

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 *Karyolysus* 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 *Karyolysus* 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.*, 2014). 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

the 18S rRNA 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 have amplified a fragment (approximately 600 bp) of the 18S rRNA gene (Ujvari *et al.*, 2004). PCR reactions were run in a 25 µl reaction mixture using 12.5 µl 2X, Thermo Scientific DreamTaq PCR master mix (Contain: 2X, DreamTaq 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 accession 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 jModelTest 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*.

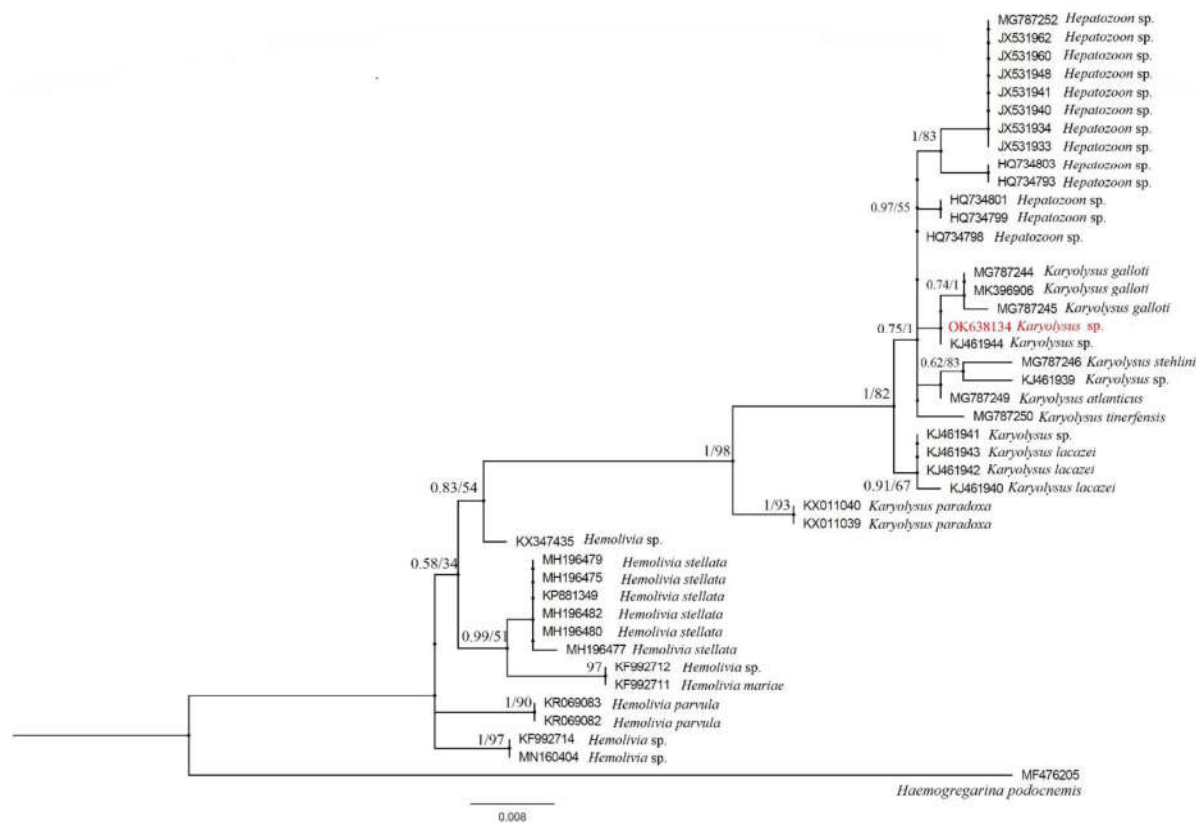


Fig. 1. The phylogenetic tree of *Karyolysus*, *Hepatozoon*, and *Hemolivia* species implemented in PhyML and MrBayes based on the 18S rRNA gene. PhyML and MrBayes trees showed the same topology; therefore, only the PhyML tree was presented. Bayesian posterior probability values are on the left of the 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 | AC No* | Locality | Origin |
|----|----------------------------------|----------|-----------------------|---------------------------------------|
| 1 | <i>Karyolysus</i> sp. | OK638134 | Rasht, Iran | This study |
| 2 | <i>Karyolysus galloti</i> | MG787244 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 3 | <i>Karyolysus galloti</i> | MG787245 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 4 | <i>Karyolysus galloti</i> | MK396906 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 5 | <i>Karyolysus stehlini</i> | MG787246 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 6 | <i>Karyolysus atlanticus</i> | MG787249 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 7 | <i>Karyolysus tinerfensis</i> | MG787250 | Canary Islands | Tomé <i>et al.</i> (2019) |
| 8 | <i>Karyolysus paradoxa</i> | KX011040 | South Africa | Cook <i>et al.</i> (2016) |
| 9 | <i>Karyolysus paradoxa</i> | KX011039 | South Africa | Cook <i>et al.</i> (2016) |
| 10 | <i>Karyolysus</i> sp. | KJ461939 | Slovakia | Haklová-Kočíková <i>et al.</i> (2014) |
| 11 | <i>Karyolysus lacazei</i> | KJ461940 | Poland | Haklová-Kočíková <i>et al.</i> (2014) |
| 12 | <i>Karyolysus</i> sp. | KJ461941 | Hungary | Haklová-Kočíková <i>et al.</i> (2014) |
| 13 | <i>Karyolysus lacazei</i> | KJ461942 | Romania | Haklová-Kočíková <i>et al.</i> (2014) |
| 14 | <i>Karyolysus lacazei</i> | KJ461943 | Hungary | Haklová-Kočíková <i>et al.</i> (2014) |
| 15 | <i>Karyolysus</i> sp. | KJ461944 | Hungary | Haklová-Kočíková <i>et al.</i> (2014) |
| 16 | <i>Hepatozoon</i> sp. | HQ734801 | Northern Portugal | Maia <i>et al.</i> (2014) |
| 17 | <i>Hepatozoon</i> sp. | HQ734799 | Northern Portugal | Maia <i>et al.</i> (2014) |
| 18 | <i>Hepatozoon</i> sp. | MG787252 | Canary Islands | Maia <i>et al.</i> (2014) |
| 19 | <i>Hepatozoon</i> sp. | JX531962 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 20 | <i>Hepatozoon</i> sp. | JX531960 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 21 | <i>Hepatozoon</i> sp. | JX531948 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 22 | <i>Hepatozoon</i> sp. | JX531941 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 23 | <i>Hepatozoon</i> sp. | JX531940 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 24 | <i>Hepatozoon</i> sp. | JX531934 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 25 | <i>Hepatozoon</i> sp. | JX531933 | Western Mediterranean | Maia <i>et al.</i> (2014) |
| 26 | <i>Hepatozoon</i> sp. | HQ734803 | North Africa | Maia <i>et al.</i> (2014) |
| 27 | <i>Hepatozoon</i> sp. | HQ734793 | North Africa | Maia <i>et al.</i> (2014) |
| 28 | <i>Hepatozoon</i> sp. | HQ734798 | North Africa | Maia <i>et al.</i> (2014) |
| 29 | <i>Hemolivia</i> sp. | KX347435 | Thailand | Ahantarig <i>et al.</i> (2016) |
| 30 | <i>Hemolivia stellata</i> | MH196480 | Northeastern Colombia | Cotes-Perdomo <i>et al.</i> (2018) |
| 31 | <i>Hemolivia stellata</i> | MH196479 | Northeastern Colombia | Cotes-Perdomo <i>et al.</i> (2018) |
| 32 | <i>Hemolivia stellata</i> | KP881349 | Brazil | Karadjian <i>et al.</i> (2015) |
| 33 | <i>Hemolivia stellata</i> | MH196475 | Northeastern Colombia | Cotes-Perdomo <i>et al.</i> (2018) |
| 34 | <i>Hemolivia stellata</i> | MH196482 | Northeastern Colombia | Cotes-Perdomo <i>et al.</i> (2018) |
| 35 | <i>Hemolivia stellata</i> | MH196477 | Northeastern Colombia | Cotes-Perdomo <i>et al.</i> (2018) |
| 36 | <i>Hemolivia</i> sp. | KF992714 | Nicaragua | Kvičerová <i>et al.</i> (2014) |
| 37 | <i>Hemolivia</i> sp. | KF992712 | Australia | Kvičerová <i>et al.</i> (2014) |
| 38 | <i>Hemolivia mariae</i> | KF992711 | Australia | Kvičerová <i>et al.</i> (2014) |
| 39 | <i>Hemolivia</i> sp. | MN160404 | - | Nordmeyer <i>et al.</i> (2020) |
| 40 | <i>Hemolivia parvula</i> | KR069083 | Southern Africa | Cook <i>et al.</i> (2015) |
| 41 | <i>Hemolivia parvula</i> | KR069082 | Southern Africa | Cook <i>et al.</i> (2015) |
| 42 | <i>Haemogregarina podocnemis</i> | 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 *Karyolysus* 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 phylogenetic tree. Based on the molecular characteristics, we suggest that these two *Karyolysus* 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 *Karyolysus* (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). To 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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