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Phylogenetic relationships of *Isospora*, *Lankesterella*, and *Caryospora* species (Apicomplexa: Eimeriidae) infecting lizards

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Abstract In this study, several species of *Isospora* infecting lizards were genetically characterized. Specifically, five described and four newly described species of *Isospora* were included in a phylogeny of the family Eimeriidae. These species were isolated from hosts originally inhabiting all geographic continents except Europe. Phylogenetic analyses of the *18S* ribosomal RNA (rRNA) gene grouped these nine species of *Isospora* with *Lankesterella* species and *Caryospora ernsti*. Therefore, within this clade, different evolutionary strategies in oocyst development and transmission occurred. Although the characteristic endogenous oocyst development of the genus *Lankesterella* may have arisen only once, the reduction in the number of sporocysts observed in the genus *Caryospora* occurred at least twice during coccidian evolution, as evidenced by the phylogenetic position of *Caryospora bigenetica* as the sister taxon of the group formed by reptilian *Isospora*, *Lankesterella*, and *C. ernsti*. Within this group, *C. ernsti* was the sister taxon to the genus *Lankesterella*. Overall, our results contradict the proposed monophyly of the genus *Caryospora*, highlighting the need for a thorough taxonomic and systematic revision of the group. Furthermore, they suggest that the recent ancestor of the genus *Lankesterella* may have been heteroxenous.

Keywords Coccidian · Evolution · Oocyst · Parasite · Phylogeny · Squamata

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Introduction

The Squamata (Reptilia) have five major genera of Eimeriidae Minchin, 1903, that infect them. These genera are distinguished by the structure of their sporulated oocysts and their life cycles. Specifically, the Squamata host eimeriids with dizoic, tetrasporocyst oocysts that develop on the epithelial surface of the gall bladder or in the microvillous zone of the intestine (i.e., genera *Choeleoeimeria*, *Acroeimeria*, and *Eimeria* (i.s.) sensu Paperna and Landsberg 1989); parasites with single, octozoic sporocyst oocysts with known extraintestinal development, including the formation of fully sporulated oocysts (i.e., genus *Caryospora* Léger, 1904); and parasites with tetrasporozoic, diplosporocystic oocysts (i.e., genus *Isospora* Schneider, 1881). However, the phylogenetic relationships among these groups of parasites remain unknown. In this sense, recent studies have shown that intestinal parasites of the families Lankesterellidae Nöller, 1920, and Schellackiidae Grassé, 1953, with blood stages of transmission in reptile hosts are evolutionarily closely related to genera of the family Eimeriidae (Megía-Palma et al. 2014).

More than 100 species of *Isospora* have been described infecting reptiles around the world, but to date, none have been molecularly characterized (e.g., Finkelman and

Paperna 1994a, b, 1995, 2002; Modrý et al. 1997, 1998, 2004; McQuiston et al. 2001; Abdel-Baki et al. 2013). Therefore, the evolutionary relationships among *Isospora* species infecting reptiles with those infecting birds and mammals are unknown (Carreno et al. 1998; Barta et al. 2005). Here, we molecularly characterized nine *Isospora* species detected in native lizards from four continents. Five of the species correspond to known *Isospora* species, while four are described here for the first time. Furthermore, we molecularly characterized two other apicomplexan parasites isolated from the green anole: *Caryospora ernsti* Upton et al. 1984 and one species of *Lankesterella* Labbé 1899. This study contributes to the unraveling of the phylogenetic relationships between the genera *Isospora*, *Caryospora*, and *Lankesterella* infecting lizards.

Materials and methods

Sample origin and processing

Lizard species in which some isosporoid parasites have already been described were chosen for the present study in order to include the described species in the first phylogeny for these reptile-infecting parasites. Furthermore, other Squamata species were also included because they are suspected coccidian hosts, since related species host parasites of the genus *Isospora* and *Caryospora*. The full list of reptile species studied is shown in Table 1. In an attempt to include representatives of the genus *Isospora* from all geographic continents containing reptiles, we looked for *Isospora* parasites in potential Iberian host species. To date, no *Isospora* species have been described in endemic Iberian reptiles. To have a broad representation of coccidia in the phylogeny, we also included reptile species belonging to different taxonomic families, namely Agamidae, Chamaeleonidae, Colubridae, Gekkonidae, Lacertidae, Opluridae, Polychrotidae, Pythonidae, Scincidae, Sphaerodactylidae, and Trogonophidae. Some fecal samples were obtained directly from recently imported individuals for sale in pet shops. All fecal samples were collected directly from the cloaca with a standard 1.5-ml vial (Eppendorf Tubes® 3810X; Eppendorf Ibérica, Madrid, Spain) filled with 1 ml of 2 % (*w*/*v*) potassium dichromate (Duszynski and Wilber 1997). Reptiles were stimulated to defecate by briefly massaging the belly. To enhance the sporulation of coccidian oocysts in the samples, we adapted the protocol described by Duszynski and Wilber (1997). For a week, vials were opened twice a day for 15 min each and then closed and vortexed, allowing the air to mix with the sample. After a week, the samples were homogenized with a plastic pipette. Some of the sample was taken for microscopic identification of sporulated oocysts. The remaining sample was stored at 4 °C for subsequent molecular characterization. We also took blood samples, following the protocol described by Megía-Palma et al. (2013), from 15 green anoles *Anolis carolinensis* Duméril and Bribon, 1837 (Squamata: Polychrotidae), recently imported from the USA by a pet shop.

Microscopic methods

For the microscopic screening of fecal samples, we followed the standard protocol for parasite concentration using Sheather's sugar flotation technique (Levine 1973). In Table 1, the prevalence (as a percentage) for each surveyed coccidian species is shown. Each sample was screened at ×200 magnification with an optic microscope (BX41TF; Olympus, Japan). The images used to measure sporulated oocysts of *Isospora* and *Caryospora* and the sporozoites of *Lankesterella* sp. in *A. carolinensis* were taken at ×1000 magnification using an adjustable camera on an Olympus SC30 microscope. Always that it was possible, we took at least 20 photographs for each species. Sporulated oocysts and corresponding structures were measured using the MB-Ruler 5.0 free software (http://www.markus-bader.de/MB-Ruler/). To compare the size of the oocyst of the species found infecting the Canarian lizards (i.e., *Gallotia* and *Tarentola* lizards), we used the nonparametric Mann–Whitney *U* test. For the newly described species, we considered the recommendations of Duszynski and Wilber (1997), and for the description of the morphology of the exogenous oocysts of the new species, we attended the standard nomenclature proposed in Berto et al. (2014). The conventional abbreviations for the different oocyst structures were used accordingly. Measurements, including the mean in micrometers, standard deviation, and range, of the morphological characteristics of oocysts for each species are given in the taxonomic section and in Table 2.

Molecular methods

We extracted genomic DNA from the blood preserved on FTA cards following the protocol described by Megía-Palma et al. (2013). The DNA was then purified using the NZYGelpure kit (NZYTech, Lda. - Genes and Enzymes, 1649-038 Lisbon, Portugal). The PowerFecal® DNA Isolation Kit was used to extract the DNA from fecal samples (MO BIO Laboratories, Inc., Carlsbad, CA 92010, USA). Partial amplification of the *18S* ribosomal RNA (rRNA) gene sequence (1626 bp) was performed using the primers BT-F1 (5′-GGT TGA TCC TGC CAG TAG T-3′) and hep1600R (5′-AAA GGG CAG GGA CGT AAT CGG-3′). These primers were previously used to amplify other coccidian species (see Megía-Palma et al. 2014). Due to the insectivorous diet of some reptilian species,

Table 1 Reptile species included in this study and the coccidian parasites found in each species. The origin of the reptile species and the microscopic prevalence of the coccidia found are also shown

Species	Family	No. of sampled individuals	Origin	Locality	Coccidian species found	Prevalence of coccidiasis in the sample (%)
Chlamydosaurus kingii	Agamidae	1	Captivity	Originally from Australia[a]	–	0
Pogona vitticeps	Agamidae	1	Captivity	Originally from Australia[a]	*Isospora amphiboluri*	100
Chamaeleo calyptratus	Chamaeleonidae	1	Captivity	Originally from Yemen[a]	–	0
Chamaeleo melleri	Chamaeleonidae	1	Captivity	Originally from Africa[a]	–	0
Coronella austriaca	Colubridae	2	Wild	Segovia and Huesca, Spain	–	0
Coronella girondica	Colubridae	2	Wild	Segovia, Spain	–	0
Hemorrhois hippocrepis	Colubridae	1	Wild	Segovia, Spain	–	0
Natrix maura	Colubridae	5	Wild	Segovia, Spain	–	0
Rhinechis scalaris	Colubridae	3	Wild	Segovia, Spain	–	0
Gekko vittatus	Gekkonidae	1	Captivity	Originally from Southeast Asia	–	0
Phelsuma madagascariensis grandis	Gekkonidae	1	Captivity	Originally from Madagascar[a]	*Isospora gekkonis*	100
Tarentola delalandii	Gekkonidae	2	Wild	Tenerife, Canary Islands	*Isospora tarentolae*	50
Acanthodactylus boskianus	Lacertidae	64	Wild	North Tunisia	*Isospora abdallahi*	10
Acanthodactylus erythrurus belli	Lacertidae	34	Wild	North Morocco	*Isospora fahdi* n. sp.	10
Acanthodactylus erythrurus erythrurus	Lacertidae	24	Wild	Almería, Navarra, Granada, Huelva, and Zaragoza, Spain	–	0
Podarcis bocagei	Lacertidae	10	Wild	León, Spain	–	0
Podarcis hispanica	Lacertidae	10	Wild	Segovia, Spain	–	0
Podarcis muralis	Lacertidae	10	Wild	Segovia, Spain	–	0
Gallotia galloti galloti	Lacertidae	50	Wild	Tenerife, Canary Islands, Spain	*Isospora tarentolae*	6
Iberolacerta cyreni	Lacertidae	40	Wild	Madrid, Spain	–	0
Lacerta schreiberi	Lacertidae	200	Wild	Segovia, Spain	–	0
Psammodromus algirus	Lacertidae	10	Wild	Segovia, Spain	–	0
Takydromus sexlineatus	Lacertidae	13	Captivity	Imported from Indonesia	*Isospora takydromi* n. sp.	23
Timon lepidus	Lacertidae	20	Wild	Segovia, Spain	–	0
Oplurus cyclurus	Opluridae	1	Captivity	Originally from Madagascar[a]	–	0
Anolis carolinensis	Polychrotidae	15	Captivity	Imported from the USA	*Caryospora ernsti*	20
Anolis carolinensis	Polychrotidae	15	Captivity	Imported from the USA	*Lankesterella* sp.	7
Anolis equestris	Polychrotidae	2	Captivity	Imported from the USA	–	0
Python reticulatus	Pythonidae	10	Captivity	Originally from Africa[a]	–	0
Chalcides parallelus	Scincidae	13	Wild	Chafarinas Islands, North Africa	*Isospora chafarinensis* n. sp.	46
Chalcides striatus	Scincidae	3	Wild	Segovia, Spain	–	0
Gonatodes albogularis fuscus	Sphaerodactylidae	2	Captivity	Imported from Central America	*Isospora albogulari*	100
Gonatodes ocellatus	Sphaerodactylidae	2	Captivity	Originally from Central America[a]	–	0

Table 1 (continued)

Species	Family	No. of sampled individuals	Origin	Locality	Coccidian species found	Prevalence of coccidiasis in the sample (%)
Gonatodes vittatus	Sphaerodactylidae	2	Captivity	Originally from Central America[a]	–	0
Sphaerodactylus nigropunctatus ocujal	Sphaerodactylidae	2	Captivity	Originally from Cuba[a]	–	0
Sphaerodactylus notatus	Sphaerodactylidae	2	Captivity	Originally from Central America[a]	–	0
Sphaerodactylus torrei	Sphaerodactylidae	2	Captivity	Originally from Cuba[a]	–	0
Trogonophis wiegmanni	Trogonophidae	71	Wild	Chafarinas Islands, North Africa	*Isospora wiegmanniana* n. sp.	52

[a] Imported/bred in captivity

in some fecal samples, we also amplified DNA sequences from haemogregarines found in insects, together with *Isospora*. To avoid this undesired amplification, *Isospora*-specific reverse primers, EimIsoR1 (5′-AGG CAT TCC TCG TTG AAG ATT-3′) or EimIsoR3 (5′-GCA TAC TCA CAA GAT TAC CTA G-3′), were used. The size of the amplicons obtained with reverse primers EimIsoR1 and EimIsoR3 were 1580 and 1528 bp, respectively. PCR reactions (total volume of 20 μl) contained between 20 and 100 ng of the DNA template. Supreme NZYTaq 2× Green Master Mix (NZYTech, Lda. - Genes and Enzymes, 1649-038 Lisbon, Portugal) and 250 nM of each primer were generally used. Using a Veriti thermal cycler (Applied Biosystems), reactions were run using the following conditions: 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 120 s, and a final extension at 72 °C for 10 min.

The 11 DNA sequences (*18S* rRNA) obtained from parasites of lizards were aligned together with 79 other sequences included in a previous study (Megía-Palma et al. 2014). The alignment was performed using PROBCONS (http://toolkit.tuebingen.mpg.de/probcons). Poorly aligned positions and divergent regions of the alignment were removed using gBlocks (Talavera and Castresana 2007), selecting the following options: "minimum number of sequences for a conserved position" to 36, "minimum number of sequences for a flank position" to 36, "maximum number of contiguous nonconserved positions" to 8, "minimum length of a block" to 5, and "allowed gap positions" to "with half." The final alignment contained 1500 positions and 90 sequences. The substitution model GTR+I+G was selected 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 5,500,000 generations per run and a burn-in of 13,750 generations (41,250 trees for consensus tree). The final standard deviation of the split frequencies was 0.01 in both runs. Convergence was checked using Tracer v1.5 (Rambaut and Drummond 2007). All model parameters were greater than 100.

In addition, the alignment was analyzed using a maximum-likelihood inference (PhyML program; Guindon et al. 2010), using the same substitution model mentioned above. The subtree pruning and regrafting (SPR) and the nearest-neighbor interchange (NNI) tree rearrangement options were selected, and a Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support (Anisimova et al. 2011).

Type photographs and DNA derived from all the material used in this study were deposited in specific collections of the Museo Nacional de Ciencias Naturales-CSIC (Madrid, Spain). The *18S* rRNA gene sequences were deposited in GenBank and are available on request (see the "Results" section).

Table 2 Relevant *Isospora* and *Caryospora* species described from reptiles

Species	Oocyst				Sporocyst				Host	Locality	Authors
	Length mean	Length range	Width mean	Width range	Length mean	Length range	Width mean	Width range			
I. abdallahi	25.4	24.5–29.0	23.9	23.0–25.5	15.4	14.0–16.0	9.4	9.0–10.0	*Acanthodactylus boskianus*	Northern Egypt	Modrý et al. (1998)
I. abdallahi[a]	25.5	22.7–27.9	23.1	20.3–26.1	14.3	11.6–17.0	9.9	9.0–11.4	*A. boskianus*	Tunisia	Present study
I. acanthodactyli	17.2	16.4–18.8	16.4	15.0–17.2	9.3	7.4–10.4	5.9	5.0–6.3	*A. boskianus*	Egypt	Sakran et al. (1994)
I. fahdi n. sp.[a]	25.6	23.1–29.2	22.0	18.2–27.1	13.7	11.6–16.2	9.7	8.3–10.9	*Acanthodactylus erythrurus belli*	Northern Morocco	Present study
I. acanthodactyli (*I. alyousifi*)	27.9	25.1–29.0	25.5	22.7–27.8	11.6	11.2–12.6	8.0	7.5–8.4	*Acanthodactylus schmidti*	Saudi Arabia	Al Yousif and Al-Shawa (1997)
I. alyousifi	24.6	17–29	21	16–26	13.5	8–16	9.0	6–11	*Acanthodactylus schmidti*	Saudi Arabia	Abdel-Baki et al. (2012)
Caryospora ernsti	12.5	11.0–14.5	12.5	11.0–14.5	10.7	10.0–12.5	8.3	7.5–9.0	*Anolis carolinensis*	United States of America	Upton et al. (1984)
C. ernsti[a]	12.4	11.4–13.5	12.0	11.0–12.7	9.4	8.5–10.1	7.2	6.3–7.6	*A. carolinensis*	Imported from the USA	Present study
Caryospora natchitochesensis	13.1	11.0–15.0	12.3	10.0–14.0	10.1	7.0–13.0	7.4	6.0–10.0	*A. carolinensis*	United States of America	McAllister et al. (2014)
I. capanemaensis	14.8	13.3–18.0	14.5	12.6–16.3	8.6	7.4–10.4	6.6	5.9–7.4	*Amphisbaena alba*	Capanema, Pará, North Brazil	Lainson (2003)
I. chalchidis	19.0	18.0–20.5	19.0	18.0–20.5	12.2	9.5–13.0	6.5	5.0–8.0	*Chalcides ocellatus*	Egypt	Amoudi (1989)
I. eimanae	18.5	17.0–19.5	18.5	17.0–19.5	12.0	11.0–13.0	8.5	7.5–9.0	*Chalcides ocellatus*	Egypt	Amoudi (1989)
I. arabica	32.5	27.5–34.0	25.0	24.5–26.5	19.0	17.5–21.0	13.5	11.0–14.5	*Chalcides ocellatus*	Saudi Arabia	Amoudi (1993)
I. chafarinensis n. sp.[a]	21.5	10.8–24.9	20.1	17.6–22.0	11.6	9.3–14.9	8.5	6.9–9.8	*Chalcides parallelus*	Chafarinas Islands (North Africa)	Present study
I. viridanae	21.6	17.6–23.4	–	–	13.2	11.7–14.0	9.5	8.2–10.5	*Chalcides viridanus*	Tenerife, Canary Islands	Matuschka (1989)
I. riyadhensis	23.0	18.0–26.0	20.0	17.0–22.0	13.0	11.0–15.0	8.0	7.0–9.0	*Diplometopon zarudnyi*	Central Saudi Arabia	Abdel-Azeem and Al-Quraishy (2011)
I. diplometoponi	33.3	28.6–35.2	30.9	26.8–32.7	20.1	17.5–22.3	13.8	12.2–15.4	*Diplometopon zarudnyi*	Eastern Saudi Arabia	Al Yousif and Al-Shawa (1998)
I. wiegmanniana n. sp.[a]	15.7	13.9–18.2	15.2	13.1–17.1	8.4	6.1–10.4	6.6	5.5–7.6	*Trogonophis wiegmanni*	Chafarinas Islands (North Africa)	Present study
Isospora gallotiae	16.5	15.3–17.6	16.5	15.3–17.6	11.5	10.2–12.2	7.3	5.2–6.6	*Gallotia galloti*	Tenerife, Canary Islands	Matuschka and Bannert (1987)
I. albogularis	29.5	26.4–32.0	26.9	22.4–30.8	14.9	13.6–16.0	10.8	10.2–11.4	*Gonatodes albogularis*	Guanacaste, Costa Rica	Upton and Freed (1990)
I. albogularis[a]	29.3	27.3–30.9	23.9	22.6–25.9	13.7	12.8–15.0	10.3	9.3–11.6	*G. albogularis*	Imported from Central America	Present study
I. gekkonis	24.2	21.6–26.4	22.0	20.0–23.6	12.2	11.2–12.8	9.4	8.4–10.0	*Phelsuma madagascariensis grandis*	Madagascar	Upton and Barnard (1987)
I. gekkonis[a]	23.3	21.9–24.7	19.1	17.8–20.7	11.3	10.4–12.3	7.9	7.1–8.5	*P. madagascariensis grandis*	Captive Bred	Present study
I. amphiboluri	25.3	23.0–26.0	25.1	23.0–26.0	17.0	16.0–18.0	11.4	11.0–12.0	*Pogona vitticeps*	Bred in California (originally from Australia)	McAllister et al. (1995)
I. amphiboluri[a]	23.5	21.6–25.4	23.5	22.1–25.4	14.2	12.0–16.4	9.4	8.1–10.2	*P. vitticeps*	Captive Bred	Present study

Table 2 (continued)

Species	Oocyst				Sporocyst				Host	Locality	Authors
	Length mean	Length range	Width mean	Width range	Length mean	Length range	Width mean	Width range			
I. canariensis	44.4	39.8–47.9	37.6	32.8–42.1	20.0	18.7–22.2	14.8	13.8–16.4	*Tarentola delalandii*	Tenerife, Canary Islands	Matuschka and Bannert (1986)
I. tarentolae	26.9	23.4–29.6	24.7	22.2–27	13.8	12.2–15.2	10.2	9.2–10.7	*T. delalandii*	Tenerife, Canary Islands	Matuschka and Bannert (1986)
Isospora tarentolae[a]	26.3	24–29.2	23.6	21.3–25.4	11.5	10.2–12.5	9.1	8.4–10.1	*T. delalandii* and *G. galloti*	Tenerife, Canary Islands	Present study
Isospora kaschkadarinica	15.1	11.9–18.7	12.2	8.5–15.3	–	–	–	–	*Eremias lineolata*	Southern Uzbekistan	Davronov (1985)
I. takydromi n. sp.[a]	23.9	16.6–30.5	19.4	15.3–24.4	12.5	14.5–9.7	8.6	9.8–7.5	*Takydromus sexlineatus*	Imported from Southeast Asia	Present study
I. nagasakiensis	24.6	22.5–25.0	21.7	20.0–22.5	12.1	10.0–13.8	9.3	7.5–10.0	*Takydromus tachydromoides*	Japan	Miyata (1987)

[a] Species included in the phylogeny in the present study

Results

Microscopy and morphology

We found oocysts of nine different *Isospora* species in ten lizard host species belonging to the families Agamidae, Gekkonidae, Lacertidae, Scincidae, Sphaerodactylidae, and Trogonophidae from Africa, South America, Asia, and Australia (Table 1). Five of the *Isospora* species have been previously described (*Isospora abdallahi* Modrý et al., 1998; *Isospora albogularis* Upton and Freed, 1990; *Isospora amphiboluri* McAllister et al., 1995; *Isospora gekkonis* Upton and Barnard, 1987; and *Isospora tarentolae* Matuschka and Bannert, 1986). *I. tarentolae* was originally described from the Canarian gecko *Tarentola delalandii* Duméril and Bribon, 1836 (Matuschka and Bannert 1986). However, in this study, this parasite was found in two sympatric host species: *T. delalandii* and *Gallotia galloti* Oudart, 1839 (see Fig. 1a, b). Conspecificity was confirmed by both morphology (Mann–Whitney *U* test: $U=14.0$, $p=0.9$, for oocyst length; $U=11.0$, $p=0.5$, for oocyst width) and molecular analysis of fecal samples that resulted in two sequences with 100 % coincidence.

In addition, we found four new *Isospora* species, which are described in the taxonomic section below. Although we were unable to statistically compare the morphological measures of these species with related ones (the original descriptions lacked some measures, e.g., the standard deviation and/or the number of measured oocysts), the internal structures and general morphology of oocysts were compared.

Taxonomic section

Isospora takydromi sp. nov.

Description: The sporulated oocysts ($N=26$) had a measure of 23.9±3.0 (16.6–30.5)×19.4±2.3 (24.4–15.3) μm, with a shape index (length/width) of 1.2±0.10 (0.9–1.4). The ellipsoidal oocysts had a bilayered wall with a smooth surface. It has a measure of 0.76 (mean)±0.1 and ranged from 0.5 to 1.0 μm thick. There was no micropyle on the surface, and the polar granule (PG) was absent. The tetrasporozoic sporocysts ($N=25$) were 12.5±1.3 (14.5–9.7)×8.6±0.6 (9.8–7.5) μm, with a shape index of 1.4±0.1 (1.1–1.6). Specimens presented a knob-like flattened stieda body (SB) on one side of the smooth surface; a rounded substieda body (SSB) was also present (1.5×1.0 μm). The sporocyst residuum (SR) was visible among the sporozoites (SPs), which were elongated and had two refractile bodies (RBs) at either end.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Takydromus sexlineatus* Daudin, 1802.

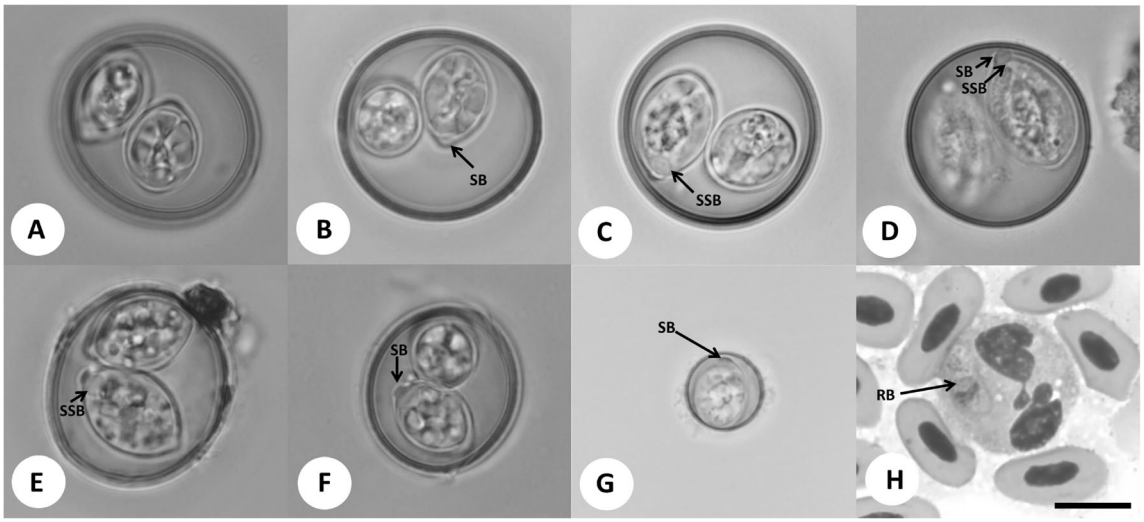

Fig. 1 Infective stages of the different coccidian species found in the present study. All images were taken at the same magnification. **a**–**g** Exogenous oocysts of coccidian species included in the phylogeny. **a** *Isospora tarentolae* from *Tarentola delalandii*. **b** *I. cf. tarentolae* from *Gallotia galloti*. **c** *Isospora abdallahi* from *Acanthodactylus boskianus*. **d** *Isospora amphiboluri* from *Pogona vitticeps*. **e** *Isospora albogulari* from *Gonatodes albogularis fuscus*. **f** *Isospora gekkonis* from *Phelsuma madagascariensis grandis*. **g** *Caryospora ernsti* from *Anolis carolinensis*. **h** Sporozoite of *Lankesterella* sp. infecting a polymorphonuclear leukocyte in the blood of *A. carolinensis*. *SSB* substieda body, *SB* stieda body, *RB* refractile body. *Scale bar*=10 μm

Origin of the sample: Imported to Spain from Indonesia in 2013. No type locality was available.

Prevalence of the parasite: 6/13 (46.1 %) of examined individuals were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65269. No lizards were euthanized, and therefore, a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: KU180238).

Etymology: The nomen triviale is derived from the generic part of the scientific name of the host, in the genitive singular ending, meaning of *Takydromus*. The first parasite species described for a genus of hosts is usually named after the host's generic name. In this case, however, the name was available because the only other species of *Isospora* described in the genus *Takydromus* received the name of the locality where it was discovered (i.e., *Isospora nagasakiensis* Miyata, 1987).

Taxonomic remark

The size of the oocyst of *I. nagasakiensis* from *Takydromus tachydromoides* Schlegel, 1838, was similar to *I. takydromi* n. sp. (see Fig. 2 and Table 2). Both species lacked a PG and oocyst residuum (OR) but had a granular SR. However, the exogenous oocyst of *I. takydromi* n. sp. presented a bilayered oocyst wall whereas *I. nagasakiensis* presented a monolayered wall. However, previous evidences suggest that the oocyst wall within the Eimeriidae consists of two layers (Belli et al. 2006). Although the presence and morphology of SB and SSB is an important character in *Isospora* species differentiation (Duszynski and Wilber 1997; Berto et al. 2014), no data on the morphology of the SB of SSB of the oocysts in *I. nagasakiensis* was given in its original description (Miyata 1987). Therefore, molecular analyses of *I. nagasakiensis* are needed to compare with *I. takydromi* n. sp. to confirm if they are, in fact, distinct species.

Isospora fahdi sp. nov.

Description: The sporulated oocysts (N=28) were ellipsoidal and had a measure of 25.6 (mean)±1.7 (SD) (range=23.1–29.2)×22.0±2.2 (18.2–27.1)μm with a shape index (length/width) of 1.17±0.07 (1.01–1.28). The oocyst wall was bilayered with a smooth surface. It has a measure of 1.1±0.1 (0.8–1.3)μm thick. The micropyle, OR, and PG were absent. Sporocysts (N=26) were ellipsoidal, had a measure of 13.7±1.2 (11.6–16.0)×9.7±0.6 (8.3–10.9)μm, and had unpigmented and smooth walls. The shape index was 1.4±0.1 (1.1–1.7). It presents a knob-like SB, and the SSB was rounded (1.5×1.9 μm). The SR was composed of numerous granules of irregular sizes. SPs were elongated with distinct anterior and posterior RB.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Acanthodactylus erythrurus belli* Grey, 1845.

Type locality: Martil, Tétouan, and North Morocco (UTM 30 S 293258, 3946654).

Prevalence: 3/34 (8 %) of examined lizards were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN:

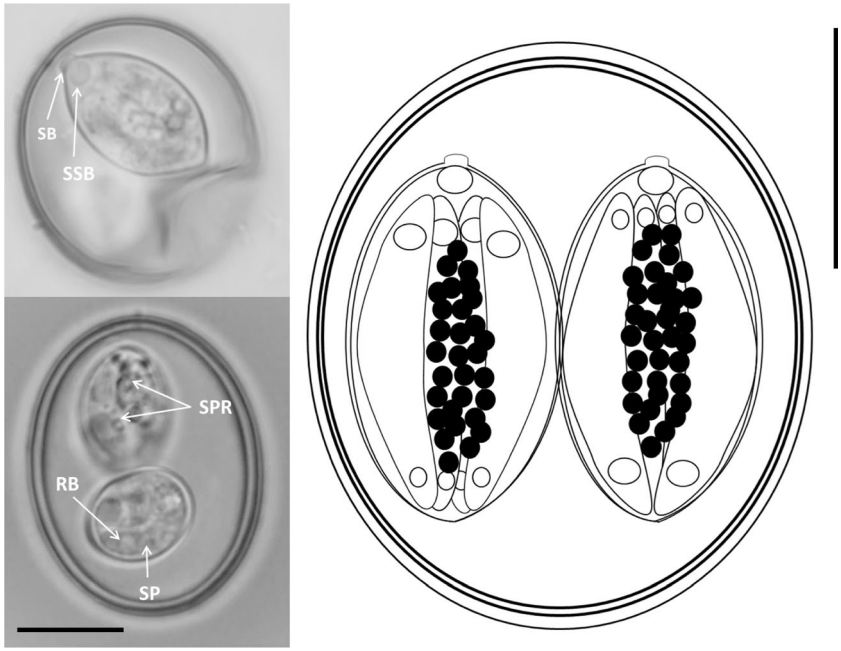

Fig. 2 Microphotographs and line drawing of *Isospora takydromi* n. sp. from *Takydromus sexlineatus*. *SB* stieda body, *SSB* substieda body, *SPR* sporocyst residuum, *RB* refractile body, *SP* sporozoite. *Scale bars*=10 μm

65270. No lizards were euthanized, and therefore, a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: KU180239).

Etymology: The specific epithet *fahdi* is a genitive (possessive) Latin name (g. masculine). This patronym (eponym) honors Pr. Dr. Soumia Fahd from the University of Tétouan, Morocco, for her lifelong dedication to herpetological studies of North Africa and in expression of our thanks for her help and hospitality during our field work in Morocco.

Taxonomic remark

The size and morphological characteristics of the oocyst of *I. abdallahi* Modrý et al., 1998, overlap with those of *I. fahdi* n. sp. (see Fig. 3 and Table 2). However, the molecular data presented here show that the *18S* rRNA gene sequences of *I. abdallahi* and *I. fahdi* n. sp. differ. Therefore, we consider *I. fahdi* as a new species based on molecular and host species differences.

Isospora chafarinensis sp. nov.

Description: The sporulated oocysts (N=62) were subspherical and had a measure of 21.5 (mean)±2.2 (SD) (range=10.8–24.9)×20.1±0.9 (17.6–22.0) μm; the index shape (length/width) was 1.07±0.10 (0.50–1.20). The micropyle, PG, and OR were absent. The sporocysts (N=62) were ellipsoid and had a measure of 11.6±1.2 (9.3–14.9)×8.5±0.6 (6.9–9.8) μm; the shape index was 1.3±0.1 (1.0–1.8). The SR (N=35) appeared as a granular sphere among the SP and has a measure of 3.7±0.5 (2.4–4.6) μm. A flattened SB and an irregularly rounded SSB were present. A banana-shaped SP had two RBs at either end.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Chalcides parallelus* Doumergue, 1901.

Type locality: Rey Francisco Island, Chafarinas Archipelago (Spain), and North Africa (UTM 30 S 552523, 3893242).

Prevalence: 6/13 (46.1 %) of examined skinks were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65272. No lizards were euthanized, and therefore, a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: KU180244).

Etymology: The specific name is a toponymic variable adjective related to the type locality.

Taxonomic remark

Four species of *Isospora* were previously described in the host genus *Chalcides*: *Isospora viridanae* Matuschka, 1989; *Isospora chalchidis* Amoudi, 1989; *Isospora eimanae* Amoudi, 1989; and *Isospora arabica* Amoudi, 1993 (see Table 2). The most similar species in size to *I. chafarinensis* n. sp. (Fig. 4) is *I. viridanae*. Indeed, the oocyst sizes of these species overlap. However, *I. chafarinensis* n. sp. presents sporocysts which are in mean 1.6 μm shorter and 1 μm narrower. Furthermore, there are geographic barriers between the host species: *Chalcides viridanus* Gravenhorst, 1851, is a Canarian endemism in the Atlantic Ocean, whereas *C. parallelus* is a

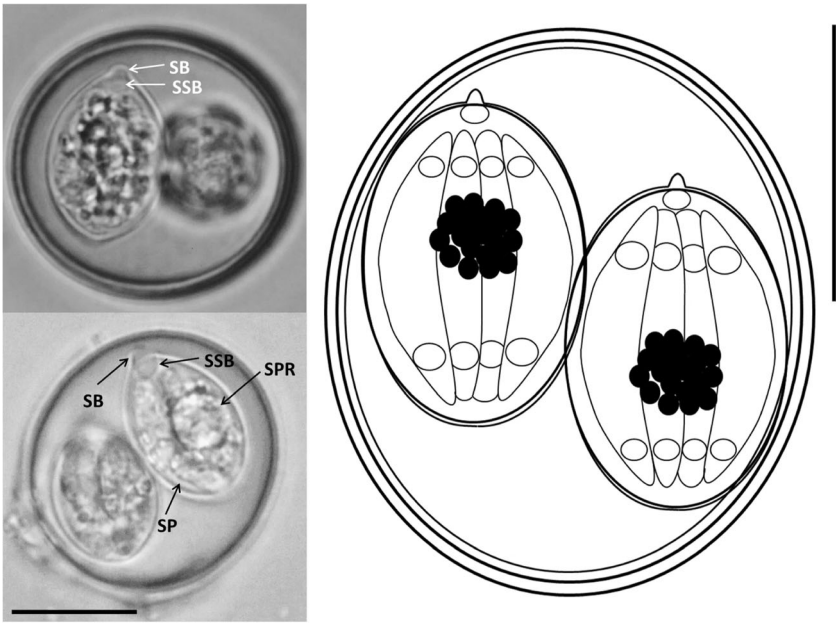

Fig. 3 Microphotographs and line drawing of *Isospora fahdi* n. sp. from *Acanthodactylus erythrurus belli. SB* stieda body, *SSB* substieda body, *SPR* sporocyst residuum, *SP* sporozoite. *Scale bars*=10 μm

Mediterranean endemism. In addition, the Egyptian species differs in morphology too with *I. chafarinensis* n. sp. The oocyst size of *I. chalchidis* and *I. eimanae* from *Chalcides ocellatus* Forskål, 1775, is respectively 2.6 and 3.1 μm shorter in mean to *I. chafarinensis* n. sp. Last, the oocyst of *I. arabica* from the Arabian Peninsula is 11 μm longer and 5 μm wider in mean whereas the sporocyst is 7.4 μm longer and 5 μm wider in mean. *I. arabica* has a fairly large SR consisting of diffuse granules, whereas *I. chafarinensis* n. sp. presents a granular and dense SR. In addition, *I. chafarinensis* n. sp. is described from Chafarinas infecting *C. parallelus* while *I. arabica* was described from the Arabian Peninsula infecting *C. ocellatus*. Given these morphological, geographic, and host species differences, we consider *I. chafarinensis* as a new species.

***Isospora wiegmanniana* sp. nov.**

Description: The sporulated oocysts (N=20) were spherical to subspherical and had a measure of 15.2 (mean)±1.0 (SD) (range=13.1–17.1)×15.6±1.1 (13.9–18.2)μm, with an index shape (length/width) of 1.04±0.02 (1.01–1.09). Transversal septa were visible in the oocyst wall. A thick monolayered wall of 0.8±0.1 (0.7–1.0)μm was observed. However, there is a growing consensus about the consistency in the structure of the coccidian oocyst wall. Thus, likely, two

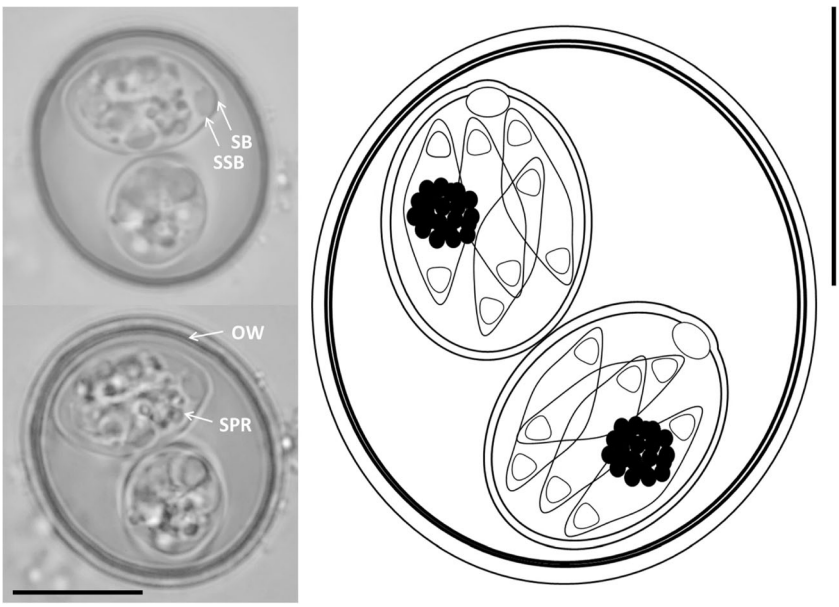

Fig. 4 Microphotographs and line drawing of *Isospora chafarinensis* n. sp. from *Chalcides parallelus. SB* stieda body, *SSB* substieda body, *OW* oocyst wall bilayered, *SPR* sporocyst residuum. *Scale bars*= 10 μm

thin or fused layers may form the wall of apparently monolayered walls of coccidian oocysts (Belli et al. 2006; Mai et al. 2009; Berto et al. 2014). The micropyle, PG, and OR were absent. Sporocysts (N=20) were ellipsoid and had a measure of 8.4±1.2 (6.1–10.4)×6.5±0.5 (5.5–7.6) μm; the shape index was 1.2±0.1 (1.0–1.5). An irregular SR, a flattened SB, and a widely flattened SSB were present. Two rounded RBs were visible at either end of the SP.

Sporulation: Probably exogenous. The time of sporulation was not recorded.

Type host: *Trogonophis wiegmanni wiegmanni* Kaup, 1830.

Type locality: Congreso, Isabel II and Rey Francisco Islands; Chafarinas Archipelago (Spain), and North Africa (UTM 30 S 551837, 3893225).

Prevalence: 37/71 (52.1 %) of the examined amphisbaenians were infected.

Type material: Phototypes and DNA voucher were deposited at the Museo Nacional de Ciencias Naturales-CSIC in Madrid, Spain, under the accession number MNCN/ADN: 65271. No lizards were euthanized, and therefore, a symbiotype was not deposited. The *18S* rRNA sequence was deposited in GenBank (accession number: KU180242).

Etymology: The nomen triviale was given after the host specific name and therefore is a variable adjective.

Taxonomic remark

Prior to this study, only one species of *Isospora*, *Isospora diplometoponi* Al Yousif and Al Shawa, 1998, found in *Diplometopon zarudnyi* Nikolsky, 1907, was known to parasitize the family Trogonophidae. However, this species differs in size from *I. wiegmanniana* n. sp. (see Fig. 5 and Table 2). In addition, contrary to *I. wiegmanniana* n. sp., *I. diplometoponi* has an obvious bilayered oocyst wall with no visible septum and a clearly visible SSB (Al Yousif and Al-Shawa 1998). One amphisbaenian species from South America, *Isospora capanemaensis* Lainson, 2003, is similar to *I. wiegmanniana* in oocyst size. However, in *I. capanemaensis*, the SB is inconspicuous and the oocyst wall shows no striation (Lainson 2003). Therefore, given the differences in morphology, geographic distribution and host families infected, we propose *I. wiegmanniana* as a new species in the genus *Isospora*. Molecular analyses of these three species are necessary to further support *I. wiegmanniana* n. sp. as a distinct species.

Phylogenetic results

Phylogenetic analysis using the *18S* rRNA gene showed that all nine *Isospora* species found in reptiles are closely related to *Lankesterella* and *C. ernsti* (Fig. 6). Within this group, a well-supported monophyletic clade grouped eight of the nine *Isospora* species close to *C. ernsti* and the genus *Lankesterella*. The ninth species, *I. wiegmanniana* n. sp., is the sister taxon to the group compounded by the genus *Lankesterella*, *C. ernsti*, and the former eight species of *Isospora*. Furthermore, *Caryospora bigenetica* Wacha and Christiansen, 1982, is the sister taxon to the group formed by reptilian *Isospora*, *Lankesterella*, and *C. ernsti*. *Lankesterella* obtained from *A. carolinensis* grouped with other *Lankesterella* species isolated from *A. erythrurus* Schinz, 1833. These two species are closely related to *Lankesterella minima* (Chaussat, 1850) Nöller, 1912, and *Lankesterella valsainensis* Martínez et al., 2006, isolated from frogs and birds, respectively (Fig. 6).

Discussion

Eimeriid coccidia are not expected to be host specific because it would not be to the parasite's advantage to limit its reproductive opportunities to a single host (Duszynski and Couch 2013). However, *Isospora* species that infect lizards show a high degree of host specificity evidenced by the high diversity

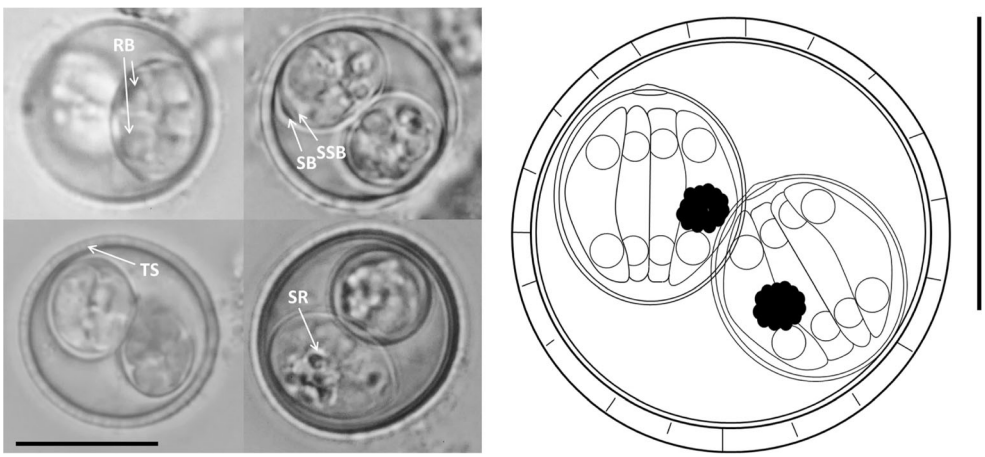

Fig. 5 Microphotographs and line drawing of *Isospora wiegmanniana* n. sp. from *Trogonophis wiegmanni wiegmanni*. *RB* refractile body, *SB* stieda body, *SSB* substieda body, *TS* transversal septum in the wall, *SR* sporocyst residuum. *Scale bars*=10 μm

Fig. 6 Phylogenetic tree derived from Bayesian inference using the GTR+I+G substitution model. This analysis consisted of two runs of four chains each, with 5,500,000 generations per run and a burn-in of 13,750 generations (41,250 trees for consensus tree). Support values less than 50 % are not shown, and these nodes were not collapsed into polytomies. Where two numbers are shown on the branch, the first one indicates the support value obtained by Bayesian inference and the second one by maximum-likelihood (ML) inferences. The ML inference was performed in PhyML also using the GTR+I+G substitution model. Bayesian-like transformation of aLRT (aBayes) was used to obtain the clade support. The length of the alignment was 1500 bp

of species described in reptiles (Duszynski et al. 2008). The species of *Isospora* isolated from *Acanthodactylus boskianus* Daudin, 1802, and *A. erythrurus belli* are a good example of the host specificity in this genus. The habitat and distribution of these two phylogenetically closely related host species overlap (Fonseca et al. 2009), but they are parasitized by two different *Isospora* species. This example of host specificity supports the description of new species of coccidian parasites when isolated from different hosts, even when hosts are evolutionarily closely related (e.g., Daszak et al. 2009; Finkelman and Paperna 2002; Modrý et al. 1997, 2004). Therefore, following the criteria of previous studies (e.g., Upton and Barnard 1987; Modrý et al. 1997, 2004; Modrý and Jirků 2006; Daszak et al. 2009) and given that *T. sexlineatus*, *A. erythrurus belli*, *T. wiegmanni*, and *C. parallelus* represent new host species for *Isospora* parasites, we consider these tetrasporozoic, diplosporocystic coccidia as a new species of *Isospora*. However, as each host-parasite system has different physiological and immunological peculiarities, molecularly characterizing parasites before describing a new species is desirable.

Supporting this recommendation, we report the occurrence of the same species of *Isospora* in two phylogenetically distant lizards that occupy in sympatry the island of Tenerife (Canary Islands). *I. tarentolae* was previously described from the geckonid *T. delalandii* (Matuschka and Bannert 1986). The occurrence of this species in the lacertid *G. galloti* might represent a host-switching event or, alternatively, a case of pseudoparasitism (Ghimire 2010). Previously, other species of *Isospora* were described in more than one host lizard species in islands (Upton and Barnard 1987; Modrý et al. 1997). However, the conspecificity of these parasites was only based on morphology. In the present case, we could not confirm if the primary host for *I. tarentolae* is the lacertid or the geckonid

species because it would have implied to kill the host lizards. However, we hypothesize that *T. delalandii* is the primary host for *I. tarentolae*, given the high prevalence of this parasite in *T. delalandii* in this study (50 %) and in imported Delalandi's geckoes (60 %) from which *I. tarentolae* was originally described (Matuschka and Bannert 1986), together with the low prevalence found in *G. galloti* (6 %).

Phylogenetic analyses of isosporoid parasites infecting bird and lizard hosts show the polyphyletic origin of the genus *Isospora* (Barta et al. 2005; Carreno and Barta 1999; Franzen et al. 2000; Frenkel and Smith 2003; Modrý et al. 2001; Morrison et al. 2004). These results emphasize the artificiality of the genus *Isospora* (Modrý et al. 2001), which was described solely based on the number of sporocysts and sporozoites per oocyst and the presence of a SB (Box et al. 1980; Frenkel et al. 1987). Therefore, the common morphological characteristics of the tetrasporozoic, diplosporocystic exogenous oocysts, and the presence of a SB in these parasites with separate origins may represent a homoplasy rather than a plesiomorphy (Jirků et al. 2002). The limitations of using morphological or life cycle characteristics for inferring evolutionary relationships among the Eimeriorina have been previously highlighted (Modrý et al. 2001; Barta et al. 2005; Ghimire 2010). For example, the genus *Isospora* (*Atoxoplasma* Garnham, 1950, pro parte) isolated from birds and the tetrasporozoic, diplosporocystic genera *Besnoitia* Henry, 1913; *Cystoisospora* Frenkel, 1977; *Frenkelia* Biocca, 1968; *Neospora* Dubey et al., 1988; *Sarcocystis* Lankester, 1882; and *Toxoplasma* Nicolle and Manceaux, 1909, all found in mammals, include extra intestinal stages in their life cycles but belong to different families (Eimeriidae and Sarcocystidae Poche, 1913, respectively) (Atkinson et al. 2008; Frenkel and Smith 2003).

The independent evolutionary origin of isosporoids from lizards would justify the creation of a new generic name for these parasites. However, despite most of the analyzed *Isospora* species infecting lizards having a recent common ancestor, *I. wiegmanniana* is placed as the sister taxon to the group compounded by *Caryospora*, *Lankesterella*, and the named monophyletic group of *Isospora* suggesting the paraphyletic origin of *Isospora* in lizards (Fig. 6). Therefore, it is inappropriate to propose a new generic name for this group (see Morrison 2009). Similarly, the phylogenetic position of *C. bigenetica* as the sister taxon of the group formed by reptilian *Isospora*, *Lankesterella*, and *C. ernsti* suggests that the reduction in the number of sporocysts observed in the genus *Caryospora* occurred at least twice during evolution and that *Caryospora* does not have a monophyletic origin. However, the characteristic endogenous development of oocysts of the genus *Lankesterella* and its transmission by vectors to the next host seem to have arisen only once during evolution in this lineage of parasites. The phylogenetic results here support the polyphyletic origin of the family Lankesterellidae as recently proposed (Megía-Palma et al. 2013, 2014). Therefore, the lack of external oocysts in both *Lankesterella* and *Schellackia* may be a case of convergent evolution, likely driven by behavioral changes in definitive host species that threatened the successful transmission of the parasite (Barta et al. 2001). These changes in host species may act as evolutionary forces favoring the selection of new parasite transmission strategies. This study reveals, for the first time, the close phylogenetic relationship between the genus *Lankesterella*, *C. ernsti*, and the reptilian *Isospora*. Our results suggest that avian *Lankesterella* species may have evolved from parasites of reptilian hosts and that the recent ancestor of the genus *Lankesterella* may have been heteroxenous. Several studies have shown that some species of *Caryospora* are heteroxenous, with predatory reptiles or birds serving as primary hosts and rodents serving as secondary hosts (Upton et al. 1984, 1986). This variability within the same clade suggests the existence of different selective forces modeling features such as the number of sporocysts per oocyst or the occurrence of endogenous development with naked sporozoites. These changes in developmental stages might lead to species-specific morphological adaptations, as previously suggested for other coccidian parasites (Jirků et al. 2009).

Conclusions

Our results suggest that the evolutionary origin of *Isospora* species infecting reptiles is independent from parasites with tetrasporozoic, diplosporocystic oocysts infecting birds, mammals, and frogs. They also confirm the artificiality of the genus *Isospora* based on morphological characteristics (see also Modrý et al. 2001). Furthermore, the phylogenetic analysis revealed that the genus *Lankesterella* is closely related to the genera *Caryospora* and *Isospora* found in reptiles. The phylogenetic positions of *C. bigenetica* and *C. ernsti* suggest that the genus *Caryospora* is not monophyletic.

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Compliance with Ethical Standards All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no competing interests.

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