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Full length article

Eimeria collieie n. sp. (Apicomplexa:Eimeriidae) from the western long-necked turtle (*Chelodina colliei*)

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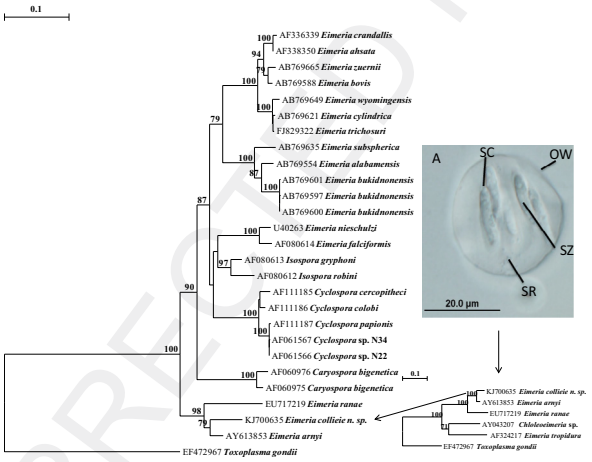
HIGHLIGHTS

- A new *Eimeria* species from the western long-necked turtle (*Chelodina colliei*).
- Morphological characterisation: Unique from all other reported *Eimeria* species.
- Molecular characterisation at 18S, 28S rRNA and COI loci: Related to *Eimeria arnyi*.

GRAPHICAL ABSTRACT



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ABSTRACT

A new species, *Eimeria collieie* n. sp., is described from the western long-necked turtle (*Chelodina colliei*). Sporulated oocysts (n = 35) are spherical to subspherical, with colourless single layer oocyst wall, 0.6 ± 0.2 (0.4–0.7) µm thick. Oocyst with elongated ellipsoid sporocysts. Oocyst length, 29.8 ± 0.4 (28.2–31.0) µm; oocyst width, 29.4 ± 0.3 (28.0–30.8) µm; oocyst length/width (L/W) ratio, 1.0 ± 0.03 (1.0–1.05). Micropyle, oocyst residuum and polar granule were absent. Sporocysts with sporocyst residuum and 2 sporozoites. Sporocyst length, 21.6 ± 0.4 (21.2–22.0) µm; sporocyst width, 6.0 ± 0.3 (5.7–6.3) µm; sporocyst L/W ratio, 3.6 ± 0.2 (3.4–3.8). Stieda, parastieda and substieda bodies absent. Sporozoite length, 14.0 ± 0.2 (13.8–14.2) µm; sporozoite width, 2.6 ± 0.2 (2.4–2.8) µm; sporozoite L/W ratio, 5.46 ± 0.10 (5.4–5.6). Molecular analysis was conducted at three loci; the 18S and 28S ribosomal RNA (rRNA) and the mitochrondial cytochrome oxidase gene (COI). At the 18S rRNA locus, *E. collieie* n. sp. shared 96.4% and 98.3% genetic similarity to *E. ranae* (GenBank accession number: EU717219) and *E. arnyi* (AY613853). At 28S rRNA locus, *E. collieie* n. sp. shared 91.6% genetic similarity to *E. papillata* (GenBank accession number: GU593706) and phylogenetic analysis at this locus placed *E. collieie* n. sp. in a separate clade. At the COI locus, *E. collieie* n. sp. shared 92.7% genetic similarity to *Eimeria setonicis* (GenBank accession number: KF225638) from a

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quokka (*Setonix brachyurus*) in Western Australia. However reptile-derived sequences were not available for the 28S rRNA and the COI loci. Based on morphological and molecular data, this isolate is a new species of coccidian parasite that, to date, has only been found in western long-necked turtles.

1. Introduction

Eimeria is a common coccidian parasite among vertebrate hosts, with over 1700 species described (http://biology.unm.edu/biology/coccidia/marsup.html). Some *Eimeria* species are a significant threat to animals, causing severe clinical disease and economic loss in poultry and production animals (Aarthi et al., 2010; Sun et al., 2009).

Traditionally, identification of *Eimeria* species has been based largely on oocyst morphological characters, host species, pathology and geographic distribution (Duszynski and Wilber, 1997; Tenter et al., 2002). However, some species of *Eimeria* are morphologically identical and numerous exceptions to host specificity have been reported (Barker et al., 1989; Zhao and Duszynski, 2001; Zhao et al., 2001). Due to the limitation of *Eimeria* identification based on traditional methods, molecular tools are necessary to accurately delimit *Eimeria* species (Austen et al., 2014; Hill et al., 2012; Tenter et al., 2002; Yang et al., 2012, 2013).

More than 50 named species of *Eimeria* have been described from turtles worldwide (Duszynski and Morrow, 2014), but to date, no *Eimeria* species have been reported from Australian turtles. Relatively little is known about life cycles, biology and genetic diversity of *Eimeria* species infecting turtles, and no genetic sequences of turtle-derived *Eimeria* species are available. In the present study, we characterised a new species of *Eimeria* from western long-necked turtles (oblong turtle) (*Chelodina colliei*), both morphologically and genetically, and propose the species name *Eimeria collieie*.

2. Materials and methods

2.1. Sample collection

Faecal samples were collected from 25 different wild western long-necked turtles that had been admitted to the Turtle Oblonga Rescue and Rehabilitation Network (TORRN) in Perth, Western Australia, over an 11 month period (February 2013–January 2014). None of the *Eimeria* positive turtles exhibited clinical signs of disease. One of the turtles positive for coccidian oocysts was found to be shedding oocysts over a 4 week period (2 samples positive at weeks 1 and 4). A further 2 samples taken a week later (week 5) were negative. This adult female turtle was highly stressed, having been found stabbed in the neck. Fishing line had been threaded through the holes in her neck and she had been tethered to a stake and left exposed on the bank of a river. After several surgeries, this female was successfully released 70 days later.

2.2. Morphological analysis

Microscopic examination of wet mounts 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 1 cm and kept at room temperature in the dark to facilitate sporulation. Sporulated oocysts were observed using an Olympus DP71 digital micro-imaging camera and images were taken using Nomarski contrast imaging system with a 100× oil immersion objective.

2.3. Isolation of Eimeria oocysts with a micromanipulator

Of the five turtles that were positive for *Eimeria*, three were selected for molecular characterisation. Two of these samples had more than one type of *Eimeria* oocysts present as identified by microscopy and molecular analysis. A 3 axis hydraulic micromanipulator (MO-102, Nirashige, Japan) was used to isolate morphologically identical oocysts for bulk DNA extraction.

2.4. DNA isolation

Total DNA was extracted from 200 mg of each faecal sample using a Power Soil DNA Kit (MolBio, Carlsbad, California) with some modifications as described by Yang et al. (2013). Briefly, the faeces for DNA extraction were subjected to four cycles of freeze/thaw (liquid nitrogen followed by boiling water) to ensure efficient lysis of oocysts before being processed using the manufacturer's protocol. A negative control (no faecal sample) was used in each extraction group.

2.5. PCR amplification and sequencing

Generic apicomplexan primers (CRYPTOF 5'-AAC CTG GTT GAT CCT GCC AGT and CRYPTOR 5'-GCT TGA TCC TTC TGC AGG TTC ACC TAC) were used to amplify the almost full-length 18S rRNA gene as described by Eberhard et al. (1999). The expected PCR product was ~1584 bp. The PCR reaction contained 2.5 μL of 10× Kapa PCR buffer, 3 μL of 25 mM $MgCl_2$, 1.5 μL of 10 nM dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 μL of DNA (about 50 ng) and 14.9 μL of H_2O. PCR cycling conditions were 1 cycle of 94 °C for 3 min, followed by 45 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 2 min and a final extension of 72 °C for 5 min.

The PCR for the 28S rRNA locus was carried out using a nested PCR with the external primers: 28SExF: 5'-TAC CCG CTG AAC TTA AGC-3' and 28SExR: 5'- CMA CCA AGA TCT GCA CTA G-3' as previously described (Schrenzel et al., 2005), which produced a PCR product size of ~1495 bp. The internal primers (28InF: 5'-ACT ATG TTC CCT AGT AAC G-3' and 28SInR 5'- AAC GCT TCG CCA CGA TCC-3') were designed for the present study using Primer 3 (http://frodo.wi.mit.edu/) and produced an amplicon size of 1420 bp. The PCR reaction contained 2.5 μL of 10× Kapa PCR buffer, 2 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTP's, 10 pM of each primer, 1 unit of KapaTaq (Geneworks, Adelaide, SA), 1 μL of DNA (about 50 ng) and 16.9 μL of H_2O. Both primary and secondary PCRs were conducted with the same cycling conditions: 1 cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 sec and a final extension of 72 °C for 5 min.

Amplification of a 465 bp region of the COI locus from samples that were positive at the 18S and 28S loci was conducted as described by Ogedengbe et al. (2011) and Yang et al. (2013).The results of the sequencing reactions were analysed and edited using FinchTV (Version 1.4), compared to existing *Eimeria* spp. 18S and 28S rRNA and COI sequences on GenBank using BLAST searches and aligned with reference genotypes from GenBank using Clustal W in BioEdit (V7.2.5).

2.6. Phylogenetic analysis

Phylogenetic trees were constructed for *Eimeria* spp. at the 18S, 28S and COI loci with additional isolates from GenBank. Distance

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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 6). Bootstrap analyses were conducted using 1000 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).

2.7. 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 35 sporulated oocysts were analysed using Microsoft Office Excel 2010, and results are presented in micrometers as the mean ± SD, with the observed range in parentheses.

3. Results

3.1. Description

3.1.1. Eimeria collieie *n. sp. (Fig. 1)*

3.1.1.1. Diagnosis. Oocysts are spherical to subspherical and measure 29.8 × 29.4 (28.2–31.0 × 28.0–30.8) µm in size with a width to length ratio of 1.01 (1.0–1.05). Micropyle, oocyst residuum, and polar granule were absent. Sporocysts were with compressed sporocyst residuum and 2 sporozoites. Sporocyst size was 21.6 × 6.0 (21.2–22.0 × 5.7–6.3); sporocyst L/W ratio, 3.60 ± 0.20 (3.40–3.80). Stieda, parastieda and substieda bodies were absent. Sporozoite length was 14.0 × 2.6 (13.8–14.2 × 2.4–2.8) µm; sporozoite L/W ratio, 5.46 ± 0.10 (5.36–5.56) (Table 1 and Fig. 1a,b).

3.1.1.2. Type hosts. Chelodina colliei (Gray 1856), oblong snake-necked turtle or south west snake-necked turtle (Kuchling, 2010).

3.1.1.3. Type locality. Perth, Western Australia (31.9522° S, 115.8589° E).

3.1.1.4. Prevalence. Eimeria sp. were detected in 5/25 samples screened, an estimated prevalence of 20.0% (6.8–40.7 CI).

3.1.1.5. Other hosts. Unknown.

3.1.1.6. Prepatent period. Unknown.

3.1.1.7. Patent period. Unknown.

3.1.1.8. Site of infection. Unknown.

3.1.1.9. Sporulation time. 4 to 6 days.

3.1.1.10. Material deposited. Oocysts in 10% formalin and oocyst phototypes were deposited in the Western Australian Museum under the reference number WAM Z68797. DNA sequences were deposited in GenBank under the accession numbers KJ700635, KJ700636 and KJ700637 for the 18S, 28S and COI loci, respectively.

Table 1
Comparison of the morphological features, hosts and known geographical distribution of *Eimeria* spp. in tortoises.

Species and Reference	Host	Distribution	Oocyst	Sporocyst
E. amazonensis n. sp. Lainson et al., 2008	*Geochelone carbonaria*	Pará, North Brazil	Ovoid 11.7 × 9.1 (10.0–13.0 × 8.0–10.0) W: 1 layer, smooth, colourless; PB: –ve	Ellipsoid 6.5 × 3.7 (5–7 × 3.0–4.0) S: +ve
E. broderi Cerruti, 1930	*Testudo graeca*	Greece	Ovoid 30.0 × 19.0 (28.0–32.0 × 18.0–20.0) W: 2 layers, smooth, with micropyle	Ellipsoid 10 × 6–7 S: not reported
E. carbonaria n. sp. Lainson et al., 2008	*G. carbonaria*	Pará, North Brazil	Spherical-subspherical 18.7 × 18.1 (16.3–21.5 × 16.3–21.5) W: 2 layers, brownish, striated; PB: 1 or 2	Ovoid 11.0 × 7.0 (10–11.8 × 6.6–7.4) S: +ve
E. carajasensis n. sp. Lainson et al., 2008	*G. carbonaria*	Pará, North Brazi	Ellipsoidal-subspherical 22.4 × 19.3 (20.0–32.0 × 18.0–25.0) W: 2 layers, brownish, striated; PB: 1	Ovoid 12.0 × 8.0 (11.0–13.0 × 7.0–8.0) S: +ve
E. collieie n. sp. This study	*Chelodina colliei*	Perth, Australia	Spherical to subspherical 29.8 × 29.4 (28.2–31.0 × 28.0–30.8) W: 1 layer, smooth, colourless; PB: –ve	ellipsoid 21.6 × 6.0 (21.2–22 × 5.7–6.3) S: –ve
E. geochelona Couch et al., 1996	*G. nigra*	Galápagos Islands	Ellipsoid-ovoid 21.6 × 18.1 (18–25 × 16–20) W: 2 layers, smooth, colourless; PB: 1	Ellipsoid 10.7 × 7 (8–12 × 5–8) S: +ve
E. jaboti Carini, 1942	*G. denticulata*	São Paulo, Brazil	Spherical-subspherical 17.0–19.0 × 15.0–17.0 W: 3 layers, colourless; PB: 1	Oval 10.0–11.0 × 6.0–6.6 S: –ve
E. jirkamoraveci Siroký et al. (2006)	*B. heliostemma*	Iquitos Peru	Ovoid to almost spherical 10.6 × 8.9 (8–12 × 7–10) W: 1 layer, smooth, colourless; PB: –ve	ellipsoidal 7.2 × 4.1 (6.0–8.0 × 4–4.5) S: +ve
E. lainsoni Lainson et al., 1990 Hürkovâ et al., 2000	*G. denticulata*	Pará, North Brazil	Spherical-subspherical 19.2 × 18.6 (15.0–20.0 × 14.0–19.0) W: 1 layer, smooth, colourless; PB: –ve	Ellipsoid 8.8 × 7.3 (8.0–9.0 × 7.0–7.9) S: –ve
E. motelo Hürkovâ et al., 2000	*G. denticulata*	Iquitos, Peru	Ellipsoid 17.0 × 9.4 (15–19.0 × 8.5–11.0) W: 1 layer, smooth, colourless; PB: –ve	Ellipsoid 8.5 × 4.4 (7.5–10 × 4.4–5.0) S: +ve
E. paynei Ernst et al., 1971	*Gopherus polyphemus*	Georgia, USA	Ellipsoid 23.2 × 18.6 (19.0–26.0 × 16.0–20.0) W: 2 layers, brownish-yellow; PB: 1–3	Ovoid 13.2 × 8.1 (12.0–14.0 × 7.0–9.0) S: +ve
E. wellcomei n. sp. Lainson et al., 2008	*G. carbonaria*	Pará, North Brazil	Ellipsoid-cylindrical 30.3 × 16.4 (28–32.0 × 15.0–17.0) W: 1 layer, smooth, colourless; PB: –ve	Subspherical-ellipsoidal 9.6 × 7.9 (9.0–10.0 × 7.0–9.0) S: –ve

PB = polar body; S = Stieda body; W = oocyst wall.

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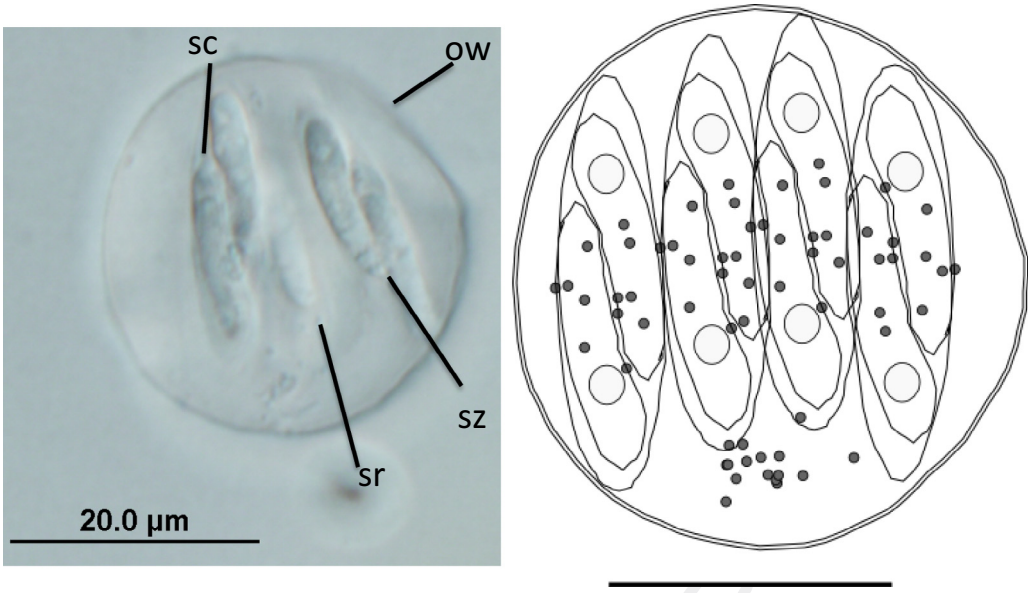

Fig. 1. (a) Nomarski interference-contrast photomicrographs of *E. collieie* n. sp. oocyst showing 4 elongated sporocysts. Abbreviations: ow = oocyst wall, sc = sporocyst, sr = sporocyst residiuum, sz = sporozoite. Note the abundant sporocyst residuum, which occupies much of the sporocyst volume. Scale bar = 20 µm. (b) Composite line drawing of *Eimeria collieie* n. sp. sporulated oocyst. Scale bar = 10 µm.

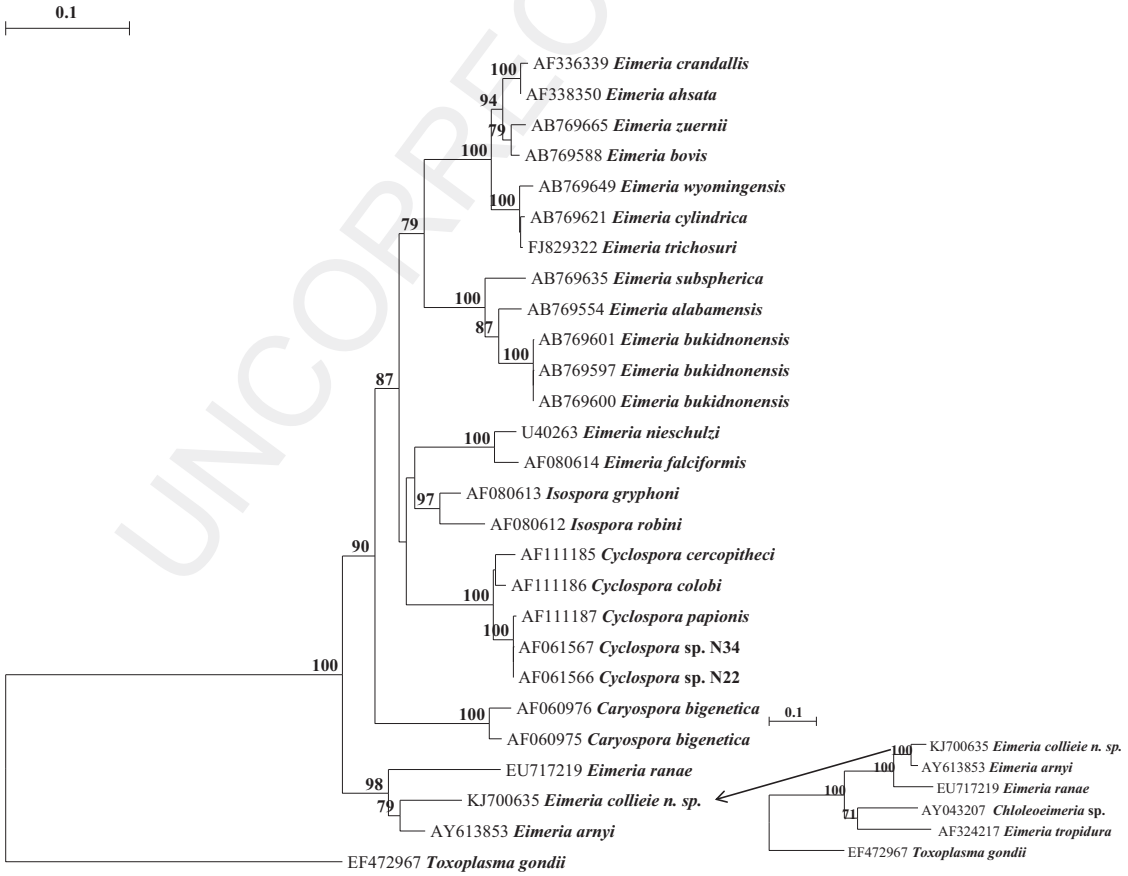

Fig. 2. Evolutionary relationships of *E. collieie* n. sp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

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3.1.1.11. Etymology. This species is named *Eimeria collieie* n. sp. after its host *Chelodina colliei* (western long-necked turtle).

3.2. Phylogenetic analysis of E. collieie n. sp. at the 18S locus

Initial sequencing of PCR amplicons from three *Eimeria* positive turtles produced mixed chromatograms from two of them, indicating infection with multiple *Eimeria* species. As a result of this, morphologically identical oocysts were isolated using a micromanipulator, and molecular characterisation was conducted on two groups of oocysts that appeared to be morphologically identical. The first group comprised *E. collieie* oocysts. The second group still exhibited mixed chromatograms and therefore was not described further. Phylogenetic analyses of the partial nucleotide sequences from *E. collieie* n. sp. at the 18S locus using Distance, Parsimony and ML analyses produced similar results (Fig. 2 NJ tree shown). *Eimeria collieie* n. sp. shared 98.3% genetic similarity with *Eimeria arnyi* from a prairie ringneck snake (Upton and Oppert, 1991, GenBank accession no: AY613853). It also showed 96.4% genetic similarity with *E. ranae* from a common frog (GenBank accession number: EU717219) and 93.0% genetic similarity with *Eimeria tropidura* (GenBank accession number: AF324217). *Eimeria collieie* n. sp., *Eimeria arnyi* and *E. ranae* formed a separate clade (Fig. 2). A 18S rRNA sequence from *Chloleoeimeria* sp. (GenBank accession number: AY043207) was also available, but only 1071 bp of common sequence could be used for the phylogenetic analysis. Therefore, an insert tree (Fig. 2a) was generated including the 18S rRNA sequences from *Chloleoeimeria* sp., *Eimeria collieie* n. sp, *Eimeria arnyi*, *Eimeria tropidura* and *E. ranae*. This analysis revealed that *Chloleoeimeria* sp. was most closely related to *Eimeria tropidura* with 93.0% genetic similarity while *Eimeria collieie* n. sp. shared 92.9% genetic similarity with this *Chloleoeimeria* sp.

3.3. Phylogenetic analysis of E. collieie n. sp. at the 28S locus

Three identical 28S rRNA PCR amplicons from three separate western long-necked turtles were obtained. Unfortunately, no 28S rRNA reptile-derived *Eimeria* sequences were available in GenBank and therefore phylogenetic analysis could only be conducted on available *Eimeria* 28S rRNA sequences and other coccidian 28S sequences including *Isospora* spp., *Goussi spp.*, *Benoitia besnoiti*, *Neospora caninum*, *Hammondia hammondi*, *Toxoplasma gondii* and *Sarcocystis arctica*. *Frenkelia microti* (GenBank accession number: AF044252) was used as an outgroup. *Eimeria collieie* n. sp. formed a clade by itself (Fig. 3) and shared the highest genetic similarity (92.2%) with *E. papillata* from chickens (*Gallus gallus*) (GenBank accession number: GU593706), followed by 90.2%–89.5% similarity with *Isospora* isolates (GenBank accession numbers: AY283866 and AY283865).

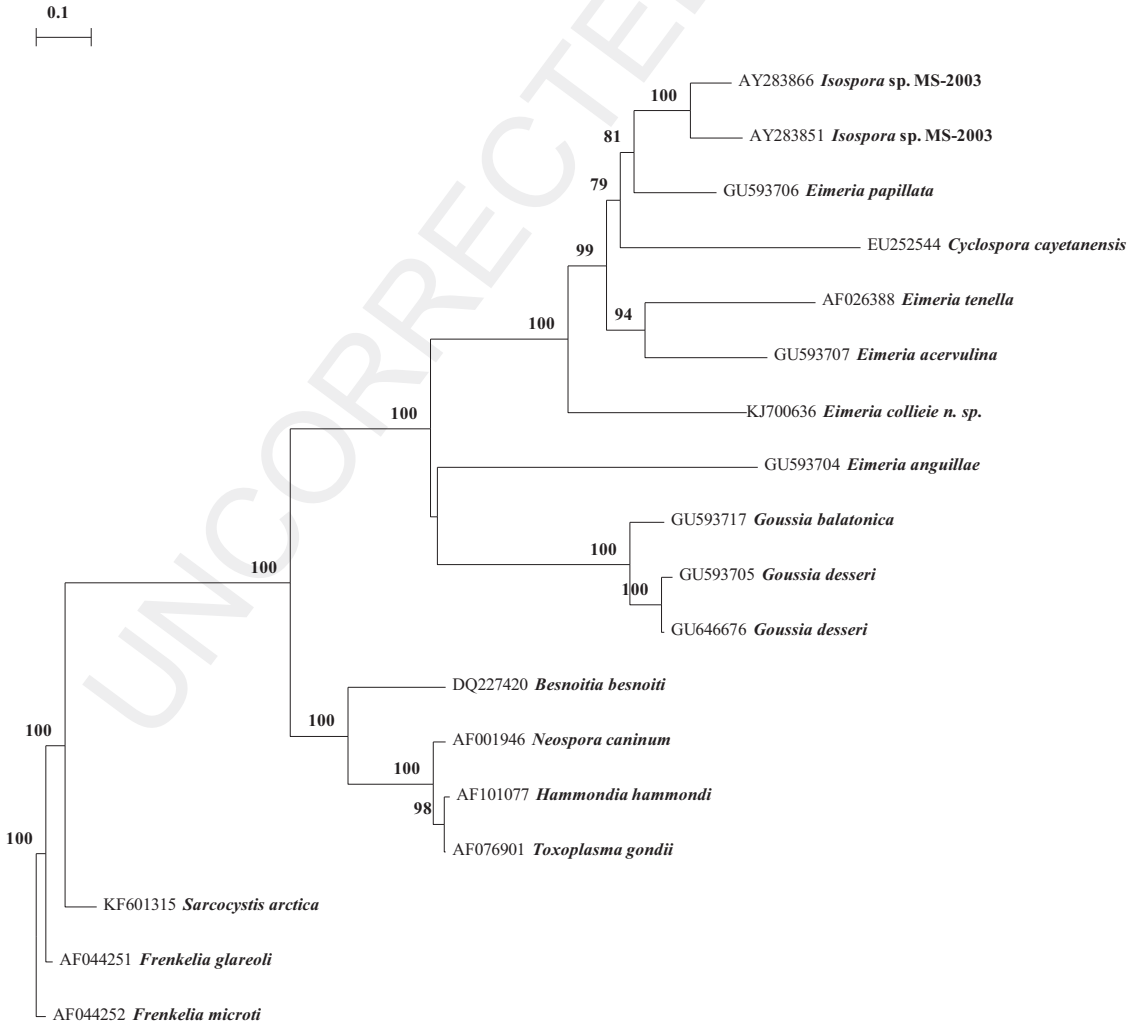

Fig. 3. Evolutionary relationships of *E. collieie* n. sp. inferred by distance analysis of 28S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

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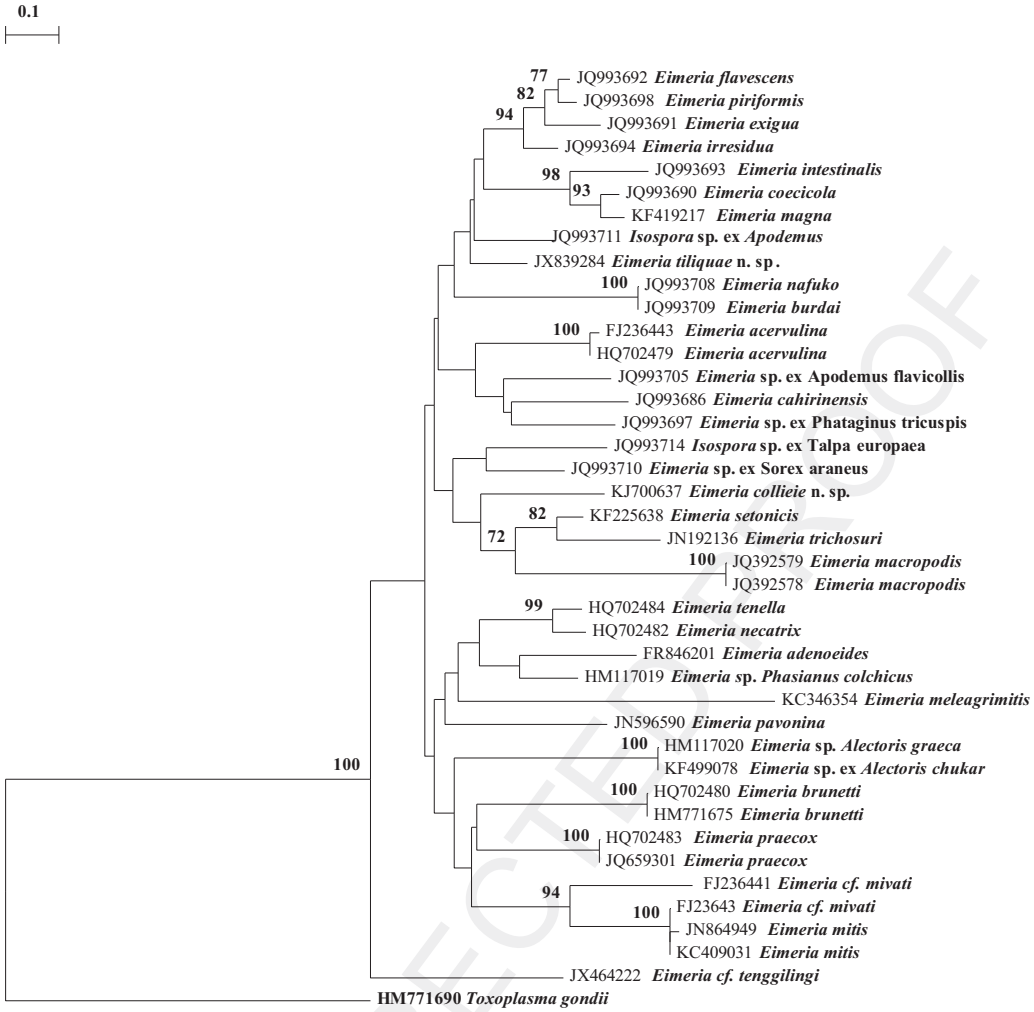

Fig. 4. Evolutionary relationships of *E. collieie* n. sp. inferred by distance analysis of mitochrondial cytochrome oxidase gene (COI). Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

3.4. *Phylogenetic analysis of* E. collieie *n. sp. at the COI locus*

Direct sequencing of the COI gene fragment from 3 turtles produced a clean chromatogram, indicating that only one sequence was present. Sequences from the 3 isolates were 100% identical. Reptile-derived sequences were not available at the COI locus and phylogenetic analysis placed *E. collieie* n. sp. in a separate clade. It shared 92.7% genetic similarity to *Eimeria setonicis* (GenBank accession number: KF225638) from a quokka (*Setonix brachyurus*) from Western Australia (Justin et al., 2014) and other marsupial-derived isolates (Fig. 4).

4. Discussion

Chelodina are a genus of chelid turtle found in Australia, East Timor, Indonesia and New Guinea (Georges and Thomson, 2010). These turtles are easily distinguished from other Australian chelids by their long necks, clawed forelimbs and contacting gular scutes on the plastron (Cogger, 2014; Georges and Thompson, 2010). The head and long neck fold sideways under the shell.

The western long-necked turtle (*Chelodina colliei* Gray, 1856 previously *Chelodina oblonga*) (ICZN, 2013) is also known as the narrow-breasted snake-necked turtle or oblong turtle. This carnivorous freshwater turtle is a native to the Perth metropolitan area and is also found throughout the south-west of Western Australia. It has an oval shaped black carapace (shell), which has a flat profile. Adults may have a carapace length of 31 cm. The western long-necked turtle shares its range with the critically endangered western swamp tortoise (*Pseudemydura umbrina*) (Cogger, 2014).

The western long-necked turtles examined in the present study were housed at the home of an experienced turtle carer in Perth, Western Australia, having been rescued by TORRN. Four of the five turtles that were positive for *Eimeria* were released back into the wild. Wild turtles frequently end up in care due to a variety of reasons including motor vehicle collisions, dog attacks, loss of habitat, immersion in salt water or illness. Approximately 85% of all turtles that go into home care with a TORRN carer are released back into the wild.

The overall prevalence of *Eimeria* sp. in the western long-necked turtle was approximately 20.0%. A previous study by Lainson and Naiff (1998) reported a prevalence of 9/18 (50.0%) in the freshwater turtle *Peltocephalus dumerilianus* from Brazil. Another study on *Eimeria* in the Arakan forest turtle (*Heosemys depressa*) by Široký and Modrý (2006) reported a prevalence of 3/9 (33.3%).

Sporulated oocysts of *Eimeria collieie* n. sp. measured 29.8 × 29.4 (28.2–31.0 × 28.0–30.8) µm with a L/W ratio of 1.0 (1.0–1.05). Široký et al. (2006) mophologically described an eimarian species from the toad-headed, side-necked turtle *Batrachemys heliostemma* (Testudines: Chelidae) in Peru, named as *E. jirkamoraveci*, which possesses mitra-shaped oocysts. The oocysts are ovoid to almost spherical, 10.6 (8–12) × 8 (7–10) µm, and are therefore considerably

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smaller than oocysts of *Eimeria collieie* n. sp. Lainson et al. (2008) reported four new *Eimeria* species in *Geochelone* spp. (Chelonia: Testudinidae) from Amazonian, Brazil. Along with other six *Eimeria* species reported earlier, the morphological features of 12 *Eimeria* species including *E. collieie* n. sp. were listed in Table 1 for comparison. There is no other *Eimeria* species from turtles with the similar morphological features as *E. collieie* n. sp. (Table 1). Unfortunately, genetic sequences for all the 11 *Eimeria* species were not available and therefore it was not possible to compare them genetically. Phylogenetic analysis at the 18S locus confirmed the validity of *E. collieie* n. sp. It shared its closest genetic similarity with *E. ranae* (98.3%). The genetic similarity between *E. collieie* n. sp. and *E. arnyi* was 96.4%, which was isolated from the gall bladder of a prairie ringneck snake, *Spalerosophis diadema* (Jirku et al., 2002).

The genetic similarity between *E. collieie* n. sp. and *E. ranae* 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. collieie* n. sp. is clearly a separate species.

In the present study, morphological and molecular data were used to describe *E. collieie* n. sp. found in the faeces of western long-necked turtles in Western Australia. Future studies need to concentrate on obtaining morphologically characterised *Eimeria* species derived from other turtle species in Australia and generating sequence data that are directly related to described species. Analysing the isolates at multiple gene loci will also provide a more in-depth analysis of the evolution of turtle-derived *Eimeria* spp.

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