019). The prevalence of Karyolysus cf. lacazei Labbé, 1894, in engorged ticks isolated from the Iberian lizard host, Lacerta schreiberi was estimated 50% (Zechmeisterová et al., 2019). In this study based on the molecular data, we introduced green bellied lizards, D. chlorogaster as a new host of Karyolysus. 10% of the individual was infected by parasites.

According to a molecular study, Karyolysus species in the present study have been completely different from other species described by Haklová-Kočíková et al. (2014), Cook et al. (2016) and Tomé et al. (2019) and Zechmeisterová et al. (2019). According to phylogenetic trees and p-distances (0.00%) analysis, Karyolysus sp. identified in the present study was closely related and had equal p-distance values to the Karyolysus sp. (KJ461944) from Ophionyssus saurarum mites (Acari: Mesostigmata) collected from the European green lizard, Lacerta viridis, in Hungary (Haklová-Kočíková et al., 2014). Results of the phylogenetic analysis in our study showed that these two haplotypes belong to the same clade identified as Karyolysus sp. They were completely different from sequences of other Karyolysus species in the phylogenic tree. Based on thr molecular characteristics, we suggest that these two Karvolvsus haplotypes can be the same species (p-distances = 0.00%). Species of Karyolysus are recognizable by sporozoites within oocysts localized in the gut cells of the final hosts (Barta et al., 2012). The studies of experimental transmission demonstrated that Karyolysus is transmitted only by Ophionyssus mites (Svahn, 1975; Telford, 2009). Transovarial transmission by definitive invertebrate hosts has been shown in Karvolvsus (Beyer and Sidorenko, 1984). The mites of the genus Ophionyssus generally prefer reptiles as hosts. Fifteen out of sixteen known Ophionyssus species have been reported as parasites of reptiles (Moraza et al., 2009). In Europe O. saurarum and O. natricis have been found to parasitize various reptilian hosts (Masan et al., 2009). The distribution of Ophionyssus mites is poorly known in Iran. Amanatfard et al. (2014) reported O. natricis from a snake in the north of Iran where D. chlorogaster is distributed. Due to the low host specificity of haemogregarines, the occurrence of host switches and the use of multiple hosts are expected (Haklová-Kočíková et al., 2014). date, no more information about Ophionyssus mites was published in Iran. In this study, we have not observed any ectoparasites in D. chlorogaster. However, identification of the Karyolysus parasite may suggest the occurrence of Ophionyssus mites in this area.

Conclusions

Using phylogenetic approaches, we detected a new record of *Karyolysus* parasites in Iran. Species of *Karyolysus* has not been previously identified in lizards of the genus *Darevskia*. Therefore, this study is the first report of this parasite in *D. chlorogaster*. Future studies, including the examination of morphology, the life cycle and stages in definitive hosts, are required for taxonomic identification.

Conflicts of interest

The authors declared no conflicts of interest.

Ethics approval

All stages of this research complied with Iranian laws and authorized the University of Guilan.

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