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Genetic diversity, phylogenetic position, and co-phylogenetic relationships of *Karyolysus*, a common blood parasite of lizards in the western Mediterranean ☆

Rodrigo Megía-Palma^{a,b,c,d,*}, Javier Martínez^a, Patrick S. Fitze^{e,f}, José J. Cuervo^e, Josabel Belliure^b, Octavio Jiménez-Robles^{g,h}, Carlos Cabidoⁱ, José Martín^e, Santiago Merino^e

^a Universidad de Alcalá (UAH), Microbial and Parasitic Biotechnology and Ecology, E-28805 Alcalá de Henares, Madrid, Spain

^b Universidad de Alcalá (UAH), Global Change Ecology and Evolution Research Group (GloCEE), E-28805 Alcalá de Henares, Madrid, Spain

^c CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, P-4485-661 Vairão, Portugal

^d BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, P-4485-661 Vairão, Portugal

^e Museo Nacional de Ciencias Naturales (MNCN-CSIC), Calle José Gutiérrez Abascal 2, E-28006 Madrid, Spain

^f Instituto Pirenaico de Ecología (IPE-CSIC), Av. Nuestra Señora de la Victoria 16, E-22700 Jaca, Spain

^g The Australian National University, Division of Ecology and Evolution, Research School of Biology, 46 Sullivans Creek Rd, ACT 2601, Australia

^h Institut de Biologie, École Normale Supérieure, 46 Rue d'Ulm, 75005 Paris, France

ⁱ Sociedad de Ciencias Aranzadi, Departamento de Herpetología, Calle Alto de Zorroaga 11, E-20014 San Sebastián, Spain

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ABSTRACT

The genus *Karyolysus* was originally proposed to accommodate blood parasites of lacertid lizards in Western Europe. However, recent phylogenetic analyses suggested an inconclusive taxonomic position of these parasites of the order Adeleorina based on the available genetic information. Inconsistencies between molecular phylogeny, morphology, and/or life cycles can reflect lack of enough genetic information of the target group. We therefore surveyed 28 localities and collected blood samples from 828 lizards of 23 species including lacertids, skinks, and geckoes in the western Mediterranean, North Africa, and Macaronesia, where species of *Karyolysus* and other adeleorine parasites have been described. We combined molecular and microscopic methods to analyze the samples, including those from the host type species and the type locality of *Karyolysus bicapsulatus*. The phylogenetic relationship of these parasites was analyzed based on the 18S rRNA gene and the co-phylogenetic relationship with their vertebrate hosts was reconstructed. We molecularly detected adeleorine parasites in 37.9% of the blood samples and found 22 new parasite haplotypes. A phylogenetic reconstruction with 132 sequences indicated that 20 of the newly detected haplotypes clustered in a well-supported clade with another 18 sequences that included *Karyolysus galloti* and *Karyolysus lacazei*. Morphological evidence also supported that *K. bicapsulatus* clustered in this monophyletic clade. These results supported the taxonomic validity of the genus. In addition, we found some parasite haplotypes that infected different lizard host genera with ancient diverging histories, which suggested that *Karyolysus* is less host-specific than other blood parasites of lizards in the region. A co-phylogenetic analysis supported this interpretation because no significant co-speciation signal was shown between *Karyolysus* and lizard hosts.

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1. Introduction

Parasites within the order Adeleorina (Apicomplexa: Coccidia) complete their life cycle after infecting both vertebrate and inver-

tebrate hosts (O'Donoghue, 2017; Adl et al., 2019). They can infect ectotherms and endotherms worldwide. However, the mode of transmission and final host species are unknown for most adeleorine parasites (Tomé et al., 2012; Maia et al., 2014; O'Donoghue, 2017). The diversity of their life cycles suggests that co-adaptive evolution with arthropod vectors may have played an important role in the diversification of these parasites (O'Donoghue, 2017). The genus *Hepatozoon* sensu lato accommodates a large proportion of the parasites described within the order Adeleorina (Miller, 1908; Smith and Desser, 1997; Hrazdilová et al., 2021). However,

* Note: Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers: [OQ093596](https://doi.org/10.1016/j.ijpara.2022.12.006) - [OQ093614](https://doi.org/10.1016/j.ijpara.2022.12.006), and [OQ077708](https://doi.org/10.1016/j.ijpara.2022.12.006) - [OQ077710](https://doi.org/10.1016/j.ijpara.2022.12.006).

* Corresponding author.

E-mail address: rodrigo.megia@uah.es (R. Megía-Palma).

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the previously erected genus *Karyolysus* (Labbé, A., 1894. Recherches zoologiques et biologiques sur les parasites endoglobulaires du sang des vertébrés. Ph.D. Thesis, Faculté des Sciences de Paris, Paris, France) and the posterior erection of the genus *Hemolivia* (Petit et al., 1990) are clustered within *Hepatozoon* (Karadjian et al., 2015). Based on the assumption that systematic taxonomy should be based on monophyletic clades (Morrison, 2009; Ghimire, 2010; Megía-Palma et al., 2015), the paraphyletic nature of *Hepatozoon* suggests that its current systematic status should no longer be accepted (Ghimire, 2010; Haklová-Kočíková et al., 2014; Karadjian et al., 2015; Kvičerová et al., 2014; Zechmeisterová et al., 2021). Indeed, Karadjian et al. (2015) proposed the erection of the genus *Bartazoon* and the re-erection of *Karyolysus* in order to reduce the paraphyly of *Hepatozoon*. However, a recent phylogenetic analysis of *Hepatozoon* cf. *muris*, the type species of the genus, compromised the taxonomic validity of the genus *Bartazoon* sensu Karadjian et al. (2015), suggesting that some of the sequences in this large clade belong to *Hepatozoon* sensu stricto (Hrazdilová et al., 2021). Clearly more information is still needed prior to the formal reclassification of some of the clades within Adeleorina (Maia et al., 2016).

Previous surveys of blood parasites in lizard hosts of the western Mediterranean revealed a high diversity of adeleorine parasites (Maia et al., 2011, 2012; Tomé et al., 2014, 2016). Recent systematic revisions proposed that the genus *Karyolysus* should be used to reaccommodate adeleorine parasites found in lacertid hosts from Central and Eastern Europe (Haklová-Kočíková et al., 2014; Dajčman et al., 2022). Phylogenetic relationships of these parasites and differential features of their natural history supported this view; motile sporokinets are formed in the oocyst by a single germinal center and released in definitive hosts, where they encyst as sporocysts of *Karyolysus* (Reichenow, 1920; Haklová-Kočíková et al., 2014). In contrast, the genus *Hepatozoon* is characterized by a large polysporocystic oocyst and, in *Hemolivia*, intraerythrocytic merogony occurs and gametocytes in the peripheral blood have a stain-resistant vacuole (Telford, 2009). Moreover, the genus name '*Karyolysus*' comes from the Greek and refers to the fact that invaded blood cells can have their nucleus distorted (Labbé, 1894, PhD Thesis, cited earlier), which is a key characteristic of the genus that does not occur in *Hemolivia* or *Hepatozoon*. Another difference between these genera is that the only known definitive hosts of *Karyolysus* are mites of the genus *Ophionyssus* (Mesostigmata: Macronyssidae), where they undergo both asexual and sexual parts of their life cycle prior to transmission to vertebrate hosts (Haklová-Kočíková et al., 2014). Microscopic surveys reported that these parasites are common in the blood of lacertid hosts across Europe, North Africa, and Macaronesia (Labbé 1894, PhD Thesis, cited earlier; Reichenow, 1919; Svahn, 1975; Aparicio-Sánchez and Cordero del Campillo, 1980; Khairutdinov and Sokolina, 2007; Mihalca et al., 2008; Majláthová et al., 2010; Haklová-Kočíková et al., 2014; Kopena et al., 2021; Megía-Palma et al., 2020a, 2020b, Rutschmann et al., 2021; Dajčman et al., 2022). So far, 18 species have been proposed to belong to the genus *Karyolysus*.

Previous analyses of the phylogenetic relationships of adeleorine parasites of lizards provided weak or no phylogenetic support for monophyly of the genus *Karyolysus* (Haklová-Kočíková et al., 2014; Kvičerová et al., 2014; Karadjian et al., 2015). We therefore investigated these phylogenetic relationships by studying adeleorine parasites in the blood of lacertid lizards, skinks, and geckoes from Western Europe, North Africa, and Macaronesia. We combined both molecular and microscopic tools to analyse (i) the diversity of these adeleorines; (ii) their taxonomic positions based on the phylogenetic relationships; and (iii) a co-phylogeny between them and their lizard hosts. We increase our knowledge on the genetic diversity of blood parasites of lizards in the western

Mediterranean and reassess the taxonomic status of *Karyolysus* based on molecular, morphological, and host data.

2. Materials and methods

2.1. Sampling

We analysed blood samples from 23 lizard species collected in 28 localities between 2011 and 2014. We analysed samples from a total of 20 species of lacertid lizard hosts (family Lacertidae), two species of skink hosts in the genus *Chalcides* (family Scincidae), and one species of gecko (genus *Tarentola*, family Phyllodactylidae). We surveyed 22 localities in the Iberian Peninsula, two in Tenerife (Canary Islands), two in the northern coast of Morocco, one in the Chafarinas Islands, and one in the eastern coast of Tunisia (near Gabès city) (Supplementary Table S1).

2.2. Blood and mite collection

Using sterile needles (25 G) and heparinized capillaries, we obtained a blood sample (5 µL) from a blood vessel of each lizard's tail (Megía-Palma et al., 2018). We performed thin blood smears using microscope slides for all the lizard species. A drop of the blood sample of all the species and individuals was preserved in a Whatman paper (FTA[®] Classic Card, Buckinghamshire, UK), which was later used to extract DNA (Megía-Palma et al., 2018). For two of the lizard host species, *Psammotromus algirus* and *Gallotia galloti*, we also performed smears of engorged mites of the genus *Ophionyssus* in an attempt to visualize sporokinets (i.e., the developmental stage of *Karyolysus* that takes place in the definitive mite hosts; Haklová-Kočíková et al., 2014). Because mites detach from the host lizards after a blood meal, we kept lizards of these two species overnight in individual cotton bags during the field campaigns in Tenerife and Madrid (Spain) and thereafter collected the engorged mites (Megía-Palma et al., 2020a, 2020b, 2022). We air-dried and fixed all blood smears with 100% methanol for 5 min. We stained them in a dilution of 1:10 (v/v) of Giemsa and phosphate buffer at pH 7.2 for 40 min.

2.3. Molecular and phylogenetic analyses of parasites

Following the protocol described by Megía-Palma et al. (2013), we performed the genomic DNA extraction of all the blood samples. A preliminary molecular screening of adeleorine parasites was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reaction volume used for this PCR was 10 µL. It contained between 20 and 100 ng of DNA, 0.25 µM of each primer and SYBR[®] Select Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used here targeted short fragments (180 bp) of the 18S rRNA gene of Adeleorina (Table 1). They were designed to avoid amplification of other apicomplexans from different orders present in the blood of these lizards. The samples were considered positive when melting curves of the amplicons were observed.

We ran a second round of PCRs targeting longer sequences (from 1,700 to 1,800 bp) with 130 of those chosen based on the relatively high parasitaemia of the corresponding blood smears (see microscopic methods below). The objective of this criterion was to facilitate the molecular detection of the parasites. These PCRs were carried out using the primer set NBA1/HPF2 (Table 1) (Merino et al., 2009, 2014). DNA extraction and preparation of the reactions were conducted in separate flow cabins. Negative controls were placed on each PCR plate and none of the negative controls were positive by PCR. In addition, a positive control was

Table 1

Primers used to detect adeleorine parasites in blood samples of lizards. Sequence length (pb) and PCR thermodynamic conditions are indicated for each set of primers.

Primer	sequence 5' → 3'	pb	annealing	extension
¹ Hep900F	gtcagaggtgaaattcttagattg	188	60 °C (30 s)	60 °C (30 s)
² Hep4	taaggtgctgaaggagtcgtttat			
³ NBA1	ggttgatcctgccagtagt	1,774	58 °C (30 s)	72 °C (120 s)
² HPF2	gacttctctctgctctaaag			

¹ Also named Hep800F. Merino et al. (2006).

² Criado-Fornelio et al. (2006).

³ Also named BT-F1. Criado-Fornelio et al. (2003).

placed on each PCR plate. Amplicons were subsequently recovered from agarose gels and directly sequenced using an ABI 3730 XL automated sequencer (Applied Biosystems, Waltham, MA, USA). When sequences rendered double peaks ($n = 1$ sample of *Podarcis*), amplicons were cloned using the TOPO® TA Cloning® KitDNA (Invitrogen, Life Technologies, Waltham, MA, USA) and plasmids were purified using the NZYSpeedy Miniprep kit (NZYTech, Lisboa, Portugal) (see García-del-Río et al. (2021)). The three cloned sequences found (Pg29c2, Pg29c3, Pg29c4) were included in the phylogeny.

From the GenBank database, we selected 18S rRNA sequences of at least 1,300 bp to build a phylogenetic tree of the Adeleorina. The final alignment contained 133 sequences of the 18S rRNA gene. This included the 22 sequences newly obtained in the present survey which are available at the GenBank database (Supplementary Table S2). We included *Adelina bambarooniae* (Apicomplexa: Adeleorina), a homoxenous coccidium species, as an outgroup. The alignment was performed using the algorithm MSAProbs, which exhibits higher alignment accuracy compared with other aligners (Liu et al., 2010). Later, we evaluated the MSAProbs alignment using the transitive consistency score (TCS). This tool identifies the most correct positions in a multiple sequence alignment (MSA) by assigning a consistency score (Chang et al., 2014). As an option, TCS can generate a weighted MSA where each column is multiplied according to its consistency score. The more reliable columns are more represented, therefore improving the support of informative and reliable positions of the MSA (Chang et al., 2014). The weighted MSA contained 17,039 positions. We selected the substitution model GTR + I + G using jModelTest 2.1.4 (Darriba et al., 2012) to perform the Bayesian analysis. This analysis consisted of two runs of four chains each, with 2,000,000 generations per run. Data were collected every 100 generations, and the burn-in was set to 25%. We obtained a consensus tree from 30,000 trees. The final standard deviation of the split frequencies was lower than 0.01. In addition, we analysed the alignment using maximum likelihood inference (PhyML program; Guindon et al., 2010) and the same substitution model mentioned above. We selected the subtree pruning and regrafting (SPR) and the nearest neighbour interchange (NNI) tree rearrangement options, and we used a Bayesian-like transformation of aLRT (aBayes) to obtain the clade support. This is a compelling alternative to other, slower, conventional methods, offering not only speed advantages but also excellent levels of accuracy and power (Anisimova et al., 2011).

2.4. Host-parasite co-speciation analysis

We used the statistical method ParaFit (Legendre et al., 2002) implemented in CopyCat software (Meier-Kolthoff et al., 2007) to test the significance of a global hypothesis of host and parasite co-speciation. ParaFit tests for the congruence of the host and parasite phylogenetic trees and for individual host-parasite association links. The global null hypothesis is that evolution of the hosts and parasites has been independent. To do a co-speciation analysis between lacertid hosts and *Karyolysus*, we reconstructed

the phylogenetic relationships of the lizards based on a recent genomic study (García-Porta et al., 2019), whereas for *Karyolysus* we used a phylogenetic tree based on >1,700 bp of the 18S rRNA gene. We based the co-speciation test on the analysis of genetic distance matrices.

2.5. Microscopic analysis of blood smears

Blood smears taken from the same lizards were used to confirm the presence of adeleorine parasites. Adeleorines can be discriminated from other blood parasites by the lack of refractile bodies, which is the diagnostic morphological characteristic of haemococcidians (Apicomplexa: Coccidia: Eimeriorina), a second order of parasites (i.e., genera *Lankesterella* and *Schellackia*) that can also be found in the blood of Mediterranean lizards (Megía-Palma et al., 2014, 2018). To do this, the lizard blood smears were systematically screened for blood parasites (10,000 blood cells at $\times 1,000$ magnification) using a light-field optic microscope (BX43, Olympus, Tokyo, Japan) (Megía-Palma et al., 2018). We took microphotographs of the detected blood parasites at $\times 1,000$ magnification using an adjustable camera (SC30, Olympus, Tokyo, Japan). We used a free software (MB-Ruler - the triangular screen ruler 5.4., <https://www.markus-bader.de/MB-Ruler/index.php>) to measure the parasites (Megía-Palma et al., 2013, 2014).

2.6. Data availability

All sequences, alignments, and microphotographs of all parasite stages and haplotypes are publicly available at <https://data.mendeley.com/10.17632/b76v9g6j9y.1>.

3. Results

3.1. Molecular detection and phylogenetic consistency

The molecular screening with primers designed to amplify short sequences indicated that 37.9% (314/828) of the samples contained DNA of adeleorine parasites. Lacertids were the most affected with 100% of the species infected and a mean prevalence of 51.3%. The two skink species were infected with a mean prevalence of 21.1%. No infection was detected in the 38 samples of the gecko host *Tarentola mauritanica* from the Chafarinas Islands (Supplementary Table S1). We obtained 78 long 18S rRNA sequences in 16 of the 20 lacertid hosts and the two skink host species investigated (Table 2). We found 22 different 18S rRNA parasite haplotypes.

The phylogenetic analyses provided strong phylogenetic support for the monophyletic origin of 38 of the sequences analysed in a clade that contained 20 of the 22 newly detected haplotypes, two species of *Karyolysus*, and 16 other sequences that were labelled in the GenBank database as 'Hepatozoon' (Fig. 1 and Supplementary Table S2). It also contained haplotype MK497254, a sequence deposited in the GenBank database as *Karyolysus* cf. *lacazei* (Zechmeisterová et al., 2019). This haplotype shared 100%

Table 2

Amplification success ratio (ASR%) in blood samples of lizard hosts that were positive (IMS +) for adeleorine parasites based on the sample size (nIMS) analysed in an initial molecular screening that targeted short sequences. Sequencing effort (SE) refers to the percentage of amplicons that were sent for sequencing by the Sanger method. Long amp. (n) equals the number of long amplicons obtained (>1,700 bp). Parasite haplotypes detected in more than one host species are plotted in bold font. Asterisks indicate haplotypes that belong to a large clade of *Hepatozoon*, all the other are *Karyolysus* in Fig. 1.

Host genus	n Species	nIMS	IMS+	Prevalence (%)	SE (%)	Long amp. (n)	Parasite haplotype/s	ASR (%)
<i>Acanthodactylus</i>	2	159	18	11.3	83.3	6	Ab112*, Ae53	40
<i>Chalcides</i> (Scincidae)	2	29	5	17.2	100	5	CH186	100
<i>Gallotia</i>	1	11	11	100	100	11	Gg241, Gg235	100
<i>Iberolacerta</i>	5	114	78	68.4	50	25	IB2 , IBa55, IBm69, IBm77, P112 , Ls132	64
<i>Lacerta</i>	1	26	5	19	100	5	Ls4 , Ls132 , Ls142	80
<i>Podarcis</i>	7	224	148	66.1	18.2	24	P112 , Pm196, Pm197, Pg29c2, Pg29c3, Pg29c4	89
<i>Psammodromus</i>	2	117	35	30	45.7	10	Psa1, PSh64*	62.5
<i>Tarentola</i>	1	38	0	0	-	-	-	-
<i>Timon</i>	1	13	9	69	89	8	Tl121, Ls4 , Ls132	100
<i>Zootoca</i>	1	97	5	5.1	80	4	Zv17	100
Total	23	828	314	37.9	60.3	78	22	

nucleotide sequence identity with Ls142 (GenBank ID: OQ093598), which has been detected by us in both *Timon lepidus* and *Lacerta schreiberi* hosts (Supplementary Table S1). This clade also included haplotype MK396906, that corresponds to *Karyolysus galloti*, detected in a lizard from the Macaronesia, *Gallotia caeasaris* (Tomé et al., 2019). MK396906 was closely related to haplotypes Gg235 (OQ093613) and Gg241 (OQ093614), detected in a second species of *Gallotia* in another island of Macaronesia (Fig. 1 and Supplementary Table S1).

This well-supported clade (hereafter, *Karyolysus*) is closely related to the *Hepatozoon* lineage detected in carnivore mammal hosts, in line with previous phylogenetic hypotheses (Haklová-Kočíková et al., 2014; Maia et al., 2016). *Karyolysus* was substructured in three major subclades (Fig. 1). (i) The first subclade contained 25 parasite haplotypes. Twenty-four of them were detected in 16 lacertid species (belonging to 10 different genera) and one of them in two skink host species (belonging to a single genus). All host species of the parasites that clustered in this subclade are native to the western Mediterranean and Macaronesia. (ii) A second well-supported subclade included 12 parasite haplotypes, nine of them in *Podarcis* spp. All host species of the parasites belonging to the second subclade, including one skink and one snake host species, are also native to the western Mediterranean (Supplementary Table S2). (iii) A third subclade clustered within *Karyolysus* with strong support included two parasite haplotypes detected in the blood of *T. mauritanica* (Gekkota: Phyllodactylidae) sampled in North Africa (Tomé et al., 2016). We could not compare any sequence with these because, as mentioned, the *T. mauritanica* investigated by us were not infected.

Two of the adeleorine haplotypes obtained in this study, Ab112 (OQ093610) and PSh64 (OQ093608), clustered together with 55 of the sequences analysed with strong phylogenetic support within a different large clade of *Hepatozoon* detected in multiple reptile host species, namely, snakes and geckoes from North Africa and Asia, but also chameleons from Madagascar, lizards from Brazil, and a snake host from Australia. Many subclades within this large clade of *Hepatozoon* were well supported, for example, the parasites detected in American amphibians, South American and European rodents, and African lizards, snakes and chameleons, while others (mainly those consisting of a single sequence) were not statistically supported (Fig. 1). Parasite haplotype Ab112 clustered in this large clade and was detected in *Acanthodactylus boskianus* from Tunisia and the gametocytes observed were intraerythrocytic with a mean \pm standard error dimensions of $11.18 \pm 0.15 \times 2.25 \pm 0.06 \mu\text{m}$ ($n = 46$) (Fig. 2A-B). The cell host type that this parasite haplotype infects and its size were similar to the general morphology described for some life stages of *Karyolysus* (Svahn, 1975). However, we did not observe any infected cell that had its nucleus distorted, which is a key diagnostic characteristic of the genus. The second parasite haplotype, PSh64, was detected in the blood of five

lizards of the host genus *Psammodromus*: in one *P. algirus* and in four *Psammodromus hispanicus*. Microphotographs of their blood samples only revealed extracellular parasite stages of $28.01 \pm 0.25 \times 4.89 \pm 0.11 \mu\text{m}$ ($n = 11$) (Fig. 2C-D), which clearly differs from the cellular dimensions of the haplotypes of *Karyolysus* detected in this study (i.e., $12.58 \pm 0.07 \times 3.94 \pm 0.04 \mu\text{m}$; $n = 1,072$). These two haplotypes were closely related to a *Hepatozoon* detected in a house gecko in Brazil, *Hemidactylus mabouia* (Squamata: Gekkonidae), an invasive species originally from Africa (Harris et al., 2015).

Hemolivia was closely related to this large clade of *Hepatozoon* and grouped all sequences of parasites that were detected in land tortoises and terrapins, and in an Australian scincid. These sequences clustered together with strong phylogenetic support.

The closest ancestral clade of '*Hepatozoon*', '*Karyolysus*', and '*Hemolivia*' was a monophyletic clade grouping six sequences of the genus *Haemogregarina*, which here were specifically detected in aquatic turtles (e.g., Dvořáková et al., 2015). Basal to this one was another clade that grouped sequences of the genera *Dactylosoma* and *Babesiosoma*, which both belong to the family Dactylosomatidae (Apicomplexa: Adeleorina) and infect amphibian and fish hosts (Barta et al., 2012; Netherlands et al., 2020).

3.2. Host-parasite specificity and lack of co-speciation signal

Six out of 22 (27.3%) of the 18S rRNA adeleorine parasite haplotypes obtained in this study were found in more than one host species (Table 2). Particularly, we detected the parasite haplotype Ls132 (OQ093597) in hosts of the genera *Iberolacerta*, *Timon*, and *Lacerta*, and the haplotype P112 (OQ093600) was detected in *Iberolacerta* and *Podarcis* (Fig. 3). The 'green lizard' genera, *Timon* and *Lacerta*, also shared the parasite haplotype Ls4 (OQ093596). In addition, two of the three Pyrenean lizard species within the host genus *Iberolacerta* (*Iberolacerta aranica* and *Iberolacerta bonnali*) shared the parasite haplotype IB2 (OQ093603) (Fig. 3). None of the tested haplotype-haplotype relationships supported the co-speciation hypothesis between *Karyolysus* and its lacertid hosts (Table 3). However, the haplotype CH186 (OQ093612) detected in the host genus *Chalcides* (scincid lizards), sampled on the Chafarinas Islands, was the only one that supported the co-speciation hypothesis, although the same parasite haplotype infected both species sampled in the archipelago, *Chalcides ocellatus* and *Chalcides parallelus*.

3.3. Morphological congruence

We observed adeleorine parasites in 50.3% of the blood smears. Intracellular parasitic stages were always observed infecting erythrocytes. These intraerythrocytic stages were (i) merozoites, which are slender-shaped (Svahn, 1975) (Fig. 4A) and (ii) trophozoites, which are characterized by the presence of multiple vac-

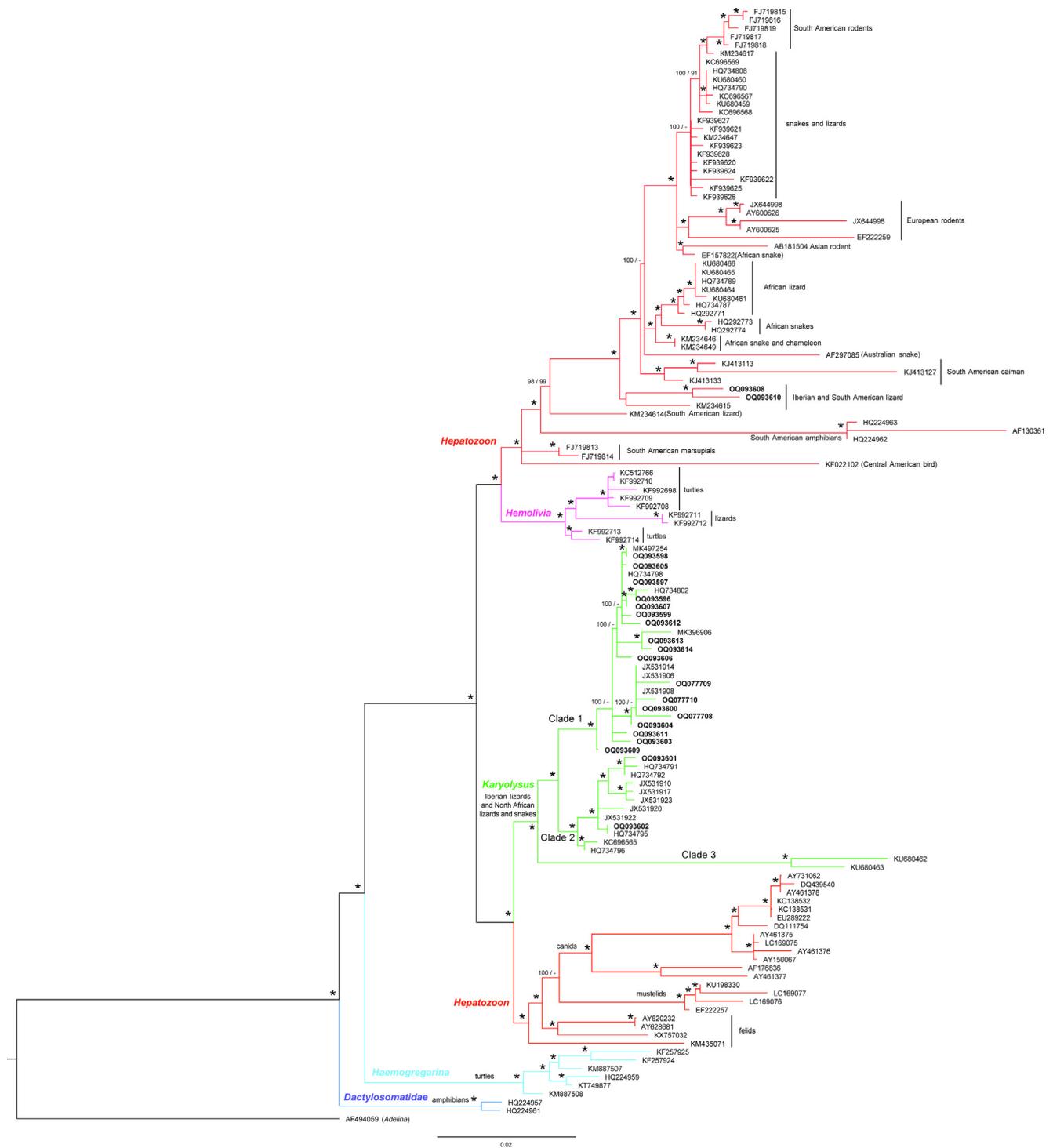


Fig. 1. Phylogenetic tree of adeleorine parasites. Support values are shown when higher than 90% for Maximum Likelihood and Bayesian inference (ML/Bayesian). Sequence names are provided and those obtained in the present study are shown in bold. The scale bar indicates number of base substitutions per site.

uoles with putative digestive function (Svahn, 1975) (Fig. 4B and Supplementary Fig. S1). We also observed (iii) gamonts, which are the sexual stages; (iv) microgametocytes, that lack vacuoles and have dense chromatin (Fig. 4C), while (v) macrogametocytes have scattered chromatin (Svahn, 1975) (Fig. 4D and Supplementary Fig. S1). Infections by the latter stages were often observed in erythrocytes with distorted nuclei (see Fig. 4C-D). Some of the parasites observed in blood smears of the host type species, *Podarcis muralis*, were morphologically compatible with *Karyolysus bicapsulatus*, which is easily recognizable because it has a central

refractive corpuscle and dense capsules on both polar ends (Reichenow, 1920) (see Fig. 4C). Moreover, the smears made of engorged mites of the genus *Ophionyssus* fed on *G. galloti* and *P. algirus* hosts revealed putative sporokinete stages and merozoites (Fig. 5).

4. Discussion

Microphotographs of blood smear demonstrated (i) that most of the erythrocytic invasions, and particularly those involving

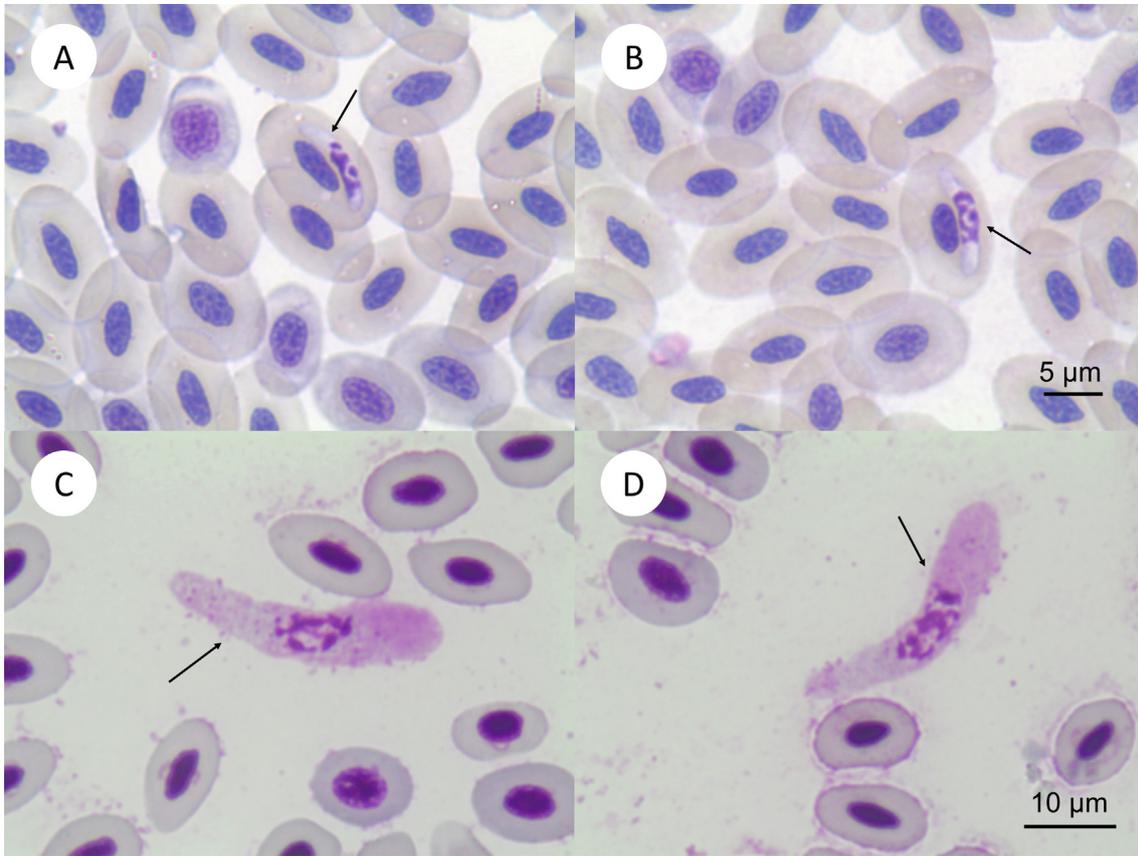


Fig. 2. Microphotographs of blood parasites of the genus *Hepatozoon* found in *Psammmodromus hispanicus* from Spain and *Acanthodactylus boskianus* from Tunisia. (A-B) Intraerythrocytic stages in blood samples from *A. boskianus* in which haplotype Ab112 was detected. (C-D) Giant extraerythrocytic stages in blood samples from *P. hispanicus* in which haplotype PSh64 was detected. Arrows in the panels indicate the parasites.

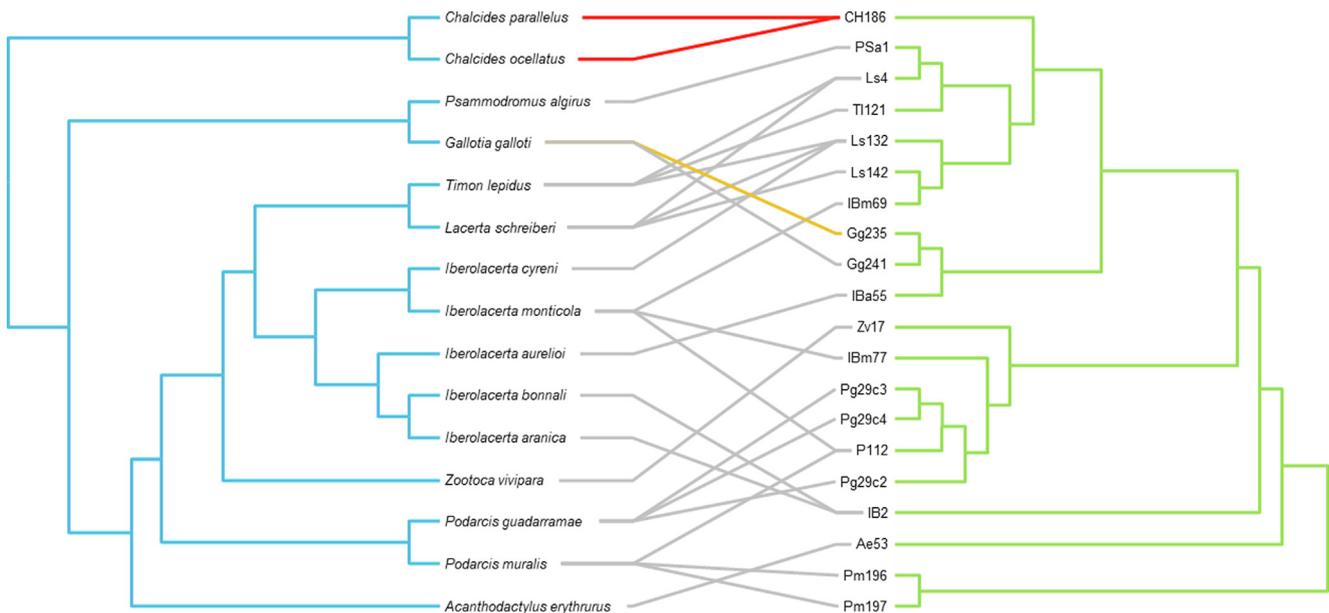


Fig. 3. *Karyolysus* and lacertid phylogenetic trees showing host-parasite link associations. A maximum likelihood tree was computed for the parasites based on long (>1,700 bp) 18S rRNA gene sequences whereas the host tree was reconstructed based on García-Porta et al. (2019). Comparison of the host-parasite trees was based on distance matrices in TreeMap software (<https://sites.google.com/site/cophylogeny/home>). Lines between trees depict the observed host-parasite associations. Red (black) lines indicate significant associations. The orange (dashed) line indicates a marginal non-significant association.

Table 3

ParaFit tests including *Karyolysus* and lizard haplotypes. Probabilities are computed after 999 random permutations on a distance matrix. The null hypothesis of the global test is that parasites select hosts at random in the host phylogenetic tree. In the tests of individual host-parasite association links, the null hypothesis is that the tested link is not due to co-speciation. Global test and individual links with $P < 0.05$ are marked in bold.

Parasite	Host	P-value
Ae53	<i>Acanthodactylus erythrurus</i>	0.105
CH186	<i>Chalcides ocellatus</i>	0.005
CH186	<i>Chalcides parallelus</i>	0.005
Geg235	<i>Gallotia galloti</i>	0.068
Gg241	<i>Gallotia galloti</i>	0.148
IB2	<i>Iberolacerta aranica</i>	0.268
IBa55	<i>Iberolacerta aurelioi</i>	0.859
IB2	<i>Iberolacerta bonnali</i>	0.252
Ls132	<i>Iberolacerta cyreni</i>	0.787
P112	<i>Iberolacerta monticola</i>	0.247
IBm77	<i>Iberolacerta monticola</i>	0.234
IBm69	<i>Iberolacerta monticola</i>	0.780
Ls4	<i>Lacerta schreiberi</i>	0.252
Ls132	<i>Lacerta schreiberi</i>	0.311
Ls142	<i>Lacerta schreiberi</i>	0.295
Pg29c2	<i>Podarcis guadarramae</i>	0.310
Pg29c3	<i>Podarcis guadarramae</i>	0.606
Pg29c4	<i>Podarcis guadarramae</i>	0.258
Pm196	<i>Podarcis muralis</i>	0.436
Pm197	<i>Podarcis muralis</i>	0.625
P112	<i>Podarcis muralis</i>	0.570
PSa1	<i>Psammodromus algirus</i>	0.481
Ls4	<i>Timon lepidus</i>	0.490
Ls132	<i>Timon lepidus</i>	0.486
TI121	<i>Timon lepidus</i>	0.728
Zv17	<i>Zootoca vivipara</i>	0.833
Global test		0.317

macrogametocytes, were associated with distortion of the host's cell nucleus. This is consistent with the original and subsequent descriptions of the genus *Karyolysus* (Labbé, 1894, PhD Thesis, cited earlier) because, as commented, the etymology of this genus name refers to the distortion of the host's cell nucleus by the parasite (Svahn, 1975; Haklová-Kočíková et al., 2014). Furthermore, the microphotographs revealed (ii) the presence of parasitophorous vacuoles, which has been proposed as an avoidance structure in *Karyolysus* developed to resist the host's immune response (Beyer and Sidorenko, 1984). (iii) We found *K. bicapsulatus* in *P. muralis* from the center of the Iberian Peninsula, i.e., in the host species and locality where *K. bicapsulatus* was first described (Reichenow, 1919). Its corresponding haplotype (Pm196; OQ093601) clustered within the '*Karyolysus*' clade. This finding indicated congruence between phylogeny and morphology and supported conclusions from previous investigations (i.e., Haklová-Kočíková et al., 2014). (iv) In addition, we observed putative sporokinetes, pre-spore stages, and free gamonts within the hemocoel of engorged mites of the genus *Ophionyssus*. The observations in mites support the concept that the parasites observed can develop to infective stages within this genus of mites, which is the specific vector of *Karyolysus* (Haklová-Kočíková et al., 2014). All these results indicated that most of the adeleorine blood parasites that infect lacertid hosts in the western Mediterranean belong to the genus *Karyolysus*.

Concerning the specificity of the genus, 25.0% (5/20) of *Karyolysus* haplotypes were found in more than one host species and three of them (13.6%) were infecting species of different host genera. This suggests a slightly higher host-switching capability of *Karyolysus* compared with haemococcidians of the genus *Schellackia*, a second parasite genus that infects lacertids in the Iberian Peninsula (Megía-Palma et al., 2018). This argument is based on a direct comparison of the same samples of lacertid hosts used here that were also used for study of the genus *Schellackia* in Megía-Palma et al.

(2018). In that previous study, a similar proportion (21.5%) of parasite haplotypes infected more than one host species. However, only 6.2% of *Schellackia* haplotypes was detected in more than one host genus (Megía-Palma et al., 2018). The subtle higher proportion of haplotypes of *Karyolysus* that infect different host genera (13.6%) suggests a more generalist ecology than in *Schellackia*, although we acknowledge that a wider screening of *Schellackia* in other regions might provide new insights on this assumption. In further support of it, *Karyolysus* haplotype Ls132 (OQ093597) was detected in the host genera *Timon*, *Lacerta*, and *Iberolacerta*, highlighting that parasites of this genus can cross the phylogenetic barrier between relatively phylogenetic distant host genera because the host genera *Timon* and *Lacerta* may have diverged from each other 17.5–20.6 million years ago (mya; Ahmadzadeh et al., 2016), whereas the divergence from other genera of the smaller Lacertini, such as *Iberolacerta*, might be even older (Ahmadzadeh et al., 2016). This suggests that genetic distances between the three host genera would be enough to have impeded infections by haplotype Ls132 if *Karyolysus* were instead a specialist parasite (e.g., Streicker et al., 2010; Gupta et al., 2019). In opposition to *Karyolysus*, the single *Schellackia* haplotype that crossed the host genus barrier was found only in *Podarcis* and *Iberolacerta* host species, despite the same samples from *Timon* and *Lacerta* hosts also being investigated (Megía-Palma et al., 2018). *Podarcis* and *Iberolacerta* are relatively closely related with an estimated diverging time of 2.5 mya (Mendes et al., 2016), which might reduce the phylogenetic barrier effect in some lineages of blood parasites (Gupta et al., 2019). Moreover, the eventual syntopic distribution of some of the lizard species in these four host genera would provide opportunities to adeleorine parasites for switching hosts (e.g., Dajčman et al., 2022). Indeed, this niche overlap can occur at the microgeographic level in some of the species of these host genera (Monasterio et al., 2010; Galán et al., 2013). Such a close contact would provide mite hosts with opportunities to infest other lizard hosts and thus the adeleorine parasites that they transmit to exploit new hosts (niches) by host-switching and posterior diversification (e.g., Fecchio et al., 2018). We thus suggest that future studies should be focused on diversity and specificity of *Ophionyssus* mites in relation to both their lizard hosts and the *Karyolysus* haplotypes that they can transmit. The study of this three-way co-adaptive relationship that includes the definitive (mite) hosts may contribute to explaining the diversification pathways of *Karyolysus*.

The parasite haplotypes found in *Podarcis* clustered in two of the major *Karyolysus* subclades. This, together with the lack of statistical support for the host-parasite co-speciation hypothesis, suggests that the parasite radiation across the region did not follow the evolutionary radiation of the host genus *Podarcis* but it is likely other factors may have favoured host-switching such as the geographic overlap and perhaps the still relatively small genetic differences among *Podarcis* spp. (Yang et al., 2021). A recent study supports our results because the same haplotype of *Karyolysus* was found in *Podarcis* and *Iberolacerta* hosts from Slovenia (Dajčman et al., 2022). Therefore, *Karyolysus* can successfully infect different host species by host-switching. This may have implications in the current scenario of climate change if *Karyolysus* and other adeleorine parasites can encounter new and thus immunologically non-adapted host populations (Rózsa et al., 2015) given that some lizard populations are effectively shifting their distribution range and are in contact with formerly isolated lizard species (Ortega et al., 2016; Gangloff et al., 2019). Although negative effects of the infection by adeleorine parasites in lacertid lizards are not evident, these can include a putative reduction of the host's oxygen carrying capacity and an impairment of their escape velocity (Oppliger et al., 1996; Garrido and Pérez-Mellado, 2014; Megía-Palma et al., 2020a, 2020b). Our results thus suggest that *Karyoly-*

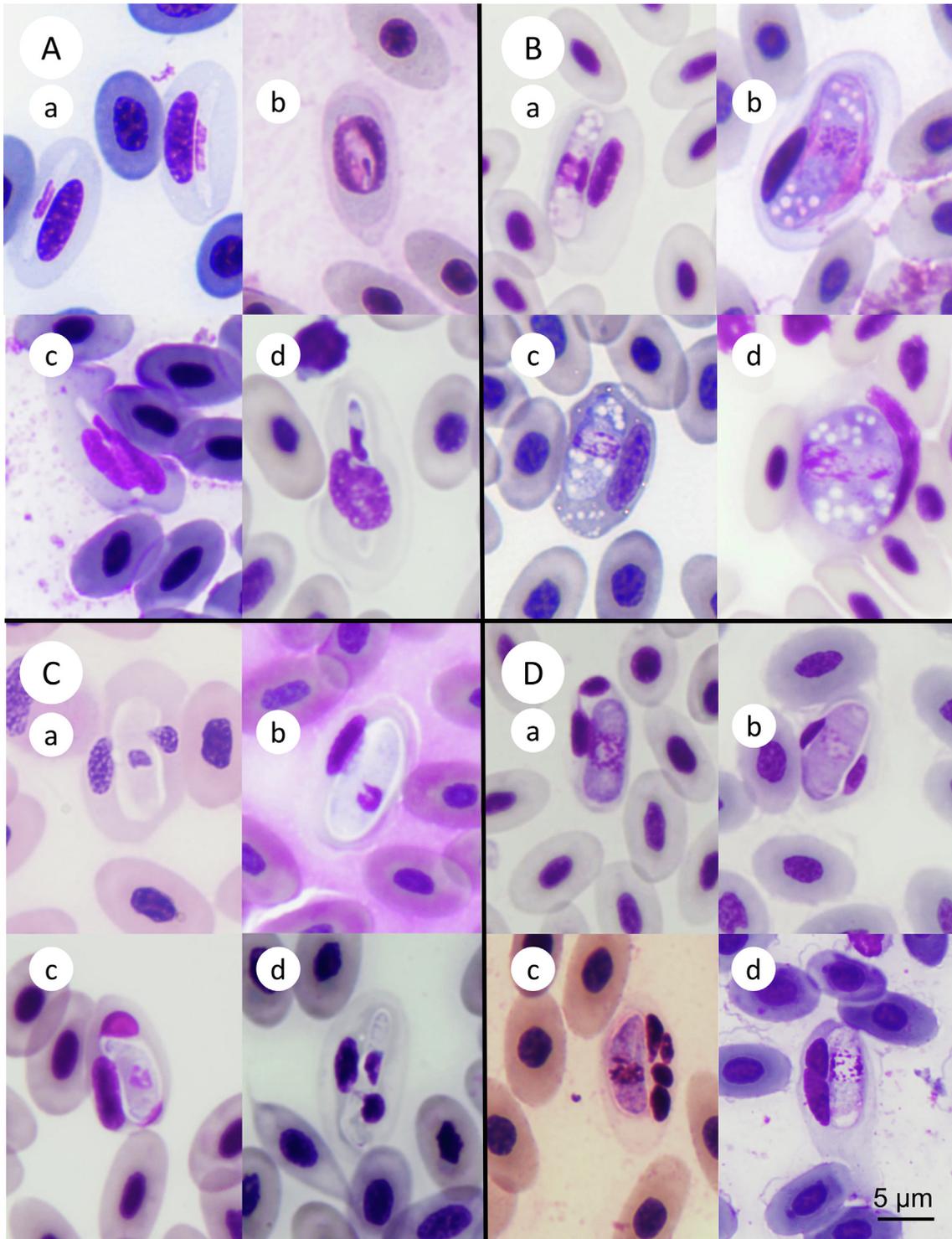


Fig. 4. Microphotographs of parasite stages of *Karyolysus* found in the blood of lacertid hosts. (A) Merozoites observed in the blood of host lizards of the genera (a) *Iberolacerta*, (b) *Gallotia*, (c) *Timon*, and (d) *Lacerta*. (B) Trophozoites with apparent vacuoles in the blood of (a) *Psammodromus*, (b) *Gallotia*, (c) *Iberolacerta*, and (d) *Podarcis*. (C) Microgametocytes in the blood of (a) *Iberolacerta*, (b and c) *Podarcis*, and (d) *Lacerta*. The parasite on the lower left (c) is morphologically compatible with *Karyolysus bicapsulatus*. (D) Macrogametocytes in the blood of (a) *Psammodromus*, (b) *Acanthodactylus*, (c) *Gallotia*, and (d) *Timon*. The nucleus of the host cell was more often distorted in C and D than in A and B.

sus, and perhaps other adeleorine blood parasites of lizards, might easily switch among relatively closely related hosts (lacertids). This may have uncertain ecological consequences that we believe deserve to be considered and further monitored.

Our analyses also provided evidence for specialist parasites in insular systems. Two host species of the genus *Chalcides* were

infected by a single parasite haplotype in the Chafarinas Islands archipelago that was not detected in the other potential lacertid and gecko hosts investigated within these islands despite their overlapping distribution (i.e., *Podarcis vaucheri* and *T. mauritanica*). A similar situation was previously observed for the Tenerife lizard *G. galloti*, which was infected by two haplotypes of *Karyolysus* that

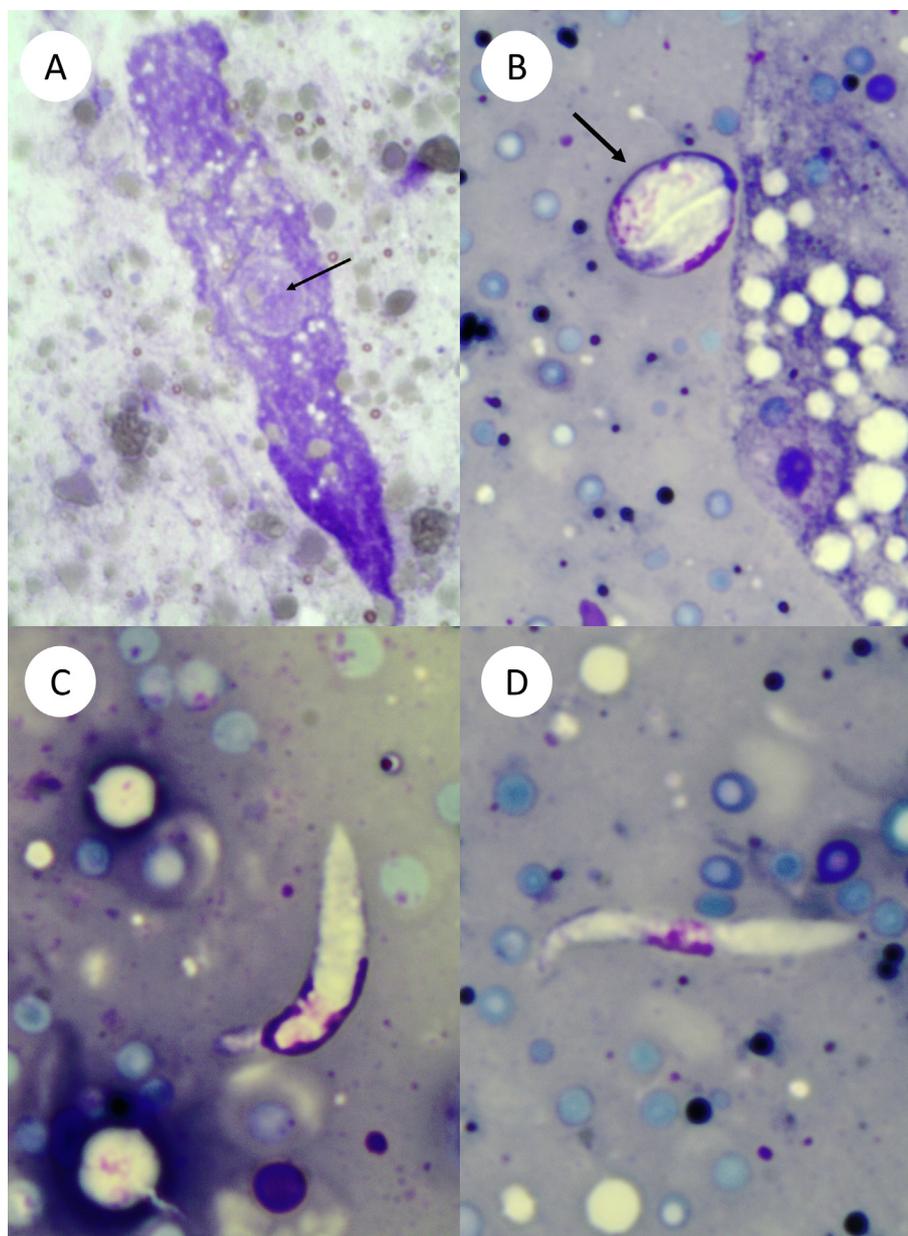


Fig. 5. Microphotographs of parasite stages in mites of the genus *Ophionyssus* that fed on lacertid hosts. (A) Putative sporokinete in a mite feeding on *Gallotia galloti*. The arrow indicates the nucleus. (B) The arrow indicates a pre-sporocystic stage in a mite feeding on *Psammodromus algirus*. (C-D) Putative free gamonts in the hemocoel of a mite feeding on *P. algirus*.

were not found anywhere else. During an extensive sampling in the Canary Islands, Tomé et al. (2018) found a high degree of parasite specialization in insular species of this archipelago where the *Karyolysus* haplotypes found in the lacertid host did not infect the endemic gecko and skink species. Despite these cases, our co-speciation analysis for the *Karyolysus*-Lacertidae assemblage revealed no significant relationships for any of the haplotypes tested. This contrasts with a similar analysis previously performed for the assemblage between the parasite genus *Schellackia* and lizard hosts in the family Lacertidae, which revealed nine significant haplotype-haplotype relationships, and provided support for the global hypothesis of host-parasite co-speciation (Megía-Palma et al., 2018). This divergence between *Karyolysus* and *Schellackia* in host-parasite co-speciation trajectories within the same vertebrate hosts, and within the same lacertid individuals, may stem from differences in the biological cycle and the different phy-

logenetic origin of these parasite genera (O'Donoghue, 2017). Given that lizards are definitive hosts for *Schellackia* but only intermediate hosts for *Karyolysus*, our findings thus support the view that significant co-adaptive relationships may be more likely to emerge between parasites and definitive hosts (i.e., where the parasite undergoes sexual reproduction) than between parasites and intermediate and/or paratenic hosts (Carreno et al., 1997; Martínez-de la Puente et al., 2011; O'Donoghue, 2017).

Our phylogenetic hypothesis supported the view of O'Donoghue (2017); adeleorine parasites would have diversified from ancestral groups of parasites with aquatic or semi-aquatic hosts, because the basal clades *Haemogregarina* and *Dactylosoma* clustered parasites detected in aquatic turtle, amphibian, and fish hosts. It also agrees with Maia et al. (2016) and Hrazdilová et al. (2021): (i) the paraphyly of the genus *Hepatozoon* remains problematic and a more extensive sampling is required to disentangle the phylogenetic

relationships within the Adeleorina. (ii) Particularly, more parasites infecting snake and rodent hosts seem to be important for a better resolution of some of the clades because they are common hosts of adeleorine parasites (Smith, 1996; Karadjian et al., 2015). Nevertheless, (iii) other hosts such as chameleons, skinks, geckoes and other lizards, and crocodylians, but also marsupials, birds, chelonians, and amphibians from different parts of the world are hosts of adeleorines that have unresolved relationships in the tree (e.g., Gutiérrez-Liberato et al., 2021). Furthermore, the genera *Cyrilia* and *Desseria* remain to be genetically characterized, which might contribute to solving some phylogenetic uncertainties (Maia et al., 2016). According to previous authors, the analysis of longer sequences and/or alternative genetic markers can also contribute to a better resolution within some of the clades (e.g., Cook et al., 2016; Hrazdilová et al., 2021).

In summary, ninety-one per cent of the adeleorine blood parasites detected in the current survey, including *K. bicipsulatus*, clustered together with *K. lacazei* and *K. galloti* and thus are considered *Karyolysus*. Given that the genera *Schellackia* and *Lankesterella* are less frequent blood parasites of lacertid hosts in the Iberian Peninsula, our analyses indicate that *Karyolysus* is the dominant genus of blood parasites in Iberian lacertids (Megía-Palma et al., 2014, 2018; Drechsler et al., 2021). In support of this view, only one of the newly sampled adeleorine blood parasites found in five Iberian lizards of a single genus (0.7% of the samples from Iberia) clustered in a clade different from *Karyolysus*. This reveals a total of four genera of protozoan blood parasites in Iberian lacertid hosts: *Karyolysus*, *Hepatozoon*, *Schellackia*, and *Lankesterella*. The life cycle of *Karyolysus*, which does not include sexual reproduction in the lizard hosts, may explain the lack of evidence for host-parasite co-speciation (Carreno et al., 1997). In this sense, future studies should include co-phylogenetic analyses between mite vectors of the genera *Ophionyssus* and *Karyolysus*, which might reveal an important diversification driver of this common blood parasite of Iberian lacertids.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpara.2022.12.006>.

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