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Two new species of *Eimeria* (Apicomplexa: Eimeriidae) in Philippine tarsier (*Tarsius syrichta*)

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Abstract

Philippine tarsier (*Tarsius syrichta*) is a small nocturnal primate from the Philippines. Little is known about tarsier parasites, including coccidia (Apicomplexa: Eimeriidae), a highly prevalent parasitic protist group in all vertebrate classes. Only 7 valid species of the genus *Eimeria*, seven species of *Isospora* and 5 species of *Cyclospora* have been described in Primates. This study extends the number of coccidia known in primates by two new species obtained from faeces of Philippine tarsiers from Bohol Island. The newly described *Eimeria syrichta* n. sp. and *Eimeria boholensis* n. sp. differ morphologically from each other as well as from other coccidia reported from primates. Partial DNA sequences of three genes were obtained from oocysts of *E. syrichta* n. sp. and *E. boholensis* n. sp., and formed clusters according to their host specificity; however, there are no other sequentional data of coccidia from primates, except for the genus *Cyclospora*, which clusters inside the chicken eimerians, and *Cystoisospora belli*, which is phylogenetically related to Sarcocystidae. More molecular data on coccidia infecting primates are needed for further discussion.

Keywords: COI; Coccidia; ORF 470; Phylogeny; Primates; 18S rDNA

Introduction

Tarsiers (Primates: Tarsiidae) are small nocturnal primates from Southeast Asia, restricted to the Greater Sunda Islands except for Java and southern Philippines. The latter archipelago is inhabited by a single extant species, the Philippine tarsier *Tarsius syrichta* Linnaeus, 1758. Tarsiers are the only strictly carnivorous primates preying on terrestrial arthropods and small vertebrates. Habitats occupied by tarsiers include mangroves, bamboo growths, and tropical rainforests with dense understorey vegetation. Although the Philippine tarsier is listed by the IUCN Red Book as Near Threatened, ongoing habitat destruction and hunting for meat and pet trade make this species susceptible to population decline (Shekelle and Arboleda 2008).

Coccidia (Apicomplexa: Eimeriidae) are obligatory intracellular parasitic protists that are highly prevalent in all vertebrate and some invertebrate classes. Interestingly, only 19 valid eimerian species are known from primates, includ-

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ing 7 species of the genus *Eimeria* (*E. coucangi*, *E. galago*, *E. lemuris*, *E. nycticebi*, *E. otolicini*, *E. pachylepyron*, and *E. tarsii*), 7 *Isospora* spp. (*I. arctopitheci*, *I. belli*, *I. callimico*, *I. cebi*, *I. endocallimici*, *I. natalensis*, and *I. saimiriae*), and 5 *Cyclospora* spp. (*C. cayetanensis*, *C. cercopitheci*, *C. colobi*, *C. macacae*, and *C. papionis*) (Duszynski et al. 1999; Li et al. 2015). The only coccidian known from tarsiers to date is *Eimeria tarsii* Duszynski, Wilson, Upton et Levine, 1999 described from the captive Philippine tarsier originating from Mindanao, the second largest island in the Philippines (Duszynski et al. 1999; Osman Hill et al. 1952).

This study presents morphologic descriptions of the infective oocyst stage, molecular sequence data, and phylogenetic analyses of two new species of *Eimeria* originating from the faecal samples of free-ranging Philippine tarsiers from Bohol Island.

Material and Methods

Sampling and coprological examination

Faecal samples of *T. syrichta* were collected in Bilar (9.7333N, 124.1000E), Bohol Island, the Philippines, between February and December 2010. In total, 12 adult radiocollared free-ranging animals (4 males and 8 females) were sampled and examined within the scope of a broader study (Lovegrove et al. 2013; Řeháková-Petrů et al. 2012a,b). All samples were collected immediately after the capture of individual animals.

Faeces of each individual animal were placed in 2.5% potassium dichromate ($K_2Cr_2O_7$) for sporulation, and in 96% molecular ethanol for PCR. After the centrifugation-flotation concentrations using modified Sheather's sugar solution (specific gravity 1.3) (Modrý et al. 2015), faecal samples were examined for the presence of endoparasites using the light microscopy. Coccidian species/morphotypes were identified based on the morphology and morphometry of the sporulated oocysts, according to generally valid criteria published by Duszynski and Wilber (1997) and Berto et al. (2014). Oocysts were measured (number of measured oocysts see below) and photographed using an Olympus BX53 light microscope and Nomarski interference contrast (NIC) microscopy, an Olympus DP73 camera, and Olympus Dimension CellSens imaging software.

Molecular analyses

Microscopic identification of oocysts was followed by DNA amplification and sequencing, and phylogenetic analyses. DNA was extracted from ~200 mg of washed sediment from both ethanol-fixed as well as from dichromate-fixed parts of representative samples using the PowerSoil DNA Isolation Kit (MO BIO Laboratories) according to the manufacturer's instructions. Three different markers, namely fragment of a gene for the small subunit of 18S rRNA, a mitochondrial gene for cytochrome c oxidase subunit I (COI), and a gene encoding the plastid ORF 470 region (ORF 470), were amplified following the PCR protocols and PCR primers published by Kvičerová and Hypša (2013). PCR reactions were performed in a 25 μl volume containing 2 μl (1–10 ng) of total DNA, 12.5 μl of commercial premix PPP master mix (Top-Bio s.r.o), 1 μl (400 μM) of each primer, and 8.5 μl of PCR H_2O. Each PCR reaction was performed with a negative control containing PCR water instead of the DNA. As positive controls, DNA of *E. ferrisi* endogenous stages from laboratory mouse, and DNA of *E. lancasterensis* oocysts from *Sciurus carolinensis* faeces, were used. PCR products were separated by electrophoresis in 1.5% agarose gel stained with GoodView (ECOLI). Amplicons were purified using ExoSAP-IT® for PCR Product Cleanup (Affymetrix). Sequencing of PCR amplicons was carried out by the commercial company Macrogen, Inc. (Amsterdam, the Netherlands).

Phylogenetic analyses

Eimerian sequences were identified by BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi), edited and aligned using the GENEIOUS Pro software package version 6.1 (Kearse et al. 2012), and deposited in the NCBI GenBank database under the accession numbers MH349726, and MH350859–MH350862. Coccidian sequences of 18S rRNA, COI, and ORF 470 genes obtained from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) together with the newly obtained sequences of our samples were used in phylogenetic analyses. Accession numbers of all sequences used in the analyses are provided in Supplementary data available online (Technical Appendix). Phylogenetic relationships were reconstructed using Bayesian inference (BI) in the program MrBayes v.3.2.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), and maximum likelihood (ML) approach in the program PHYML (Guindon and Gascuel 2003). BI was run for 10 million generations and with four chains; the trees were summarized after removing 25% burn-in. ML was carried out with bootstrap values calculated by 1000 replicates. The most suitable evolutionary model ($GTR + \Gamma + I$) for the phylogenetic analyses was selected by jModeltest (Posada 2008, 2009). Final trees were visualized and exported using TreeView v.1.6.6 program (Page 1996).

Results

Oocysts of the genus *Eimeria* were found in 3 Philippine tarsiers of the 12 examined (25%). Microscopic examination revealed morphologically similar thick-walled oocysts in all three positive animals (N9, N10, N12). Detailed morphological examination revealed the presence of two slightly distinct morphotypes of eimerian oocysts differing moder-