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Putative intranuclear coccidium in *Mauremys leprosa* (Schweigger) from Morocco

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Abstract: Although intranuclear coccidiosis was first identified in chelonians less than 30 years ago, it is now considered an important emerging disease. Symptoms include anorexia, weakness and weight loss, potentially leading to death of the infected animal. The use of molecular tools has led to improved diagnosis and has also led to an increase in known host species. Here we report a putative intranuclear coccidium in *Mauremys leprosa* (Schweigger), from Morocco, based on 18S rDNA sequence analysis. This is, to the best of our knowledge, the first report of this parasite from a freshwater terrapin species.

Keywords: Coccidia, freshwater turtles, 18s rRNA

Intranuclear coccidiosis is an emerging disease in chelonians (Hofmannová et al. 2019). Although over 30 coccidian species are known from tortoises and turtles, most of these are members of the genus *Eimeria* Schneider, 1875, and there is little evidence of pathology caused by these parasitic protists. Jacobson et al. (1994) reported the first case of intranuclear coccidiosis in chelonians, noting that the two infected radiated tortoises *Astrochelys radiata* (Shaw) were so severely afflicted that they were euthanised. Since then, intranuclear coccidiosis has been reported in various tortoises from around the world (Garner et al. 2006, Kolesnik et al. 2017), to the point of being listed among the most important emerging infectious diseases in tortoises (Gibbons and Steffes 2013).

Coproscoical detection of oocysts can be difficult due to their fragility and small dimensions (Bardi et al. 2019), whereas genetic tools such as PCR on faecal, cloacal swab or blood samples have been used to diagnose the disease, and enlarge the known host range (Stilwell et al. 2017). At the same time, genetic data are necessary to define the phylogenetic position of the parasite involved. Currently tortoise intranuclear coccidiosis (TINC) is caused by an indeterminate genus and species of coccidium (Bardi et al. 2019), and there are limited genetic data published.

Partial 18S rRNA gene sequences have been analysed, either a short fragment in quantitative PCR for diagnosis (Alvarez et al. 2013), or longer fragments to permit phylogenetic analyses (Innis et al. 2007, Hofmannová et al.

2019). The first of these phylogenetic studies indicated that TINC from a Sulawesi tortoise (*Indotestudo forstenii* (Schlegel et Müller)) formed a highly distinct lineage, sister taxon to the Eimeriidae (Innis et al. 2007). In contrast, Hofmannová et al. (2019) showed that a sample from a leopard tortoise (*Stigmochelys pardalis* (Bell)) was genetically extremely similar to the earlier isolate.

Here we report results of a molecular screening of 467 *Mauremys leprosa* (Schweigger) from Morocco and, to the best of our knowledge, the first identification of an apparent TINC in a freshwater terrapin species.

Samples of two subspecies of *M. leprosa* (*M. leprosa leprosa* (Schweigger) and *M. leprosa saharica* Schleich) were collected from 30 different localities in Morocco during the period of March–June 2018 (Fig. 1). Turtles were caught by hand or with baited fish-traps. Blood samples were collected with insulin syringes from the jugular vein (0.1–0.5 ml depending on the weight and size of each individual). These specimens were collected as part of a study on other parasites, in particular haemogregarines, and precise locality details are given in Laghzaoui et al. (2020). For genetic analysis, 467 blood samples of turtles were screened with PCR using primers HepF300 and HepR900 (Ujvari et al. 2004). Although these primers were designed to amplify haemogregarines, they are known to amplify members of the Eimeriidae (see Harris et al. 2012), as well as more distantly related organisms such as Stramenopiles (Maia et al. 2012).

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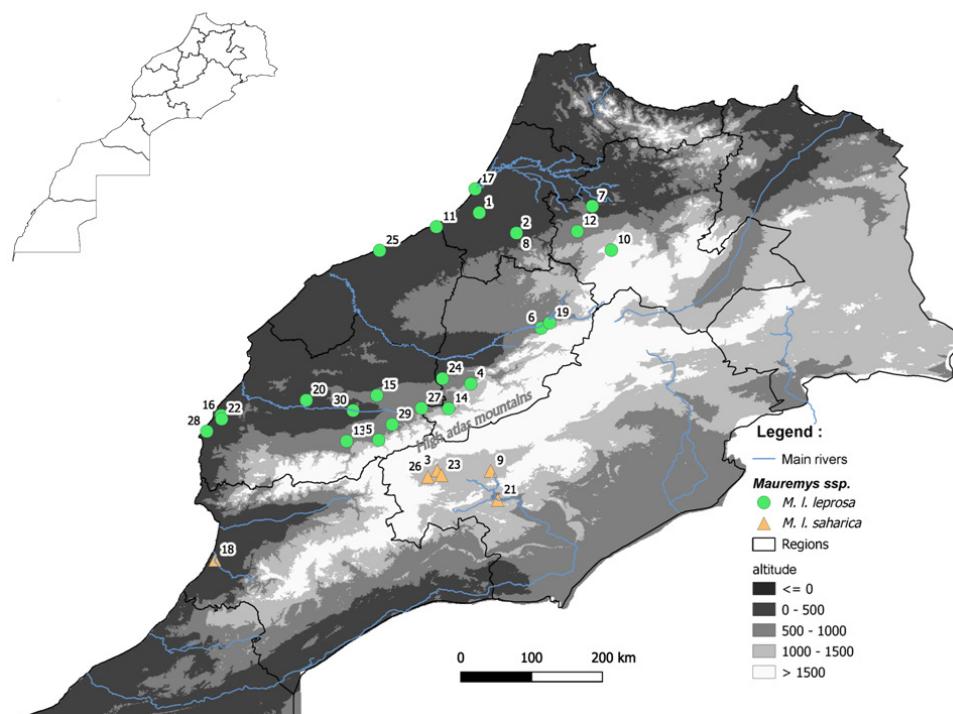


Fig. 1. Distribution of sampling localities for *Mauremys leprosa leprosa* (Schweigger) (green circle) and *Mauremys leprosa saharica* Schleich (yellow triangle) within Morocco included in this study. The single host individual (ML262) infected by a putative intranuclear coccidium was identified in population 22 (Had Dra – Essaouira).

For DNA extraction we followed a standard saline protocol (Maia et al. 2014). PCR reactions were run in a 20 μ l reaction mixture containing 11.6 μ l pure H₂O, 1.5 μ l MgCl₂, 0.4 μ l of each nucleotide (50 μ M), 2 μ l of PCR buffer (200 mM Tris-HCl, 500 mM KCl), 1 μ l of each primer (10 μ M), 0.4 mg/ml of albumin (BSA; Roche, Manheim, Germany), 2 μ l of DNA and 0.1 μ l (1 unit) of Taq DNA polymerase. The PCR reaction mix was heated to 94 °C for 3 min, and then amplification was performed through 35 cycles at 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min, followed by a final 10 min extension at 72 °C. Positive PCR products were purified and sequenced by a commercial sequencing facility (Genewiz, Takeley, UK).

Sequences were preliminarily identified by comparison against published sequences on GenBank using BLAST (Altschup et al. 1990). A single specimen (male, weighing 232 g, 125 mm carapace length – specimen sample code ML262) from the town of Had Dra, Essaouira Province, Morocco (31.64, -9.58; 94 m above sea level), gave a BLAST result with over 99% similarity with AY728896, intranuclear coccidium from *Indotestudo forstenii* (see Innis et al. 2007). Seventeen other specimens collected at this locality were all negative for this type of parasite using this screening approach, as were all other individuals from other localities.

The new sequence was combined with published data from GenBank to estimate phylogenetic relationships. Three sequences from species of *Goussia* Labb  , 1896, were included as outgroups. The resulting alignment included 62 sequences, with a total aligned length of 612 base pairs, except for the comparative sequence available for the specimen of the tortoise *S. pardalis* which is

shorter (330 bp). The new sequence has been submitted to GenBank (length 593 bp, accession number MZ508499). The Maximum likelihood (ML) method was employed to estimate a phylogeny, using PhyML 3.0. (Guindon et al. 2010) both for defining the most appropriate model of molecular evolution under the AIC criterium, and producing a phylogenetic tree. The chosen model was GTR + I + G and a phylogenetic tree was inferred with 1,000 bootstrap replicates.

Our estimate of phylogenetic relationships (Fig. 2) is similar to those of previous studies (Innis et al. 2007, Hofmannov   et al. 2019), in that the TINC are a distinct lineage that is the sister taxa to the majority of the Eimeriidae. The sample from *M. leprosa* is sister taxa to that from *S. pardalis* and *I. forstenii*. Compared to the sequence from *I. forstenii*, our sample differs by 5 or 6 nucleotides across the aligned region (there is one ambiguous position in the sequence for *I. forstenii*). The comparative sequence available for the specimen of the tortoise *S. pardalis* (KY683841) is shorter (330 bp; although the total length is 663, it only partially overlaps the region analysed in this study). However, across the aligned region, the sequence from *S. pardalis* is identical to that from *I. forstenii*, and differs from the new sequence from *M. leprosa* by 3 nucleotides. The 18S rRNA is a very slow evolving gene, and many other species of apicomplexan parasites differ from each other by less than five nucleotides across the corresponding region (e.g., Tom   et al. 2019). It is therefore not unreasonable to hypothesise that the form from *M. leprosa* may represent a distinct species of intranuclear coccidium.

To conclude, this is, to the best of our knowledge, the first report of a possible TINC within a freshwater terrapin

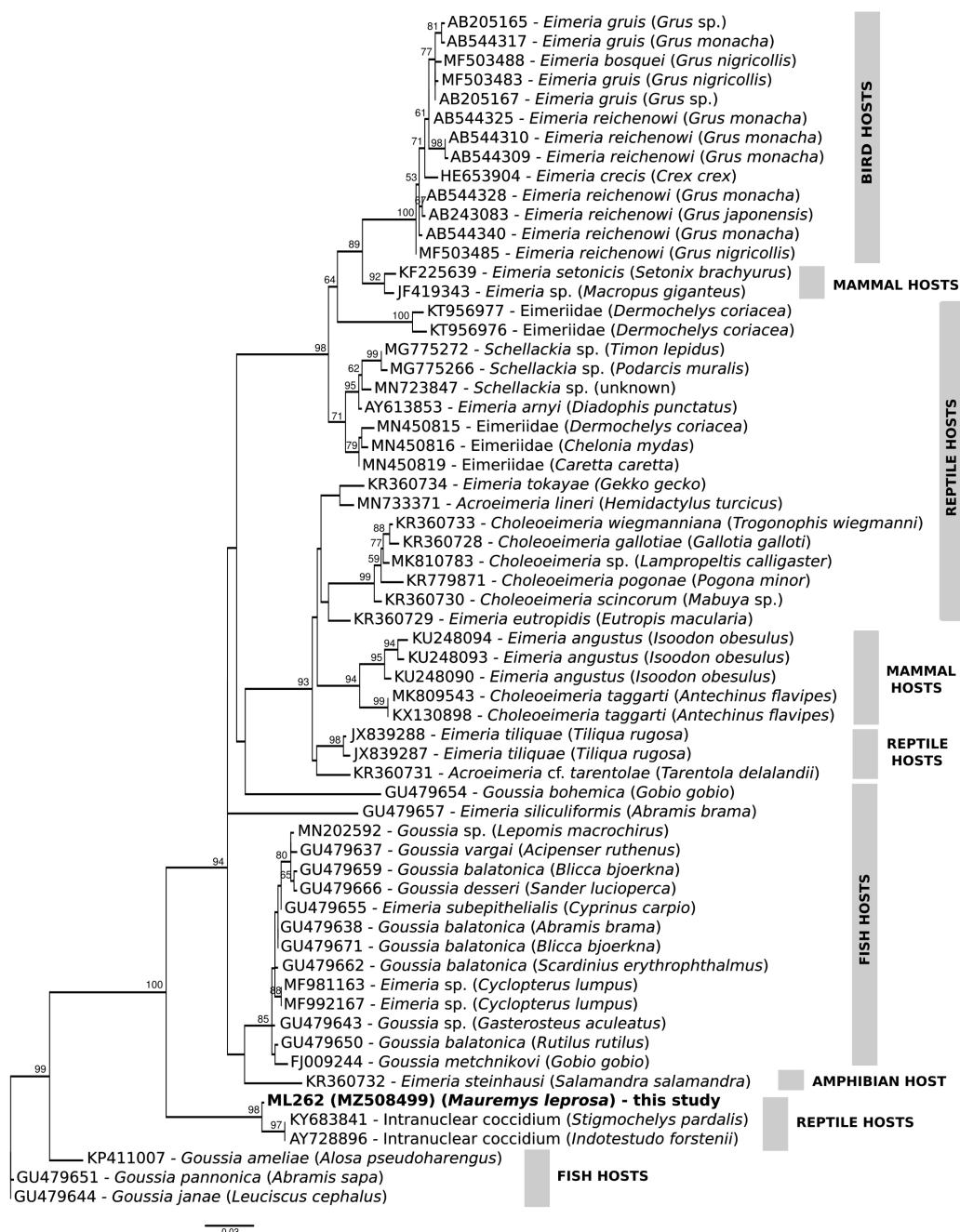


Fig. 2. Estimate of the phylogenetic relationships of the putative intranuclear coccidium (host code ML262) isolated from *Mauremys leprosa* (Schweigger) from Morocco, based on a Maximum Likelihood approach. Bootstrap support values > 50% are indicated beside nodes.

species, and the first in *M. leprosa*. The infected individual looked in good health and showed no visible symptoms. The ratio of size/weight can be used as an estimate of body condition (reviewed in Green 2000). In the population at Had Dra, adult males ($n = 9$) had an average size of 134 ± 19 mm (mean \pm SD), and average weight of 285 ± 79 g. The infected individual (125 mm length, 232 g weight) showed no evident deviation from the expected ratio, which might have indicated poor body condition.

Given the potential importance of this disease, additional screening efforts should be made on wild populations of chelonians to establish how widespread these parasites are, and to determine which the host species are. More variable genetic markers and morphological data are also needed to determine if the different genetic lineages identified may correspond to distinct species, in which case it would become imperative to assess if some species of TINC cause more severe symptoms than others.

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