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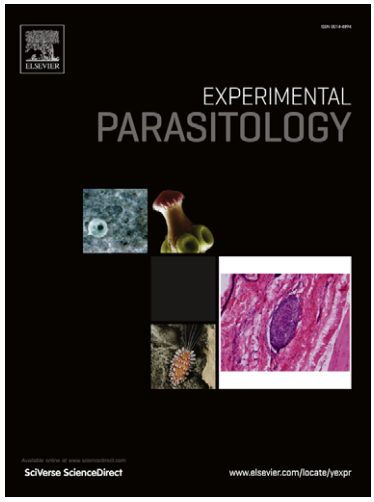

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Eimeria tiliquae n. sp. (Apicomplexa:Eimeriidae) from the shingleback skink (*Tiliqua rugosa rugosa*).

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ABSTRACT

A new species, *Eimeria tiliquae* n. sp. is described from a shingleback skink (*Tiliqua rugosa rugosa).* Sporulated oocysts (n = 50) are spherical to subspherical, with colorless trilaminate oocyst wall, 0.7±0.1 (0.5-0.75) thick. Oocyst with 4 spheroidal to subspheroidal sporocysts. Oocyst length, 13.7±0.9 (12.0-16.3); oocyst width, 12.8±0.9 (11.5-15.0); oocyst length/width (L/W) ratio, 1.07±0.05 (1.0-1.2). Micropyle, oocyst residuum and polar granule absent. Sporocysts with globular sporocyst residuum and 2 sporozoites. Sporocyst length, 6.0±0.6 (5.0-7.5); sporocyst width, 5.4±0.6 (4.0-7.0); sporocyst L/W ratio, 1.11±0.11 (1.0-1.5). Stieda, parastieda and substieda bodies absent. Phylogenetic analysis of 18S rRNA sequences indicated that *E. tiliquae* n. sp. shared 96.4-96.5% genetic similarity to *E. tropidura,* its closest relative. Reptile-derived sequences were not available for the mitochrondial cytochrome oxidase gene (COI) and phylogenetic analysis at this locus placed *E. tiliquae* n. sp. in a clade by itself but grouping closest (92% similarity) with a novel isolate from a King's skink (*Egernia kingii*) from Western Australia. Based on morphological and molecular data, this isolate is a new species of coccidian parasite that to date has only been found in shingleback skinks.

Keywords: Eimeria tiliquae n. sp.; morphology, genetic characterization; 18S rRNA; mitochrondial cytochrome oxidase gene (COI); phylogeny.

1. Introduction

Eimeria spp. are coccidian parasites that infect a wide range of vertebrate hosts (McDonald and Shirley, 2009). With more than 1,300 described species (Duszynski et al., 2000), the genus is one of the most speciose eukaryotic taxa. Pathogenic eimerian species that cause severe clinical disease and economic loss in poultry and production animals have been well characterised (Aarthi et al., 2010; Fitzgerald, 1980; Taubert et al., 2010). Traditionally, identification of *Eimeria* species has been based largely on sporulated oocyst morphology but also on host species, pathology and geographic distribution (Duszynski and Wilber, 1997; Tenter et al., 2002). However, some species of *Eimeria* are morphologically identical and occur in several hosts and it is now recognized that molecular data are essential to accurately delimit species and infer phylogenetic relationships among *Eimeria* species (Tenter et al., 2002).

Little is known about coccidia species infecting reptiles; three species of *Isospora* have been described in Australian reptiles (Cannon, 1966a), and although more than 100 named species of *Eimeria* have been described in lizards (Duszynski et al., 2000), relatively little is known about their life cycles, biology and genetic diversity. To date, only *Eimeria tropidura* (Aquino-Shuster et al., 1990), a species found in Galapagos lava lizards (*Tropidurus delanonis)*, an un-named species from a wall lizard (*Podarcis hispanica)*, from Portugal (Harris et al., 2012) and an un-named species from a King's skink (*Egernia kingii*) (Yang et al., 2012a) have been genetically characterized at the 18S locus. In the present study, we characterized a new species of *Eimeria* from shingleback skinks (*Tiliqua rugosa rugosa)*, both morphologically and genetically and propose the species name *Eimeria tiliquae*.

2. Materials and methods

2.1 Sample collection

A survey was conducted over a 4-month period (late February - June 2012), to determine the incidence of coccidean parasites in a population of shingleback skinks, (*Tiliqua rugosa rugosa*) that had been admitted to the Kanyana Wildlife Rehabilitation Centre (KWRC) in Western Australia. A faecal sample was collected from 34 individual shingleback skinks that were housed in separate vivaria at KWRC under the KWRC permit. Samples were collected into sterile containers and then labeled with a number identifying the lizard from which they came and refrigerated at 4 ºC until examined.

2.2 Morphological analysis

Microscopic examination of a wet mount, as well as faecal flotation analysis were performed on all samples. Faecal flotation was done using a saturated sodium chloride and 50% sucrose (w/v) solution. If any sample was found to contain coccidean oocysts, a portion of faeces was placed in 2% (w/v) potassium dichromate solution ($K_2Cr_2O_7$), mixed well and poured into petri dishes to a depth of less than 1cm and kept at room temperature in the dark to facilitate sporulation. Sporulated oocysts were observed using the ×100 oil immersion objective of an Olympus CH-2 binocular microscope, in combination with an ocular micrometer.

87

88 *2.3 DNA isolation*

89 Total DNA was extracted from 200mg of each faecal sample using a QIAamp
90 DNA Mini Stool Kit (Qiagen, Hilden, Germany) or from 250mg of each faecal sample
91 using a Power Soil DNA Kit (MolBio, Carlsbad, California). A negative control (no
92 faecal sample) was used in each extraction group.

93

94 *2.4 PCR amplification and sequencing*

95

96 Samples were screened at the 18S rRNA locus for *Eimeria* spp. using primers
97 and conditions described by Yang et al., (2012b).PCR contamination controls were
98 used including negative controls and separation of preparation and amplification
99 areas. A spike analysis (addition of 0.5 µL of positive control DNA from *Eimeria*
100 *crandallis* into each sample) was conducted on randomly selected negative samples
101 from each group of DNA extractions to determine if negative results were due to PCR
102 inhibition.

103 The amplified DNA fragments from the secondary PCR product were
104 separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et
105 al., 2006). Gel-purified PCR products were cloned in the pGEM-T Easy Vector
106 System II (Promega, USA). After transformation of JM109 competent cells, plasmid
107 DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) from
108 cultured clones grown overnight, and 10 colonies were sequenced with the T7 (5'
109 TAA TAC GAC TCA CTA TAG GG) and SP6 (5' ATT TAG GTG ACA CTA TAG)
110 primers in both directions, using an ABI Prism™ Dye Terminator Cycle Sequencing

kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58 ºC.

Amplification of a 465 bp region of the mitochrondial cytochrome oxidase gene (COI) locus from samples that were positive at the 18S locus was conducted as described by Ogedengbe et al., (2011) and Yang et al., (2012a).

The results of the sequencing reactions were analysed and edited using Chromas lite version 2.0 (http://www.technelysium.com.au), compared to existing *Eimeria* spp. 18S rDNA and COI sequences on GenBank using BLAST searches and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp).

2.5 Phylogenetic analysis

Phylogenetic trees were constructed for *Eimeria* spp. at the 18S and COI loci with additional isolates from GenBank. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Tamura-Nei model and grouped using Neighbour-Joining. Parsimony analyses were conducted using MEGA version 5.1 (MEGA5.1: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA). Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper et al., 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006).

At the 18S locus, the relationship between *E. tiliquae* n. sp., a wall lizard isolate (JQ762306) and a *Choleoeimeria* sp. (AY043207) was also analysed. The analysis

was only based on a short region (~352 bp) of 18S rDNA sequence because only short 18S rDNA overlapping fragments were available from GenBank from these isolates.

2.6 Statistical Analysis

Prevalences were expressed as percentage of positive samples, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Measurements of 50 sporulated oocysts were analysed using Microsoft Office Excel 2007, and results are presented in micrometers as the mean ± SD, with the observed range in parentheses.

3. Results

3.1 Morphological analysis of Eimeria tiliquae n. sp.

Sporulated oocysts (n = 50) spherical to subspherical, with colorless trilaminate oocyst wall, 0.7±0.1 (0.5-0.75) thick. Oocysts with 4 spheroidal to subspheroidal sporocysts. Oocyst length, 13.7±0.9 (12.0-16.3); oocyst width, 12.8±0.9 (11.5-15.0); oocyst length/width (L/W) ratio, 1.07±0.05 (1.0-1.2). Micropyle, oocyst residuum, and polar granule absent. Sporocysts with globular sporocyst residuum and 2 sporozoites. Sporocyst length, 6.0±0.6 (5.0-7.5); sporocyst width, 5.4±0.6 (4.0-7.0); sporocyst L/W ratio, 1.11±0.11 (1.0-1.5). Stieda, parastieda and substieda bodies absent (Figs 1-2).

156

157 *3.2 Phylogenetic analysis of E. tiliquae* n. sp. *at the 18S locus*

158 Initial sequencing of 3 isolates indicated mixed chromatograms and as a result
159 of this, the 18S PCR products were cloned and 10 colonies each were sequenced. Two
160 partial 18S sequences (1,300 and 1,302 bp respectively) were obtained from cloned
161 PCR products of *E. tiliquae* n. sp., which exhibited 8 single nucleotide
162 polymorphisms (SNP's) compared to each other. Phylogenetic analyses of the partial
163 nucleotide sequences from *E. tiliquae* n. sp. at the 18S locus using Distance,
164 Parsimony and ML analyses produced similar results (Fig. 3 NJ tree shown). *Eimeria*
165 *tiliquae* n. sp. grouped in a clade with *E. tropidura* and shared 96.3% genetic
166 similarity to *E. tropidura*. The two sequences from *E. tiliquae* n. sp. were 99.3%
167 similar to each other. The isolate from the wall lizard grouped most closely with
168 *Eimeria arnyi* from a colubrid snake (Upton and Oppert, 1991, GenBank accession
169 no: AY613853) (Fig 3a). *Eimeria tiliquae* n. sp. was genetically very distinct from a
170 recent *Eimeria* sp. identified in the faeces of a King's skink (Yang et al., 2012a).

171

172 *3.3 Phylogenetic analysis of E. tiliquae n. sp. at the COI locus*

173 Direct sequencing of the COI gene fragment from 3 isolates produced a clean
174 chromatogram, indicating that only one sequence was present. Sequences from the 3
175 isolates were 100% identical. Reptile-derived sequences were not available at the COI
176 locus and phylogenetic analysis placed *E. tiliquae* n. sp. in a clade by itself but
177 grouping closest (92% similarity) with a novel isolate from a King's skink (*Egernia*
178 *kingii*) from Western Australia (Yang et al., 2012a) and rodent-derived isolates (Fig.
179 4).

3.4 Description

3.4.1 Eimeria tiliquae n. sp. (Figs 1-2).

Diagnosis: Oocysts are spherical to subspherical and measure 13.7 × 12.8 µm in size with a width to length ratio of 1.07.

Type hosts: Tiliqua rugosa rugosa (Gray, 1825), shingleback skink.

Type locality: Jandakot, Perth, Western Australia.

Prevalence: Eimeria sp. were detected in 7/34 samples screened, an estimated prevalence of 21% (7-34.2 CI).

Other hosts: Unknown.

Prepatent period: Unknown.

Patent period: Unknown.

Site of infection: Unknown.

Sporulation time: Unknown but assumed to be very short as some of the oocysts were already sporulated in the fresh faecal samples.

Material deposited: DNA sequences have been deposited in GenBank under accession numbers JX839287 and JX839288 for the 18S locus and JX839284 for the COI locus.
Etymology: This species is named Eimeria tiliquae n. sp. after its host *Tiliqua rugosa rugosa* (shingleback skink).

4. Discussion

Shingleback skinks (*Tiliqua rugosa*) are robust, have a broad triangular head, short blunt tail and large rugose scales. They are a slow moving species that are native to Australia and are members of the Scincidae (Wilson and Swan, 2010). There are 4 recognised subspecies of *Tiliqua rugosa*, 3 of which are only found in Western Australia; *T. rugosa rugosa*; *T. rugosa konowi* and *T. rugosa palarra*. The other *T. rugosa* subspecies inhabits Eastern Australia and is known as *Tiliqua rugosa aspera*. The subspecies from which *E. tiliquae n. sp.* was isolated was *Tiliqua rugosa rugosa*, which is found in the South-West of Western Australia (Wilson and Swan, 2010).

In the present study, the shingleback skinks examined were housed at the KWRC in Perth, Western Australia, which admitted 225 shingleback skinks in 2010, 173 during 2011 and 70 until June 2012. Approximately 55% of shingleback skinks admitted during that 3 year period showed signs of an upper respiratory infection (URTI). The majority of the remaining shingleback skinks were admitted due to dog attacks, motor vehicle accidents and injuries caused by gardening equipment. Approximately 80-85% of skinks that were admitted to Kanyana were released back into the wild.

In the present study, the overall prevalence of *Eimeria* sp. in shingleback skinks was estimated to be approximately 21%. Previous studies have reported prevalence estimates of 32.5-63% in lizards (Daszak, 1995; Couch et al., 1996; Modrý et al., 2000; Leinwand et al., 2005). Other parasites identified in faecal samples of skinks in the present study included oxyurid sp. eggs (50%), *Trichomonas* spp. trophozoites (35%) and *Balantidium* spp. trophozoites (6%). Five of the seven skinks that were positive for *Eimeria* sp. had symptoms of a URTI (nasal discharge, thick mucus in throat, pale mucous membranes, eyes closed, lethargic and thin) but no gastrointestinal signs.

Sporulated oocysts of *Eimeria tiliquae* n. sp. measured 13.7 × 12.8 (12.0-16.25 × 11.5-15.0) µm with a L/W ratio of 1.07 (1.0-1.2). Four other species of *Eimeria* have been described from Australian skinks from Queensland; *Eimeria ablephari*, *Eimeria egerniae*, *Eimeria sternfeldi* and *Eimeria jamescooki* (Cannon, 1966b; McAllister et al., 1993; Paperna, 2003). *Eimeria ablephari* was described from *Ablepharus boutonii* (Scincidae) and *E. egerniae* from *Egernia whitii* (Scincidae) (Cannon, 1966). Oocysts of *E. ablephari* and *E. egerniae* measured 23.1 × 17.7 µm and 30.3 × 16.1 µm respectively and are therefore larger than *E. tiliquae*. Micropyle,

oocyst residuum and polar granule were absent in both species. *Eimeria sternfeldi* was described from two blue-tongued skinks (*Tiliqua multifasciata*) from the Dallas Zoo, Dallas TX, USA (McAllister et al., 1993). Oocysts of *E. tiliquae* n. sp. are smaller than *E. sternfeldi*, whose oocysts measured 16.6 × 15.9 µm, with a L/W ratio of 1.1 (McAllister et al., 1993). Sporocysts of *E. sternfeldi* were ellipsoidal and measured 7.9 × 6.9 (6.6-9.4 × 6.4-7.4) µm, compared to 6.0 × 5.4 µm for *E. tiliquae* n. sp. Like *E. tiliquae* n. sp., micropyle, oocyst residuum, Stieda, substieda, and parastieda bodies were absent. *Eimeria jamescooki* was identified from the wall skink (*Cryptoblepharus virgatus)* from North Queensland, Australia (Paperna, 2003). Oocysts of this species were considerably larger than *E. tiliquae* n. sp. and measured 22.1 × 17.7 µm (Paperna, 2003).

Unfortunately genetic sequences for *E. sternfeldi*, *E. jamescooki*, *E. eblephari* and *E. egerniae* were not available and therefore it was not possible to compare them genetically.

The morphological similarity of oocysts, the broad host specificity of some *Eimeria* spp. and the diversity of *Eimeria* spp. within one host complicate species delimitation (Tenter et al., 2002). Molecular data are therefore essential to accurately delimit species. Phylogenetic analysis at the 18S locus confirmed the validity of *E. tiliquae* n. sp. It shared its closest genetic similarity of 96.3% with *E. tropidura*. The genetic similarity between *E. tiliquae* n. sp. and a *Choleoeimeria* sp isolated from the gall bladder of the diadem snake, *Spalerosophis diadema* (Jirku et al., 2002) was 91.1% over a 352 bp fragment of the 18S gene. *Choleoeimeria* is a genus of protozoan parasites whose members infect the bilary tract of reptiles (Paperna and Landsberg, 1989). Morphologically they are similar to *Eimeria* spp. to which they are

closely related. The genetic similarity to the wall lizard isolate was 89.5% over 352 bp.

The genetic similarity between *E. tiliquae* n. sp. and *E. tropidura* is similar to the genetic differences between accepted species of *Eimeria*. For example, the genetic similarity between *E. arnyi* and *E. ranae* is 97.5% and the similarity between *E. tenella* and *E. necatrix* and between *E. bovis* and *E. crandallis* is 99.1% and 99.5%, respectively, across the same length of sequence. By these criteria, *E. tiliquae* n. sp. is clearly a separate species.

The two sequences from *E. tiliquae* n. sp. were 99.3% similar to each other at the 18S locus. Previous studies have reported heterozygous alleles in *Eimeria* spp. at the 18S locus (Hill et al., 2012). In that study, additional sequence analysis at the COI locus confirmed that the genetic differences were due to heterozygous alleles at the 18S locus and not to multiple *Eimeria* species within the same sample (Hill et al., 2012). In the present study, sequence analysis of 3 isolates at the COI locus indicated that they were 100% identical which also suggest the presence of heterozygous alleles at the 18S locus. Reptile-derived sequences were not available at the COI locus and phylogenetic analysis placed *E. tiliquae* n. sp. in a clade by itself but grouping closest with a novel isolate from a King's skink (*Egernia kingii*) from Western Australia (Yang et al., 2012a) and rodent-derived isolates. Studies comparing the utility of the 18S and COI genes indicate the latter has higher resolving power for *Eimeria* sp., especially with respect to recent speciation events (Ogedengbe et al., 2011). COI has become the target gene for the Barcode of Life project that aims to use the marker for rapid identification of animals, including parasites (Ratnasingham and Hebert, 2007). One drawback of using this gene in the context of wildlife studies is the paucity of

Eimeria spp.sequences available for hosts other than poultry, rodents and more recently marsupials (Hill et al., 2012).

In the present study, morphological and molecular data were used to describe *E. tiliquae* n. sp. found in the faeces of shingleback skinks in Western Australia. Future studies need to concentrate on obtaining morphologically characterized *Eimeria* species derived from lizard hosts and generating sequence data that are directly related to described species. Analyzing the isolates at multiple gene loci will also provide a more in-depth analysis of the evolution of lizard-derived *Eimeria* spp.

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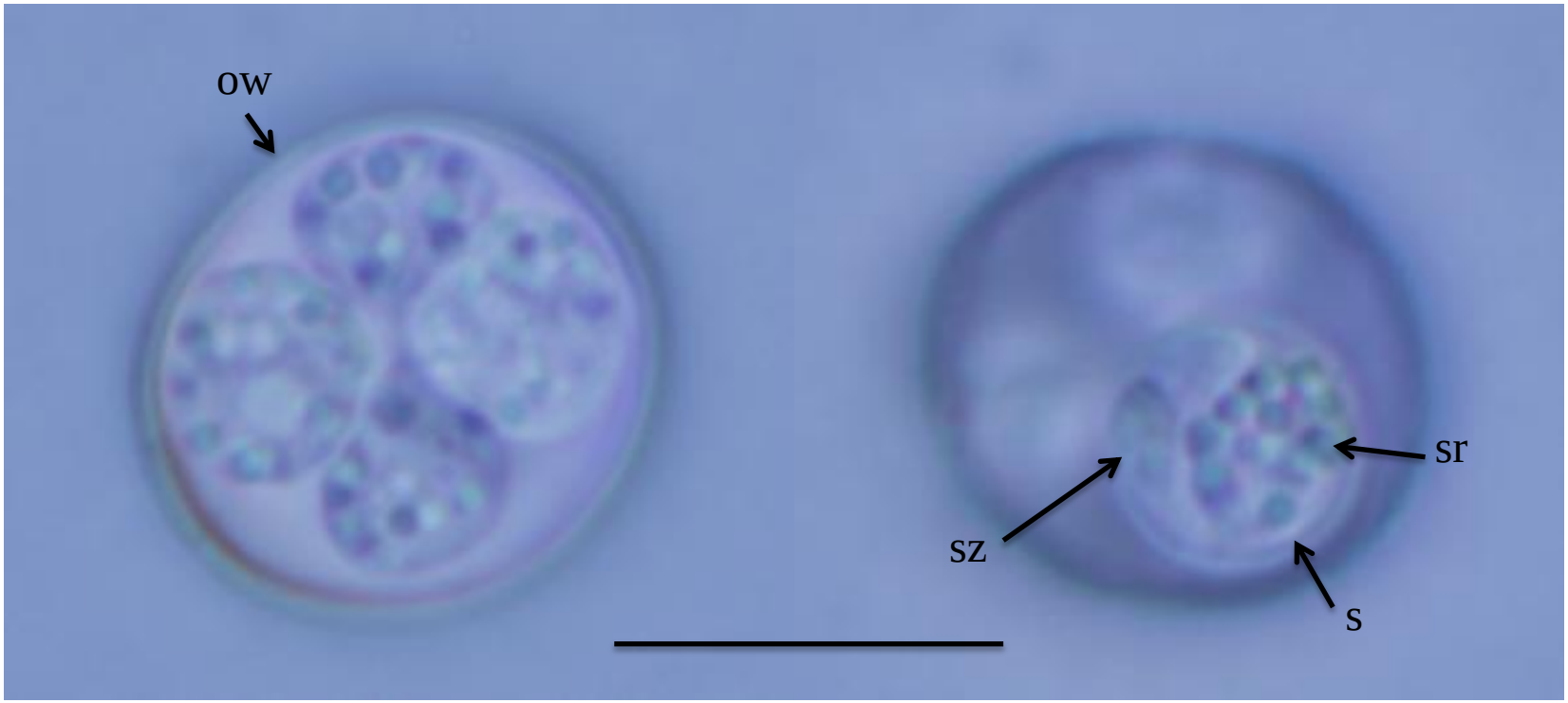
Fig. 1. Nomarski interference-contrast photomicrographs of *E. tiliquae* n. sp. oocyst showing 4 spheroidal to subspheroidal sporocysts. Abbreviations: ow=oocyst wall, s=sporocyst, sr=sporocyst residiuum, sz=sporozoite. Note the abundant sporocyst residuum which occupies much of the sporocyst volume. Scale bar = 10 µm.

Fig. 2. Composite line drawing of *Eimeria tiliquae* n. sp. sporulated oocyst. Scale bar = 5 µm.

Fig. 3. Evolutionary relationships of *E. tiliquae* n. sp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node. a) Phylogenetic position of *E. tiliquae* n. sp., *E. tropidura, Choleoeimeria sp., E. ranae, E. arnyi* and wall lizard isolate (~352 bp 18S rDNA only).

Fig. 4. Evolutionary relationships of *E. tiliquae* n. sp. inferred by distance analysis of mitochrondial cytochrome oxidase gene (COI). Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.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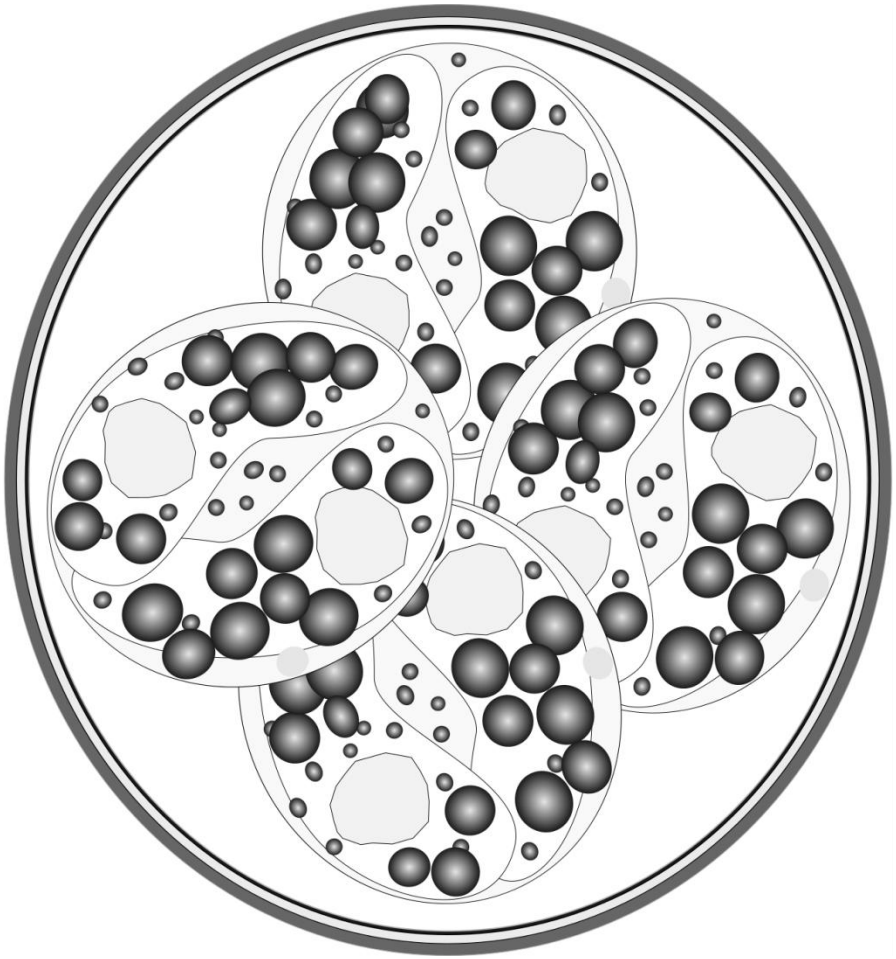
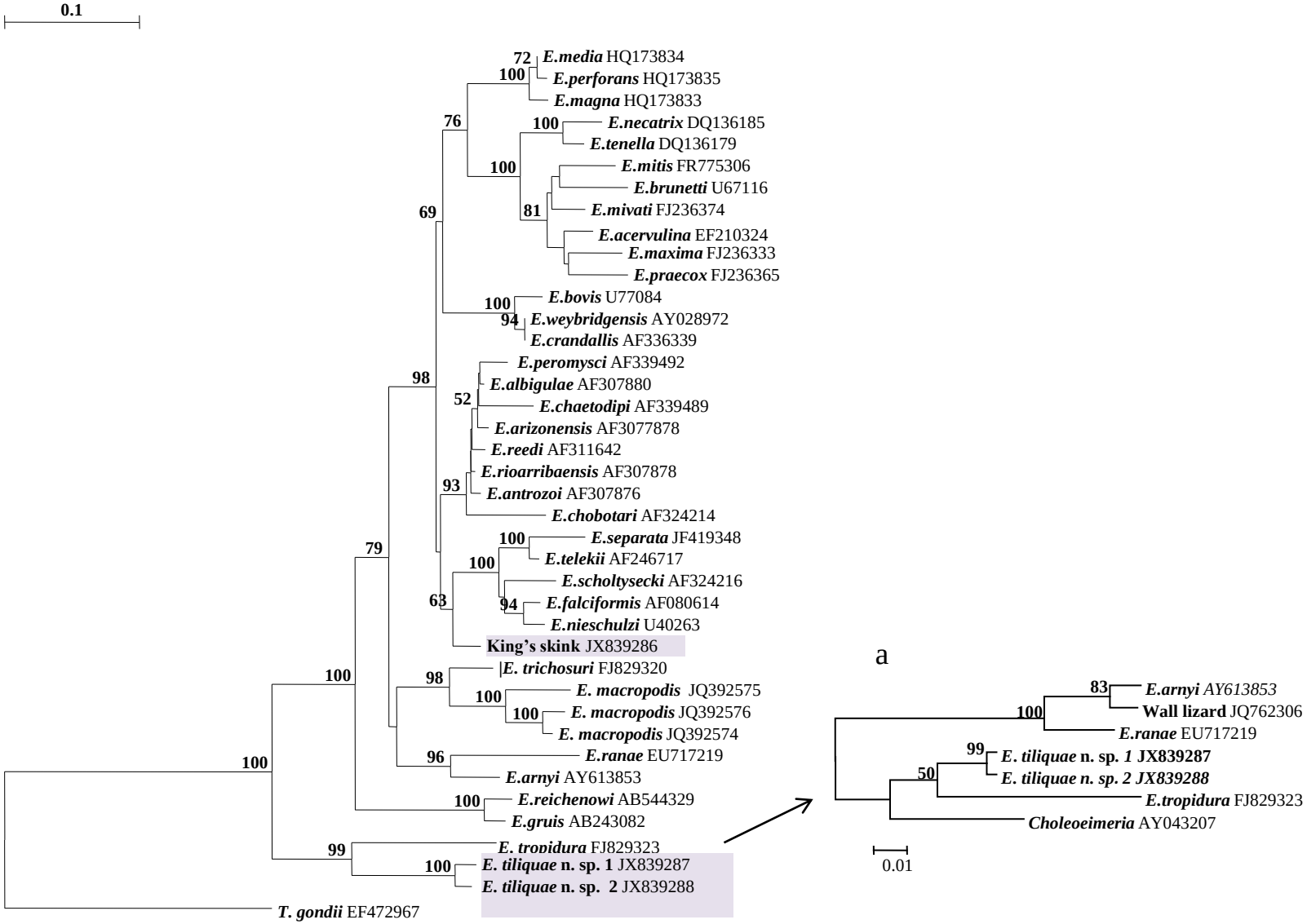
SR
S




0.1
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100
E.perforans HQ173835
E.magna HQ173833
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100
E.necatrix DQ136185
E.tenella DQ136179
100
E.mitis FR775306
E.brunetti U67116
69
81
E.mivati FJ236374
E.acervulina EF210324
E.maxima FJ236333
E.praecox FJ236365
100
E.bovis U77084
94
E.weybridgensis AY028972
E.crandallis AF336339
E.peromysci AF339492
98
E.albigulae AF307880
52
E.chaetodipi AF339489
E.arizonensis AF3077878
E.reedi AF311642
E.rioarribaensis AF307878
93
E.antrozoi AF307876
E.chobotari AF324214
100
E.separata JF419348
79
100
E.telekii AF246717
E.scholtysecki AF324216
63
94
E.falciformis AF080614
E.nieschulzi U40263
King's skink JX839286
100
98
E. trichosuri FJ829320
E. macropodis JQ392575
100
100
E. macropodis JQ392576
E. macropodis JQ392574
100
96
E.ranae EU717219
E.arnyi AY613853
100
E.reichenowi AB544329
E.gruis AB243082
99
E. tropidura FJ829323
100
E. tiliquae n. sp. 1 JX839287
E. tiliquae n. sp. 2 JX839288
T. gondii EF472967
a
83
E.arnyi AY613853
100
Wall lizard JQ762306
E.ranae EU717219
99
E. tiliquae n. sp. 1 JX839287
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Choleoeimeria AY043207
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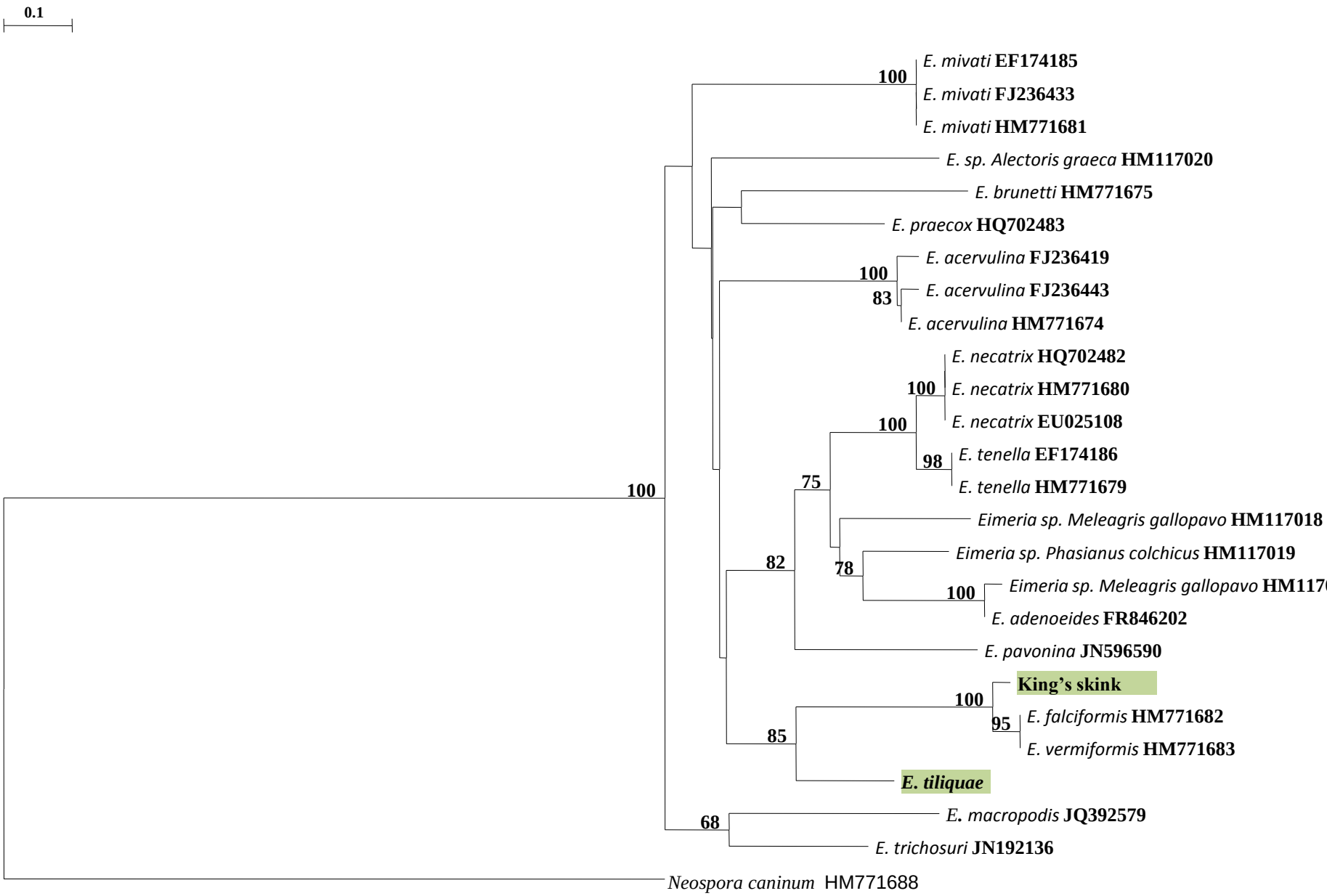
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E. mivati HM771681
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E. brunetti HM771675
E. praecox HQ702483
E. acervulina FJ236419
E. acervulina FJ236443
E. acervulina HM771674
E. necatrix HQ702482
E. necatrix HM771680
E. necatrix EU025108
E. tenella EF174186
E. tenella HM771679
Eimeria sp. Meleagris gallopavo HM117018
Eimeria sp. Phasianus colchicus HM117019
Eimeria sp. Meleagris gallopavo HM117
E. adenoeides FR846202
E. pavonina JN596590
King's skink
E. falciformis HM771682
E. vermiformis HM771683
E. tiliquae
E. macropodis JQ392579
E. trichosuri JN192136
Neospora caninum HM771688
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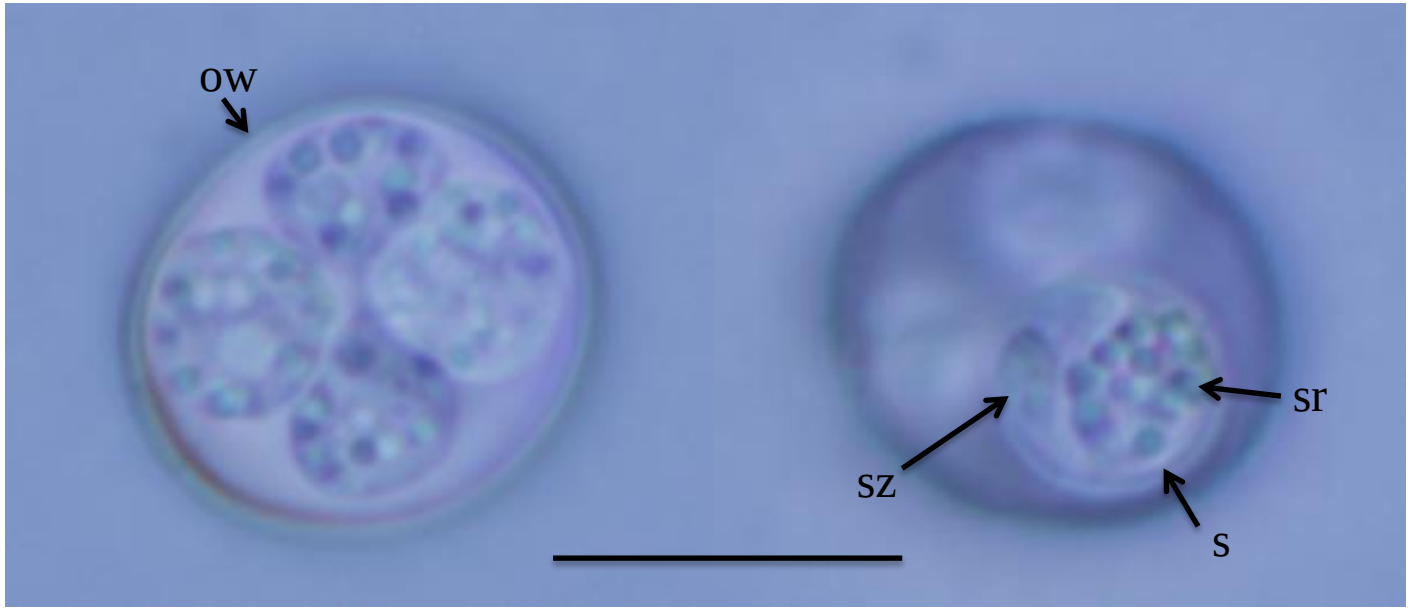
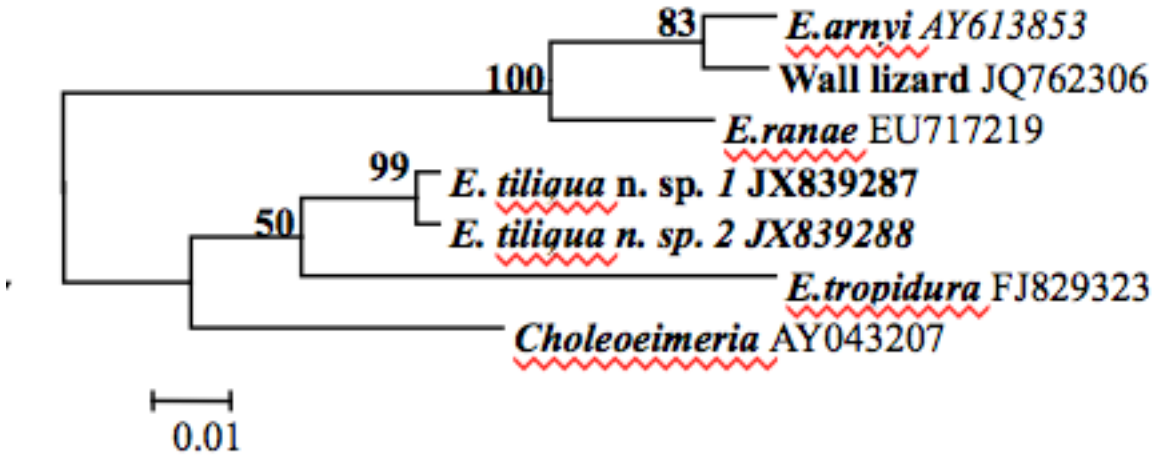
100
83
100
100
98
75
82
78
100
100
95
85
68

*Graphical Abstract (for review)





Highlights

- Description of a new species of *Eimeria* in Lizards
- Morphological characterisaton
- Molecular characterization at 2 loci