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PROTOZOOLOGY - ORIGINAL PAPER

Molecular characterization of *Eimeria* sp. from Galápagos giant tortoises (*Chelonoidis* spp.)

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Abstract

Galápagos giant tortoises are an essential component of their ecosystem and evaluation of parasites in their populations is essential for the management of conservation processes. Coccidiosis is the most common intestinal infection in free-living and captive reptiles. The aim of this study was to characterize molecularly the presence of *Eimeria* sp. in captive reared giant tortoises from Santa Cruz, Santiago, Española, and Pinzon Islands hatched and housed at the tortoise rearing center on Santa Cruz Island, Galápagos, by sequencing of the 18S rRNA gene. Galápagos. All samples were previously analyzed by coproparasitoscopic flotation technique and PCR for molecular identification. The results obtained by microscopy examination showed oocysts in all samples. PCR and sequencing indicated the presence *Eimeri*a sp., showing a similarity percentage of 98% with *Eimeria environmental*. In conclusion, we identified a group of coccidia of the genus *Eimeria* sp. (MK909931) in Galápagos tortoises.

Keywords Molecular characterization · *Eimeria* sp. · Giant tortoises · *Chelonoidis* spp. · Galápagos

Introduction

Today, giant tortoises survive in the wild only in the Galápagos Islands and the Aldabra atoll in the Indian Ocean. The extinction of tortoise populations has occurred relatively recently as a result of human activities and the introduction of invasive species into their environments (Caccone and Powell 2009). The long-lasting impact of this loss may not have been fully understood until now, as the role of giant tortoises in ecosystems has only recently been recognized (Froyd et al. 2014). However, restoration programs are carried out in the Galápagos Islands to recover giant tortoise populations to their historical distribution and abundance prior to human intervention (Tapia et al. 2017).

The government of Ecuador and the Charles Darwin Research station established a captive breeding and rearing program for depleted tortoise populations in 1965. The focus of this program was threatened species. The program initiated with the Pinzon Island tortoise (*Chelonoidis duncanensis*). Estimated 100–200-old adult tortoises remained on the island, and no juveniles or even young adults (Tapia et al. 2015).

Parasitism in wild animal populations is recognized as a fundamental factor that can affect fertility and survival. Parasitic infections in reptiles are generally asymptomatic, but they can also cause severe disease leading to mortality in chelonians (Chapman et al. 2016). Although imperceptible most of the time, parasitic infection can significantly reduce existing populations (Fournie et al. 2015; Hudson et al. 1998). Therefore, the evaluation of the parasite communities in wild animal populations is important for conservation management (Fournie et al. 2015).

Intestinal coccidiosis is very common in free-living and captive reptiles (Ras-Norynska and Sokol 2015). Coccidia are among the most common gastrointestinal parasite species in tortoises and vary among species (Innis et al. 2007). It is believed that the presence of this type of parasite in Galápagos giant tortoises is transferred orally by contamination of water

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with fecal material from infected animals or by contamination of plants (Chapman et al. 2016). Oocysts, the resistant stage, are eliminated in feces without sporulating. They have an undifferentiated cytoplasmic mass called the spore, which is surrounded by a double membrane. Under the right conditions of oxygenation, temperature, and humidity, sporocysts are formed, initiating the infective phase of the parasite (Blake and Tomley 2014).

These infections usually occur in the intestinal tract or in the associated ducts and organs. The cycle ends with the formation of oocysts, which are then passed from the host to their feces, thus ending the infection (Duszynski and Morrow 2014). However, in contaminated environments, transmission to other individuals or re-infection can occur when tortoise food or water is contaminated with oocysts.

The aim of this study was to characterize molecularly the presence of *Eimeria* sp. in captive reared giant tortoises from Santa Cruz, Santiago, Española, and Pinzon Islands hatched and housed at the tortoise rearing center on Santa Cruz Island, Galápagos, by sequencing of the 18S rRNA gene.

Materials and methods

Study area

This study was carried out on tortoises hatched and reared at the Fausto Llerena Tortoise Center of the Galápagos National Park on Santa Cruz Island in the Galápagos.

Sampling

There was a total of 110 fecal samples from juvenile tortoises (*Chelonoidis* spp.) between 2 and 3 years old. The tortoises held in the pre-adaptation corral where the fecal samples were collected included tortoises from Española (*C. hoodensis*), Santa Cruz (*C. donfaustoi*), Pinzón (*C. duncanensis*), and Santiago (*C. darwini*) Islands hatched and housed at the tortoise rearing center on Santa Cruz Island, Galápagos.

Parasitological examination

Samples were analyzed to identify coccidia by light microscopy using the flotation technique with a supersaturated solution of sodium chloride. Two grams of fecal matter was homogenized with distilled water in plastic container, and then each sample was filtered (size of the filter 0.2 cm) to remove the solid material. The filtrate was placed in a 15 ml tube and centrifuged at 3400 rom for 2 min. The supernatant was then removed and a saturated NaCl solution of 1.3 specific gravity was added (210 g of NaCl in 800 ml distilled water), until it formed a meniscus at the edge of the tube. A coverslip was placed on the top and the tube sat for 15 to 30 min to allow the oocysts to fix to the coverslip. The sample was then observed under a microscope at × 100 and × 400.

Fecal samples with the highest number of coccidia eggs from each tortoise species were selected to be analyzed by PCR.

DNA isolation, PCR, and sequencing

The selected stool samples were subjected to a DNA extraction procedure using a commercial QIAamp DNA Stool Mini kit (Qiagen, Chadstone, AU), in accordance with the manufacturer's instructions, except for the resuspension step where 50 μl of buffer AE were used. The product was quantified in the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and 2 ng placed on a 0.8% agarose gel to evaluate its quality.

The molecular technique used for the identification of coccidia was based on the protocol established by Chapman et al. (2016) for 18S rRNA of coccidia. The conditions for the PCR test were optimized with the available equipment and reagents. A generic forward eukaryotic primer (3F: 5′GTT CGG AGA GGG A-3′) and a specific reverse apicomplex primer were used (Api1R: 5′-TAA TCT ATC ATC CCC ACG ATG C-3′) (Saffo et al. 2010). The total reaction added up to a volume of 25 μl, composed of 2.5 μl of Colorless GoTaq® Reaction Buffer 5X (Promega, USA), 4 μl of the dNTP mixture (Invitrogen, USA) at a final concentration of 1.25 mM each, 2.5 μl of 10 mM each primer (Invitrogen, USA), at a concentration of 10 mM 1.25 units of GoTaq® DNA Polymerase (Promega, USA), 3 mM of magnesium chloride (MgCl 2), and 100 ng of template DNA, completing the missing volume with nuclease-free water to a final volume of 25 μl.

PCR was carried out in a C1000 touch thermal cycler Bio-Rad®. Cycling conditions comprised an initial activation step of 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, hybridization at 57 °C for 30 s, and extension at 72 °C for 2 min, with a final extension step of 72 °C for 10 min. Products were visualized using a 1% agarose gel stained using SYBR Safe (Life Technologies Pty Ltd, Grand Island, NY, USA).

PCR amplified fragments were analyzed by horizontal cube electrophoresis and the bands were visualized under an ultraviolet light transilluminator (BioPh-ItTM System UVP—Transilluminator UV/white light) and photo-documented (Logic 200 PRO Serie). The sizes of the amplified fragments were compared to a 1 Kb Plus DNA Ladder marker (Invitrogen, Thermo Fisher Scientific).

The amplified products were purified using a QIAquick Gel Extraction Kit (Qiagen, Chadstone, AU), and sequenced by Sanger method at Macrogen Inc. Company (Seoul, South Korea). The chromatograms obtained were analyzed to obtain the consensus sequences. A heuristic search was completed automatically by applying the neighbor-join algorithms, using

the maximum composite probability (MCL) method, with 2000 bootstrap replicates to infer the relationship between them and others previously reported. All bioinformatics performed were done with MEGA bioinformatics software, version 7.0.21 (Kumar et al. 2016). The 18S ribosomal RNA sequences of Apicomplexans were obtained from GenBank.

Results and discussion

Coproparasite examinations revealed the presence of oocysts in 21 of 110 fecal samples (19%) analyzed by flotation technique.

Couch et al. (1996) observed the presence of *Eimeria geochelona* in a Galápagos giant tortoise from the Fausto Llerena Tortoise Center; a high number of discharged oocysts were observed in only one of 26 (4%) individuals. This data is in contrast with results obtained in our study, where 21 of 110 (19%) samples from the juvenile tortoises presented oocysts. All sampled tortoises were kept in separate pens without contact with other animals, and coprophagy is not common in adult tortoises (Couch et al. 1996). Our results could be related to the fact that these tortoises were only fed with leaves of the "otoy" plant (*Xanthosoma sagittifolium*) and "porotillo" stems (*Erythrina* spp.), both of which could have been contaminated with oocysts.

Molecular techniques can be used for identification and molecular characterization of gastrointestinal parasites to validate microscopic results (Inacio et al. 2016); therefore, PCR was performed in all fecal samples, of which were chosen four fecal samples from different tortoise species with the highest number of oocysts observed by microscopy. These included samples SC2016, S2016, P2016, and E2014, from tortoises of Santa Cruz, Santiago, Pinzón, and Española Islands, respectively. All samples showed positive results by amplifying a fragment from approximately to 1300 bp (Fig. 1).

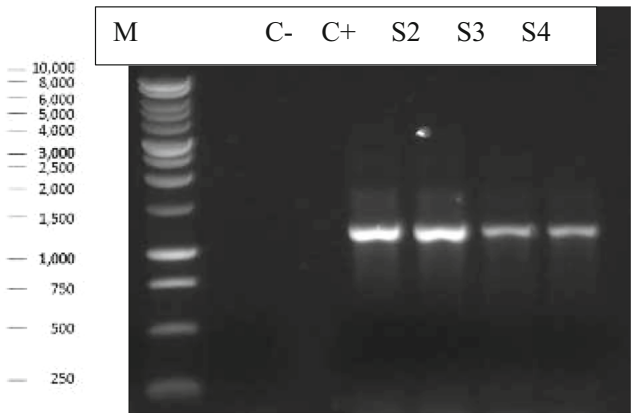

Fig. 1 Polymerase chain reaction (PCR) for identification of coccidia in fecal DNA samples from giant tortoises. M, marker; C−, negative control; C+, positive control; S2, sample 2 Santiago Island; S3, sample 3 Pinzón Island; S4, sample 4 Española Island

The sequences obtained were compared with known sequences of the GenBank NCBI (National Biotechnology Information Center). The analysis involved 22 nucleotide sequences. All positions that contained gaps and missing data were deleted. A total of 850 positions were obtained in the final set of data. Results showed the consensus sequences corresponding to the same organism. The consensus sequences were registered in the GenBank database with number accession MK909931.

BLASTN databases indicated homologous nucleotide sequences of coccidia with the closest match with *Eimeria enviromental* (GenBank accession EF024673.198% similarity).

The maximum likelihood trees showed a similar topology of the *Eimeria* sequences obtained in comparison with previous studies. The evolutionary history was deduced by the maximum-likelihood method based on the Tamura-Nei model, showing a tree with the highest probability of registration. The percentage of trees in which the associated taxa are joined is shown next to the branches, which indicate that three coccidia obtained are grouped 100% *Eimeria* sp. (Fig. 2). The sequences obtained from the samples of giant tortoises belong to the third clade, which corresponds to an unclassified group of coccidian. This indicates that a more distant group of coccidia could exist, which does not form part of the same group as *Eimeria bovis* (bovine) and *Eimeria arnyi* (snake). Phylogenetic analysis of these sequences showed a closer relationship to the Cryptosporidiidae family than to Sarcocystidae and Eimeriidae families (Fig. 2).

Previous studies of Galápagos giant tortoises reported *Eimeria geochelone* (Couch et al. 1996) and *E. iversoni* identified by morphological characterization but not confirmed by molecular characterization (McAllister et al. 2014); therefore, it is not possible to assure that those species are the same parasites found in our study. The presence of *E. iversoni* in Galápagos giant tortoises might indicate a transient parasite or even a false infection from another host living in close contact with tortoises, given that *E. iversoni* is a non-specific coccidia (McAllister et al. 2014). However, *E. geochelone* was found in a Galápagos giant tortoise on Santa Cruz Island and considered a new species of parasite found in these individuals due to the large number of oocysts discharged, the lack of contact with other animals as the tortoises were isolated in pens, and the fact that the structure of the oocysts found did not resemble oocysts described in other animals (Couch et al. 1996).

The oocysts found in our study may belong to the same previously reported species on Santa Cruz Island; however, the morphological examination of sporulated oocysts was not carried out. Thus, this was not confirmed. These oocysts have been classified in the genus *Eimeria* due to their morphological characteristics, which are similar to those of other

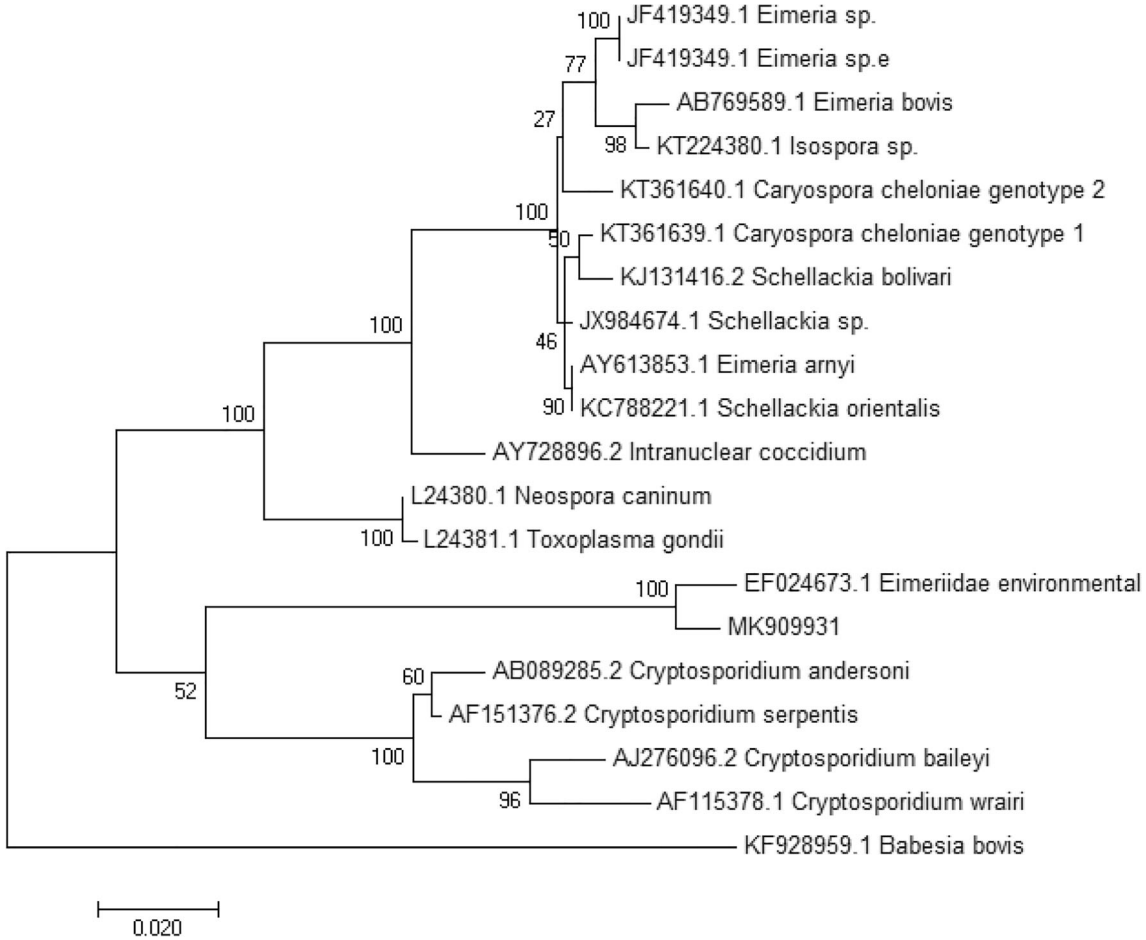

Fig. 2 Molecular phylogenetic analysis by maximum-likelihood method of nucleotide partial sequences of coccidias genus 18S rRNA

sporulated oocysts described in the National Collection of Parasites (USNPC) number 85499. Without a molecular analysis, however, it is not possible to confirm that we found the same organism.

In conclusion, this is the first report of *Eimeria* sp. in Galápagos giant tortoises using molecular characterization. The presence of coccidia is widespread in the fecal samples collected, and can be the cause of enteritis and death in young tortoises. The source of coccidiosis infection in Galápagos giant tortoises could be attributed to contamination of food and water.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors.

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