Phylogenetic analysis based on 18S rRNA gene sequences of *Schellackia* parasites (Apicomplexa: Lankesterellidae) reveals their close relationship to the genus *Eimeria*

R. MEGÍA-PALMA[1]*, J. MARTÍNEZ[2] *and* S. MERINO[1]

[1] *Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, J. Gutiérrez Abascal, 2. E-28006, Madrid, Spain*
[2] *Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Alcalá de Henares, Alcalá de Henares, E-28871, Madrid, Spain*

(*Received 30 November 2012; revised 22 February and 13 March 2013; accepted 30 March 2013; first published online 4 June 2013*)

SUMMARY

In the present study we detected *Schellackia* haemoparasites infecting the blood cells of *Lacerta schreiberi* and *Podarcis hispanica*, two species of lacertid lizards from central Spain. The parasite morphometry, the presence of a refractile body, the type of infected blood cells, the kind of host species, and the lack of oocysts in the fecal samples clearly indicated these blood parasites belong to the genus *Schellackia*. Until now, the species of this genus have never been genetically characterized and its taxonomic position under the Lankesterellidae family is based on the lack of the exogenous oocyst stage. However, the phylogenetic analysis performed on the basis of the 18S rRNA gene sequence revealed that species of the genus *Schellackia* are clustered with *Eimeria* species isolated from a snake and an amphibian species but not with *Lankesterella* species. The phylogenetic analysis rejects that both genera share a recent common ancestor. Based on these results we suggest a revision of the taxonomic status of the family Lankesterellidae.

Key words: Eimeriidae, haplotypes, haemococcidia, *Lacerta schreiberi*, lizard, phylogeny, *Podarcis hispanica*, taxonomy.

INTRODUCTION

Due to the few works published characterizing at the molecular level apicomplexan parasites from reptiles, it is not rare that relationships of many of these protozoan species are unresolved (Smith, 1996; Tenter *et al*. 2002; Jirku *et al*. 2009; Morrison, 2009). In this sense, the haemococcidia group is a paradigmatic example. According to Telford (2008), haemococcidians include three different genera, *Lankesterella* (Labbé, 1899), *Schellackia* (Reichenow, 1919) and *Lainsonia* Landau, 1973, under the Family *Lankesterellidae*, although Upton (2000) considers *Lainsonia* as a synonym of *Schellackia*. Lankesterellids are considered closely related to the intestinal parasites belonging to the Eimeriidae family (Telford, 2008), and parasites of the genus *Lankesterella*, the only genus from the family Lankesterellidae for which molecular data exist to date, falls within the Eimeriidae in recent molecular phylogenies (Barta, 2001; Barta *et al*. 2001; Jirku *et al*. 2009; Morrison, 2009; Ghimire, 2010). Biologically, gametogony and sporogony processes are similar in both haemococcidians and intestinal coccidians except in the absence of sporocyst formation in lankesterellids (Telford, 2008). However, in the intestinal coccidians the infective stages are the oocysts expelled in feces whereas in the haemococcidians the sporozoites leave the oocysts at intestinal level, pass to the bloodstream where they penetrate blood cells and then are ingested by acarine, dipteran or hirudinean haematophagous animals acting as passive vectors (Upton, 2000). At least for saurian hosts, the transmission is finally accomplished by predation of the infected invertebrate (Telford, 2008).

Traditionally the genera *Schellackia* and *Eimeria* have well-demarcated taxonomic boundaries based on their life cycles and their modes of transmission and, therefore, they have been clustered into different families (Lankesterellidae and Eimeriidae). However, there is an increasing consensus that life cycle or host associations may not reflect the evolutionary history within the Apicomplexa (Moore and Willmer, 1997; Barta, 2001). This fact, together with the scarcity of differential phenotypic traits, stimulated the use of molecular phylogenetics based on molecular data to shed light on the relationships within apicomplexan parasites (Barta, 2001; Merino *et al*. 2006; Jirku *et al*. 2009; Morrison, 2009). In this sense, recent phylogenetic analyses have shown that the genus *Eimeria* does not form a monophyletic group (Jirku *et al*. 2009; Morrison, 2009) and the term *Eimeria sensu*

* Corresponding author: Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales-CSIC, José Gutiérrez Abascal 2, E-28006 Madrid, Spain. E-mail: rodrigo.megia@mncn.csic.es

doi:10.1017/S0031182013000553

lato had been proposed for this group (Jirku *et al*. 2009). Other authors, highlighting the importance of the use of monophyletic clades in taxonomy, go even further, suggesting the 'phylogenetic destruction' of the genus *Eimeria* due to its paraphyly (see Morrison, 2009).

The life cycle of *Schellackia* lacks exogenous stages (Bristovetzky and Paperna, 1990), so identification of these parasites relies solely on detection and characterization of endogenous stages. On the other hand, little is known about the morphology of the endogenous stages of most *Eimeria* species apart from the characteristic oocysts released in feces (Upton, 2000; Atkinson *et al*. 2008). Although the occurrence of extra-intestinal stages in some species from the genus Eimeria have been previously reported (Mottalei *et al*. 1992; Carpenter, 1993; Ghimire, 2010 and references therein), these parasitic stages are unknown in more than 98% of all described species (Duszynski and Wilber, 1997; Ghimire, 2010). Interestingly, the infective blood stages of *Schellackia* are morphologically similar to certain extra-intestinal stages present in some species of Eimeria (Paperna and Ostrovska, 1989, see discussion below). However, contrary to the case in *Schellackia, Eimeria* parasites have never been detected in blood cells. These data are based on few observations because there are only 12 named species of *Schellackia* (Upton, 2000) and studies on the *Eimeria* genus are mainly based on the analysis of exogenous oocysts (Duszynski and Wilber, 1997; Alyousif *et al*. 2005; Jirku *et al*. 2009; Ghimire, 2010; Daszak *et al*. 2011). Other coccidian genera possessing blood stages in their life cycles are *Isospora* and *Atoxoplasma,* both isolated from leukocytes of passerine birds (Atkinson *et al*. 2008).

Although molecular analysis of *Eimeria* from diverse hosts (e.g. mammals, birds, amphibians and reptiles) has been carried out from fecal stages (Honma *et al*. 2007; Jirku *et al*. 2009; Power *et al*. 2009), there has been no molecular analysis of *Schellackia* which is characteristic of lizards. In the present study we describe the morphology of *Schellackia* hemoparasites in lizards from the Iberian Peninsula and, for the first time, carry out a molecular phylogenetic analysis.

MATERIALS AND METHODS

Lizard sampling

In total, 115 (78 in 2011 and 37 in 2012) Schreiber's green lizards (*Lacerta schreiberi* Bedriaga, 1878) were collected in a deciduous forest in Segovia (Spain) by noosing and hand from early spring to late summer. This is the only period when lizards are available for study because they enter hibernation for the remaining part of the year (Marco, 2011). *Lacerta schreiberi* is a dimorphic midsize lacertid endemic to the Iberian Peninsula (Portugal and Spain) inhabiting humid forests and linked to streams (Marco, 2011). Adult male snout-to-vent length (SVL) averaged 96·19 ± 7·59 (80–113) mm, $n = 42$ and adult female SVL averaged 104·04 ± 9·68 (84–123) mm, $n = 25$ in this population in 2011. In addition, 7 *Podarcis hispanica* were captured in the same area. *Podarcis hispanica* is a facultative rock-dweller midsize lacertid lizard with SVL 38–70 mm in males and SVL 37–67 mm in females (see Salvador, 1997).

Blood sampling

Blood samples were taken from the ventral vein at the base of the tail (Salkeld and Schwarzkopf, 2005) by puncture using a syringe needle (BD Microlance 3; 23G: 0·6 × 25 mm). The skin around the area of puncture was previously cleaned with ethanol 96% to avoid potential fecal contamination. Blood was collected with the help of a heparinized capillary tube. Two samples were obtained from each lizard: blood smears were made from one drop of the sample, while the remaining blood was preserved in Whatman FTA Classic Cards (FTA® Classic Card, Cat. No. WB12 0205). The FTA cards were stored in plastic bags with silica gel for later DNA extraction. All blood smears where immediately air dried and later, within the same day, fixed with absolute methanol (Svahn, 1975). At the end of the field season, all blood smears were stained with Giemsa stain (1/10 v/v) for 45 min. Slides were examined for haemoparasites following Merino and Potti (1995) and were double-checked in the few cases when we found differences in results between microscopic and molecular analyses (see Results). The intensity of infection in the sample was calculated counting the total number of cells infected per 10 000 erythrocytes divided by the number of infected individuals (Stuart-Fox *et al*. 2009). In the 3 cases where we obtained intensities of less than 1 parasite per 10 000 erythrocytes intensity was considered as 0·5 parasites per 10 000 erythrocytes. The prevalence of infection in the population was calculated as the percentage of individuals infected. Pictures of parasites were taken with an adjustable camera for microscope (Olympus SC30) incorporated with a microscope U-CMAD3 (Olympus, Japan). Length and width of the intracellular parasites were measured with the MB-ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/).

Fecal samples

In 2011, 19 fecal samples were directly collected into plastic vials (2 mL) from the cloaca of those lizards defecating spontaneously during handling. The feces were stored at −80 °C. These samples were exclusively used to perform molecular analysis

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

(see Molecular methods). During the field season of 2012, individual lizards were radiotracked by supplying them with small transmitters (BD-2 transmitters, 1·4 g; Holohil Systems Ltd., Ontario, Canada) allowing us to capture every lizard at least 3 times during a period of 24 days, thus obtaining different fecal samples from the same individual. At every capture we obtained systematically fecal samples from all individuals by briefly massaging the belly of the lizards and collecting the sample directly from the cloaca as indicated above. Following this method we collected 124 fecal samples from 37 individuals. In this way we increased the chances of detecting coccidian oocysts from individual lizards because shedding is not continuous and depends on several factors (López *et al*. 2007). In 2012, fecal samples were stored in 2% potassium dichromate for at least 48 h to allow the sporulation of oocysts and thereafter were subjected to concentration by flotation in 15 mL of sugar solution prior to microscopic examination in search of oocysts (Duszynski and Wilber, 1997). We could not obtain fecal samples from *P. hispanica*.

DNA extraction and PCR

We extracted parasite DNA from blood preserved on FTA cards corresponding to lizards captured in 2011 by applying the following protocol: FTA punches were transferred to collection vials with 250 μL of SET buffer (0·15 M NaCl, 0·05 M Tris, 0·001 M EDTA, pH = 8). Immediately, SDS 20% (7 μL) and proteinase K (50 μg) were added to the vials and incubated at 55 °C overnight using a thermo-shaker. The next day, ammonium acetate 5 M (250 μL) was added to the vials and incubated for 30 min at room temperature. Subsequently, vials were centrifuged at 13 000 ***g*** for 10 min. After removing the pellet, DNA was precipitated with ethanol and re-suspended in sterile water. DNA of the fecal samples was extracted using the UltraClean® Fecal DNA Isolation Kit (Mo Bio Laboratories, Inc).

Due to the lack of previous genetic information for *Schellackia* parasites we first tried partial amplification of the 18S rRNA gene sequence using primers for other haemococcidians as hep900F (5′ GTC AGA GGT GAA ATT CTT AGA TTT G 3′)/hep1615R (5′ AAA GGG CAG GGA CGT AAT C 3′) or hep50F (5′ GAA ACT GCG AAT GGC TCA TT 3′)/hep1600R (5′ AAA GGG CAG GGA CGT AAT CGG 3′) (see Merino *et al*. 2006). In order to obtain a larger fragment or to perform internal readings the primers hep600F1 (5′ TCG TAG TTG GAT TTC TGT CG 3′), EIMROD-R (5′ GCA TTT CCC TAT CTC TAG TCG G 3′) and Isosp-R (5′ ATT GCC TCA AAC TTC CTT GC 3′) were designed on the basis of the first sequences obtained. The primer BT-F1 (5′ GGT TGA TCC TGC CAG TAG T3′) was used in the same way (Criado-Fornelio *et al*. 2003).

To perform a systematic and specific screening of the blood samples, we used the primers hep600F1/hep1600R (~ 1000 bp). As the quality of the DNA extracted from fecal samples is lower than that extracted from blood samples, we facilitated the amplification using the primers hep600F1 and Isosp-R which yield a shorter amplicon (*c*. 800 bp). PCR reaction volume (20 μL) contained between 20 and 100 ng of template DNA, 50 mM KCl, 10 mM TRIS–HCl, 1·5 $MgCl_2$, 0·05 mM of each dNTP, 0·5 M of each primer, and 1·25 U of AmpliTaq Gold 360 (Applied Biosystems, Foster City, CA). The reactions were cycled under the following conditions using the Verity thermal cycler (Applied Biosystems): 95 °C for 10 min (polymerase activation), 40 cycles at 95 °C for 30 s, annealing temperature at 58 °C for 30 s, 72 °C for 80 s and a final extension at 72 °C for 10 min. All amplicons were sequenced to discriminate the haplotypes.

Sequences of *Schellackia* haplotypes were deposited in GenBank under the following accession numbers: haplotype Ls-A: JX984674; haplotype Ls-B: JX984675; haplotype Ph-B4: JX984676.

Phylogenetic analysis

DNA sequences were obtained from the Acacia Website (David Morrison, http://acacia.atspace.eu/Alignments.htm) where Whipps *et al*. (2012) deposited a nexus file containing most of the Eimeriorina species (454 sequences) which were initially aligned by secondary structure following the model of Gutell *et al*. (1994). Thereafter, the alignment was refined and manually optimized as indicated by Whipps *et al*. (2012). To perform the phylogenetic analyses we only used sequences belonging to the families Lankesterellidae and Eimeriidae (194 sequences). In order to decrease redundancy of the alignment, we suppressed all sequences with identity 99%, or higher, using the program JALVIEW (Waterhouse *et al*. 2009). In addition, 3 sequences of *Lankesterella* and the 3 sequences of *Schellackia* were manually aligned on this file. The final alignment contained 67 sequences. Poorly aligned positions and divergent regions of the alignment were suppressed using GBlocks program (Talavera and Castresana, 2007) selecting the following options: 'Minimum Number of Sequences for a Flank Position' to 34, 'Maximum Number of Contiguous Nonconserved Positions' to 10, 'Minimum Length of a Block' to 5, and 'Allowed Gap Positions' to 'With Half'. The GBlocks program suppressed 18% of ambiguous sites. The final alignment (1626 bp) was analysed using both Bayesian and maximum-likelihood inference. Bayesian inference was performed using the program MrBayes v3.2 (Ronquist and Huelsenbeck, 2003).

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

We used a single partition with the GTR + I + G substitution model. This analysis consisted of 2 runs of 4 chains each, with 6 000 000 generations per run and a burn-in of 600 000 generations (108 000 trees for consensus tree). The final standard deviation of the split frequencies was lower than 0·01. Convergence was checked using the Tracer v1.5 software (Rambaut and Drummond, 2007). All model parameters were higher than 100. On the other hand, the maximum-likelihood inference was performed using the PhyML program (Guindon *et al*. 2010). The substitution model used was GTR + I + G, the subtree pruning and regrafting (SPR) and the nearest-neighbour interchange (NNI) tree-rearrangements were selected, and the approximate likelihood-ratio test (aLRT) was used to obtain the clade support.

RESULTS

Blood smears

In 2011, the prevalence of *Schellackia* sp. in blood smears was 29·5% (23/78) and the mean intensity per 10 000 erythrocytes was 8·9 parasites. All the blood samples that were positive by PCR scanning were also positive by microscopy after checking again 3 smears found negative in a first check. These 3 smears showed low parasitaemia (< 1/10 000; see microscopic methods in 'Blood sampling' above). The sporozoites of *Schellackia* sp. were always found in erythrocytes but in some parasitized individuals (21·7%, 5 of 23 infected animals) parasites were also detected in leukocytes as lymphocytes, monocytes, thrombocytes and azurophyles (Fig. 1, E–F and K–O). They were never detected in granulocytes.

The general shape of the sporozoites of *Schellackia* sp. in *L. schreiberi* varied from elongated to rounded. In some cases, a single refractile body can be seen in the cytoplasm. The measures of the sporozoites infecting erythrocytes and leukocytes are shown in Table 1. The nucleus of the sporozoites infecting erythrocytes showed a variety of shapes and placements (see Fig. 1). Forty eight per cent of sporozoites showed nucleus touching the parasite membrane in the pointed end of the parasite as two narrow bands one at each side of the parasite (see Fig. 1I) or occupying a broad area only in one side of the parasite (see Fig. 1B–D). The other 52% of the sporozoites showed a band-like nucleus usually situated closer to the broader end of the sporozoite (see Fig. 1G, J, K and M). Parasites showed a soft-stained cytoplasm in 70·5% of the cases (Fig. 1, see G), being darker in the rest of the parasites (Fig. 1, see D).

In 2012 we only conducted microscopic examination of blood samples and 17 out of the 37 individuals of *L. schreiberi* sampled (45·9%) were found infected by sporozoites of *Schellackia* sp. The mean intensity was 7·2 parasites/10 000 erythrocytes. Parasites in this year showed similar characteristics to those found in 2011.

In addition, we found one individual of *Podarcis hispanica* (14·28% (1/7)) infected by *Schellackia*. In this case parasites were always found inside erythrocytes. We only found 5 parasites in the whole slide (more than 50 000 cells scanned). The size of these parasitic stages was 5·94 ± 0·73 (5·1–6·77) μm in length and 2·98 ± 0·35 (2·56–3·32) μm in width ($n = 5$) and there was a lack of a visible refractile body in the cytoplasm. They had a pyriform shape, and the nucleus appeared in contact with the cytoplasmic membrane. The cytoplasm was dark stained (see Fig. 1, P–R).

Genetic analysis of blood samples

Overall, 125 blood samples from 78 *L. schreiberi* captured during the year 2011 were analysed by means of PCR, being 29·5% (23/78) positive for *Schellackia*. All samples from the same individual yielded the same result. All amplicons were sequenced to discriminate the haplotypes. Sequencing of these amplicons revealed the occurrence of two different haplotypes (named as Ls-A and Ls-B) whose genetic identity was 99·2%. The haplotypes Ls-A and Ls-B were detected in 17·9% (14/78) and 11·5% (9/78) of the lizards sampled, respectively. We never found both haplotypes infecting the same individual as indicated by the lack of double peaks in the chromatograms from sequences obtained (see Discussion). The BLAST analysis indicated that both haplotypes are close to *Eimeria arnyi* (see Fig. 2). In addition, we detected in blood samples of *P. hispanica* a haplotype showing a high similarity with those isolated from *L. schreiberi* and identical to that recently reported in Portugal for the same host species (Harris *et al*. 2012). Specifically we amplified a DNA fragment of 1544 pb (haplotype Ph-B4 with GenBank accession number: JX984676) while Harris *et al*. (2012) report a 621 pb fragment (haplotype 667PhPO with Gen Bank accession number: JQ762306). This latter isolate corresponds to the fragment comprised between the position 256 and the position 876 in our isolate Ph-B4.

In relation with haplotype Ls-A, only 4 sporozoites were found infecting leukocytes (2 infecting lymphocytes, 1 infecting a monocyte, and 1 infecting an azurophyle; Fig. 1, E–F). However, haplotype Ls-B appears to infect white blood cells more frequently than haplotype Ls-A (haplotype A: 5·9%, $n = 68$; haplotype B: 48·9%, $n = 47$; difference between two proportions $P < 0.001$). Exceptionally, and always in samples containing the haplotype Ls-B, we detect azurophyles infected with 2 parasites. In these cases, the cytoplasm of the parasites appears more stained than in single infections in leukocytes (Fig. 1, N–O).

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

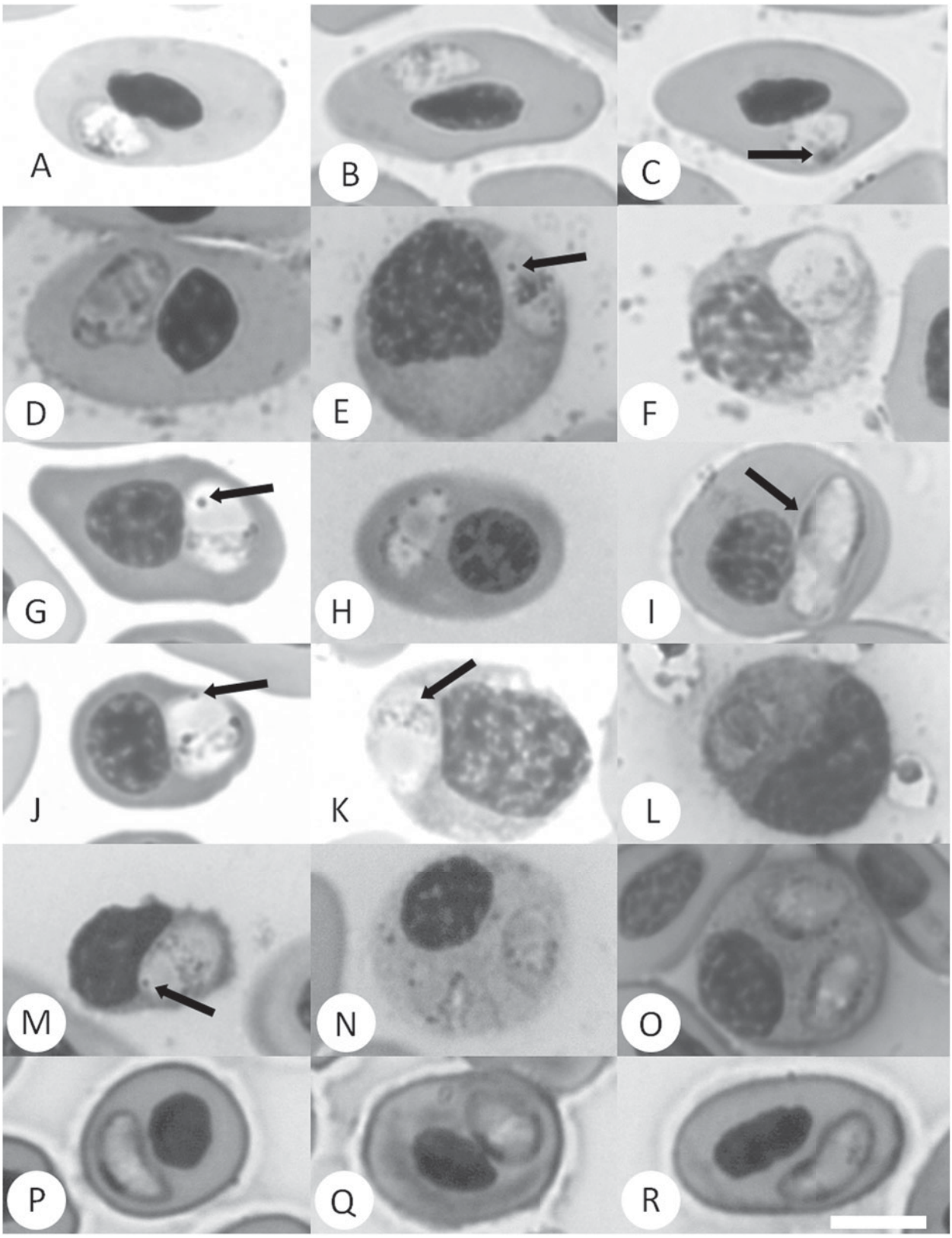

Fig. 1. *Schellackia* sporozoites infecting blood cells of *Lacerta schreiberi* and *Podarcis hispanica*. Haplotype Ls-A of *L. schreiberi* (A–F). Infecting both erythrocytes (A–C) and leukocytes (D–F). Haplotype Ls-B of *L. schreiberi* (G–O). Infecting erythrocytes (G–J) and leukocytes (K–O). Haplotype Ph-B4 infecting erythrocytes in *P. hispanica* (P–R). Black arrows in E, G, J and M indicate single refractile body; in C and I indicate bands of chromatine along the side of the cell; in K shows a band-like nucleus. All the pictures are shown at the same scale. Scale bar = 5 μm.

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

Table 1. Average length and width of *Schellackia* sp. parasites of different haplotypes infecting different types of blood cells in *Lacerta schreiberi* and *Podarcis hispanica*

	Erythrocytes				Leukocytes			
	Mean	S.D.	Range	*n*	Mean	S.D.	Range	*n*
Haplotype Ls-A								
Length	5·31	0·63	6·83–3·95	64	6·89	0·72	7·63–5·84	4
Width	3·41	0·52	4·77–2·33		4·62	0·86	5·83–3·71	
Haplotype Ls-B								
Length	5·61	0·77	8·45–4·66	24	5·77	0·72	8·14–4·82	23
Width	3·30	0·58	4·38–2·19		3·66	0·63	4·92–2·57	
Haplotype Ph-B4								
Length	5·94	0·73	5·10–6·77	5	–	–	–	–
Width	2·98	0·35	2·56–3·32		–	–	–	–

No relationship has been found between the shape of blood stages and particular haplotype.

Statistical analyses comparing the length and width of parasites corresponding with haplotypes Ls-A and Ls-B do not show significant differences (one-way ANOVA; length: $F_{1,110}=3·787$, $P=0·054$; and width: $F_{1,110}=0·004$, $P=0·947$). When comparing parasites infecting erythrocytes and leukocytes from both haplotypes, significant differences emerge (one-way ANOVA; length: $F_{3,108}=8·087$, $P<0·001$; and width: $F_{3,108}=6·869$, $P<0·001$). A *posteriori* Tukey tests show that parasites from haplotype Ls-A infecting leukocytes are longer and wider than parasites from both haplotypes when infecting erythrocytes and from haplotype Ls-B infecting leukocytes. In addition, parasites corresponding to haplotype Ls-A infecting erythrocytes are shorter than parasites from haplotype Ls-B infecting leukocytes ($P<0·05$ in all cases).

Genetic analysis of fecal samples

The 19 fecal samples from 2011 were analysed by means of PCR. Six amplicons were obtained and sequenced. The BLAST analysis showed that 4 of them were related with the genus *Eimeria* (GenBank accession numbers: KC574076, KC574077, KC574078, KC574079) and the phylogenetic analysis grouped them within the major clade of the Eimeriidae. The other two amplicons corresponded to the genera *Adelina* (GenBank accession number: KC574080) and to an unidentified Apicomplexa (GenBank accession number: KC574081). In 2012, we found sporulated oocysts of coccidians in samples from 7 individuals (7/37) by microscopical examination of fecal samples (see Methods). Four of these cases correspond to *Adelina* oocysts and the DNA sequences from the other 3 revealed that they belong to *Eimeria* species but related to species isolated from mammals (thus probably pseudoparasites). In other words, the haplotypes obtained from these fecal parasitic stages do not correspond with those obtained from blood parasites.

Phylogenetic analysis

The Bayesian and maximum-likelihood inferences (ML) showed that the species of *Schellackia* detected in the present study form a well-supported group on its own, closely related to *E. arnyi* and *Eimeria ranae* (GenBank sequence for *E. arnyi* was obtained from oocysts isolated from feces of the prairie ringneck snake *Diadophis punctatus arnyi* (Colubridae), GenBank accession number AY613853, while GenBank sequence for *E. ranae* was obtained from oocysts isolated from feces of the European common frog *Rana temporaria* (Ranidae); GenBank accession number EU717219; see Fig. 2). In addition, the haplotype detected in *P. hispanica* showed a close relation with the haplotype Ls-B from *L. schreiberi*. The topology of the phylogenetic tree indicates that the group including *Schellackia* and the clade grouping *Lankesterella* and *Caryospora* are well supported but they do not share a recent common ancestor. As shown in Fig. 2, *Lankesterella*, but not *Schellackia*, clusters with the clade containing the majority of Stieda body-bearing eimeriids infecting birds and mammals, which is sister to the *Lankesterella–Caryospora* clade (81%).

DISCUSSION

In the present study we genetically characterized *Schellackia* species for the first time. The phylogenetic analysis shows that they form a monophyletic cluster together with species of *Eimeria* isolated from a snake (*D. punctatus arnyi*) and an amphibian (*R. temporaria*). The clade where the genus *Schellackia* is placed and the clade containing species of *Lankesterella* are both robustly supported and clearly separated. However, they do not share a recent common ancestor. This fact reveals that the lack of the exogenous oocyst is a characteristic that emerged independently in these two haemococcidian genera, *Lankesterella* and *Schellackia*.

Parasites detected in *L. schreiberi* were identified as *Schellackia* species based on several factors. These

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

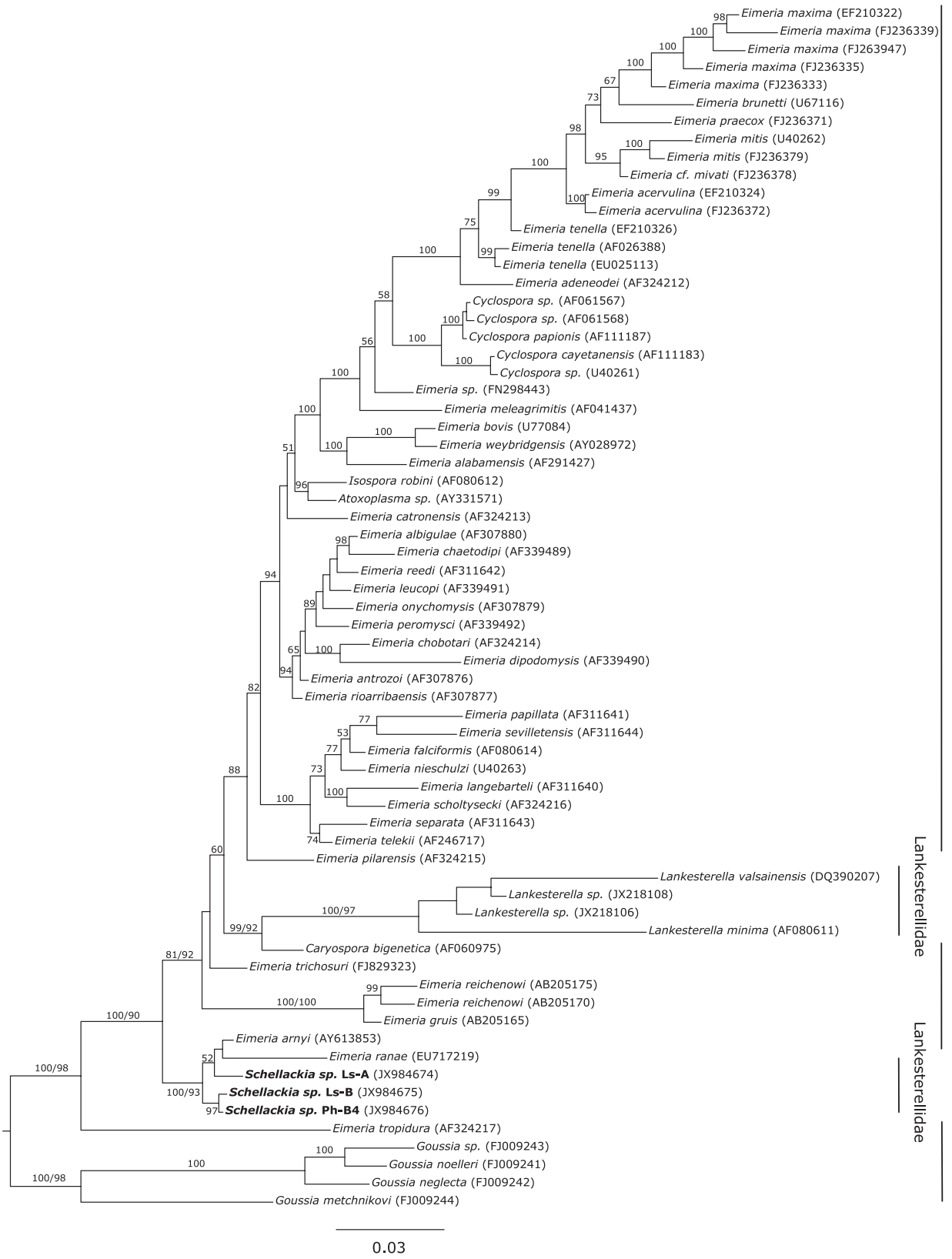

Fig. 2. Bayesian inference using the GTR + G + I substitution model. This analysis consisted of 2 runs of 4 chains each, with 6 000 000 generations per run and a burn-in of 600 000 generations (108 000 trees for consensus tree). All branches were maintained but support values less than 50% were suppressed. All support values are percentages. Where two numbers are shown in the branch, the first one indicates the supporting value achieved by Bayesian inference and the second one by maximum-likelihood inferences (ML). The ML inference was performed using PhyML program selecting the GTR + I + G substitution model. The approximate likelihood-ratio test (aLRT) was used to obtain the clade support. The length of the alignment was 1626 bp (1208 conserved residues, 418 variables and 188 singletons). Parasite families are shown on the right and follow Upton (2000).

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

factors are morphological characteristics (see Telford, 2008) but also the different types of cells infected, which include mainly erythrocytes but also leukocytes. Furthermore they were not detected in granulocytes as previously described for some species of the genus *Schellackia* (Telford, 1993, 2008). The type of host is also an important feature as *Schellackia* is recognized as a specific parasite of lizards. However, the fact that these parasites appear closely related to *Eimeria* species isolated from *D. punctatus arnyi* and *R. temporaria* (see Fig. 2), may mean that *Schellackia* parasites observed in the smears from 2011 correspond to blood stages of a novel *Eimeria* species infecting blood of *L. schreiberi*. The *Eimeria* species of *D. punctatus arnyi* and *R. temporaria* were genetically characterized from the oocyst stages expelled with feces (Upton and Oppert, 1991; Jirku *et al*. 2009), a typical phase in *Eimeria* species but absent in *Schellackia* species. Thus in 2012, we reanalysed both blood and fecal samples collected from *L. schreiberi*. However, and as expected for a *Schellackia* species, none of the fecal oocysts detected corresponded to the DNA fragment obtained from blood parasites. In every case where we detect coccidian oocysts by flotation techniques we also were able to amplify their DNA. This fact implies that the PCR performed in fecal samples was sensitive enough to detect coccidian parasites. Therefore we can be relatively confident of the absence of exogenous oocysts corresponding with parasites detected in blood as expected for a *Schellackia* species.

The genetic characterization of the sporozoites found in *L. schreiberi* showed 2 haplotypes whose genetic identity was 99·2%. Both haplotypes were mainly found in erythrocytes although in a few hosts, parasites were detected in both erythrocytes and leukocytes. Regardless of the type of cell parasitized, we never detected both haplotypes in the same host. However, this should be treated cautiously because the sequences do not come from cloned amplicons. The lack of double peaks in the chromatograms at least indicates that if another haplotype was present in the host its intensity was very low. We found statistically significant differences in size among parasites from different haplotypes and/or infecting different host cell types. These results should be taken cautiously for parasites infecting white cells, because sample size is as low as 4 parasites for haplotype Ls-A. The different haplotypes detected might correspond to different developmental stages of the same parasite (see Telford, 2008), but we observed multiple stages in the same host. In addition, one haplotype (Ls-B) clusters more closely to the *Podarcis* parasite than the other (Ls-A). So it is unlikely that haplotypes Ls-A and B come from the same species. It is surprising that both haplotypes never appear coinfecting the same host, although this may be indicative of a competitive exclusion between both parasites. Our parasite sequence from *Podarcis* is identical to a previous one that was labelled as *Eimeria* (see Harris *et al*. 2012), which emphasizes the importance of obtaining both molecular and morphological data when identifying a parasite. The absence of a visible refractile body in all of the sporozoites of the haplotype PhB4, the pyrimorphic shape presented by these sporozoites, and the bluish stain reaction of the cytoplasm of parasites found in the slide from *P. hispanica* are traits that could be indicative of parasites recently reaching blood cells (Lainson *et al*. 1976). The only previous species of *Schellackia* found in the Iberian Peninsula is *Schellackia bolivari* Reichenow 1919 which is known from lacertids of different genera, i.e. *Acanthodactylus* and *Psammodromus* (Telford, 2008). However, *S. bolivari* appears to be a different species to those found in the present study because they differ with respect to the number of refractile bodies and the sort of cells infected. Molecular characterization of *S. bolivari* will help ascertain if they are really different species because some variation in morphometrics within the same protozoan species infecting different hosts could occur (see for example Merino *et al*. 2012).

Our results, based on the 18S rRNA gene sequences, indicate that *Schellackia* species form a monophyletic group together with *E. ranae* and *E. arnyi*. This fact may indicate (i) the occurrence of haematic stages in those two species of *Eimeria* or (ii) that other unknown species of *Schellackia* have an exogenous oocyst stage. On the basis of the phylogenetic analysis, Lankesterellidae is not a monophyletic family. Thus the lack of exogenous oocysts is a characteristic arising independently for different lankesterellid parasites. Molecular characterization of *S. bolivari*, the type species of the genus, and the study of its phylogenetic position will allow the definite elucidation of the phylogenetic affinity of this parasite genus.

ACKNOWLEDGEMENTS

The Spanish Ministerio de Ciencia e Innovacion provided financial support for the research presented (project CGL2009-09439 to S. M. and J. M. and grant number BES-2010-038427 to R. M.). We also want to thank three anonymous reviewers for their helpful comments and Centro de Montes y Aserradero de Valsaín in Segovia for permission to work in the Monte Matas of Valsaín. El Ventorrillo field station provided logistical support. Authorization to capture and handling of lizards was obtained from corresponding authorities.

REFERENCES

Alyousif, M. S., Al-Shawa, Y. R. and Mubarak, M. (2005). Light and electron microscopy of gall bladder epithelium of the gray monitor, *Varanus griseus*, infected with *Eimeria griseus* (Apicomplexa: Eimeriidae). *Journal of Biological Sciences* **5**, 542–546.

Atkinson, C. T., Thomas, N. J. and Hunter, D. B. (2008). *Parasitic Diseases of Wild Birds*, pp. 108, 162. Wiley–Blackwell, Ames, IA, USA.

https://doi.org/10.1017/S0031182013000553 Published online by Cambridge University Press

Barta, J. R. (2001). Molecular approaches for inferring evolutionary relationships among protistan parasites. *Veterinary Parasitology* **101**, 175–186.

Barta, J. R., Martin, D. S., Carreno, R. A., Siddall, M. E., Profous-Juchelka, H., Hozza, M., Powles, M. A. and Sundermann, C. (2001). Molecular phylogeny of the other tissue coccidia: *Lankesterella* and *Caryospora*. *Journal of Parasitology* **87**, 121–127.

Bristovetzky, M. and Paperna, I. (1990). Life cycle and transmission of *Schellackia* cf. *agamae*, a parasite of the starred lizard *Agama stellio*. *International Journal for Parasitology* **20**, 883–892.

Carpenter, J. W. (1993). Infectious and parasitic diseases of cranes. In *Zoo and Wild Animal Medicine: Current Therapy 3* (ed. Fowler, M. E.), pp. 229–237. W. B. Saunders, Philadelphia, PA, USA.

Criado-Fornelio, A., Martínez-Marcos, A., Buling-Saraña, A. and Barba-Carretero, J. C. (2003). Presence of *Mycoplasma haemofelis, Mycoplasma haemominutum* and piroplasmids in cats from southern Europe: a molecular study. *Veterinary Microbiology* **93**, 307–317.

Daszak, P., Ball, S. J., Streicker, D. G. and Snow, K. R. (2011). A new species of *Eimeria* (Apicomplexa: Eimeriidae) from the western hognose snake, *Heterodon nasicus* (Serpentes: Xenodontidae), from Texas. *Journal of Parasitology* **97**, 463–465.

Duszynski, D. W. and Wilber, P. G. (1997). A guideline for the preparation of species descriptions in the Eimeriidae. *Journal of Parasitology* **83**, 333–336.

Ghimire, T. R. (2010). Redescription of genera of family Eimeriidae Minchin, 1903. *International Journal of Life Sciences* **4**, 26–47.

Guindon, S., Dufayard, J. F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

Gutell, R. R., Larsen, N. and Woese, C. R. (1994). Lessons from an evolving rRNA: 16 S and 23 S rRNA structures from a comparative perspective. *Microbiological Reviews* **58**, 10–26.

Harris, J., Joao, P. M., Maia, C. and Perera, A. (2012). Molecular survey of Apicomplexa in Podarcis wall lizards detects Hepatozoon, Sarcocystis, and *Eimeria* species. *Journal of Parasitology* **98**, 592–597.

Honma, H., Yokoyama, T., Inoue, M., Uebayashi, A., Matsumoto, F., Watanabe, Y. and Nakai, Y. (2007). Genetical identification of coccidia in red-crowned crane, *Grus japonensis*. *Journal of Parasitology Research* **100**, 637–640.

Jirku, M., Jirku, M., Oborník, M., Lukes, J. and Modrý, D. (2009). A model for taxonomic work on homoxenous coccidia: redescription, host apecificity, and molecular phylogeny of *Eimeria ranae* Dobell, 1909, with a review of Anuran-host *Eimeria* (Apicomplexa: Eimeriorina). *Journal of Eukaryotic Microbiology* **56**, 39–51.

Lainson, R., Shaw, J. J. and Ward, R. D. (1976). *Schellackia landauae* sp. nov. (Eimeriorina: Lankesterellidae) in the Brazilian *Polychrus marmoratus* (Iguanidae): experimental transmission by *Culex pipiens* fatigans. *Parasitology* **72**, 225–243.

López, G., Figuerola, J. and Soriguer, R. (2007). Time of day, age and feeding habits influence coccidian oocyst shedding in wild passerines. *International Journal for Parasitology* **37**, 559–564.

Marco, A. (2011). Lagarto verdinegro – *Lacerta schreiberi*. In *Enciclopedia Virtual de los Vertebrados Españoles* (ed. Salvador, A. and Marco, A.). Museo Nacional de Ciencias Naturales, Madrid. http://www.vertebradosibericos.org/.

Merino, S. and Potti, J. (1995). High prevalence of hematozoa in nestlings of a passerine species, the pied flycatcher, *Ficedula hypoleuca*. *Auk* **112**, 1041–1043.

Merino, S., Martínez, J., Martínez-de la Puente, J., Criado-Fornelio, A., Tomás, G., Morales, J., Lobato, E. and García-Fraile, S. (2006). Molecular characterization of the 18S rRNA gene of an avian *Hepatozoon* reveals that it is closely related to *Lankesterella*. *Journal of Parasitology* **92**, 1330–1335.

Merino, S., Hennicke, J., Martínez, J., Ludynia, K., Torres, R., Work, T. M., Stroud, S., Masello, J. F. and Quillfeldt, P. (2012). Infection by *Haemoproteus* parasites in four species of Frigatebirds and the description of a new species of *Haemoproteus* (Haemosporida: Haemoproteidae). *Journal of Parasitology* **98**, 388–397.

Moore, J. and Willmer, P. (1997). Convergent evolution in invertebrates. *Biological Reviews* **72**, 1–60.

Morrison, D. A. (2009). Evolution of the Apicomplexa: where are we now? (Review). *Trends in Parasitology* **25**, 375–382.

Mottalei, F., Mayberry, L. F. and Bristol, J. R. (1992). Localization of extraintestinal *Eimeria nieschulzi* (Apicomplexa: Eimeriidae) in the rat utilizing an indirect immunofluorescence technique. *Transactions of the American Microscopical Society* **111**, 61–64.

Paperna, I. and Ostrovska, K. (1989). Ultrastructural studies on sporozoite stages of *Schellackia* cf. *agamae* from liver and blood of the starred lizard *Agama stellio*. *Australian Society for Parasitology* **19**, 13–19.

Power, M. L., Richter, C., Emery, S., Hufschmid, J. and Gillings, M. R. (2009). *Eimeria trichosuri*: phylogenetic position of a marsupial coccidium, based on 18S rDNA sequences. *Experimental Parasitology* **122**, 165–168.

Rambaut, A. and Drummond, A. J. (2007) Tracer: MCMC trace analysis tool. *Institute of Evolutionary Biology*, University of Edinburgh, Edinburgh.

Ronquist, F. and Huelsenbeck, J. P. (2003). MrBayes 3: bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Salkeld, D. J. and Schwarzkopf, L. (2005). Epizootiology of blood parasites in an Australian lizard: a mark-recapture study of a natural population. *International Journal for Parasitology* **35**, 11–18.

Salvador, A. (1997). Reptiles. In *Fauna Ibérica*, Vol. 10 (ed. Ramos, M. A. *et al.*). Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain.

Smith, T. G. (1996). The genus *Hepatozoon* (Apicomplexa: Adeleina). *Journal of Parasitology* **82**, 565–585.

Stuart-Fox, D., Godinho, R., Goüy de Bellocq, J., Irwin, N. R., Brito, J. C., Moussalli, A., Siroky, P., Hugall, A. F. and Baird, S. J. E. (2009). Variation in phenotype, parasite load and male competitive ability across a cryptic hybrid zone. *PLoS ONE* **4**, e5677.

Svahn, K. (1975). Blood parasites of the genus *Karyolysus* (Coccidia, Adeleidae) in Scandinavian lizards. Description of the life cycle. *Norwegian Journal of Zoology* **23**, 277–295.

Talavera, G. and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**, 564–577.

Telford, S. R. (1993). A species of *Schellackia* (Apicomplexa: Lankesterellidae) parasitising east and southeast Asian lizards. *Systematic Parasitology* **25**, 109–117.

Telford, S. M. (2008). *Hemoparasites of the Reptilia: Color Atlas and Text*, pp. 261–273. CRC Press, Boca Raton, FL, USA.

Tenter, A. M., Barta, J. R., Beveridge, I., Duszynski, D. W., Mehlhorn, H., Morrison, D. A., Thompson, R. C. A. and Conrad, P. A. (2002). The conceptual basis for a new classification of the coccidia. *International Journal for Parasitology* **32**, 595–616.

Upton, S. J. (2000). Suborder Eimeriorina Léger, 1911. In *The Illustrated Guide to the Protozoa*, 2nd Edn, Vol. 1 (ed. Lee, J. J., Leedale,, G. F. and Bradbury, P.). Allen Press, Lawrence, KS, USA.

Upton, S. J. and Oppert, C. J. (1991). Description of the oocysts of *Eimeria arnyi* n. sp. (Apicomplexa: Eimeriidae) from the eastern ringneck *Diadophis punctatus arnyi* (Serpentes: Colubridae). *Systematic Parasitology* **20**, 195–197.

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. and Barton, G. J. (2009). Jalview Version 2 – a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189–1191. doi:10.1093/bioinformatics/btp033.

Whipps, C. M., Fournie, J. W., Morrison, D. A., Azevedo, C., Matos, E., Thebo, P. and Kent, M. L. (2012). Phylogeny of fish-infecting *Calyptospora* species (Apicomplexa: Eimeriorina). *Parasitology Research* **111**, 1331–1342.