Molecular and phylogenetic characterizations of an *Eimeria kriegsmanni* Yakimoff & Gouseff, 1938 (Apicomplexa: Eimeriidae) mouse intestinal protozoan parasite by partial 18S ribosomal RNA gene sequence analysis

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Abstract

Previously, we characterized an undocumented strain of *Eimeria kriegsmanni* by morphological and biological features. Here, we present a detailed molecular phylogenetic analysis of this organism. Namely, 18S ribosomal RNA gene (rDNA) sequences of *E. kriegsmanni* were analyzed to incorporate this species into a comprehensive *Eimeria* phylogeny. As a result, partial 18S rDNA sequence from *E. kriegsmanni* was successfully determined, and two different types, Type A and Type B, that differed by 1 base pair were identified. *E. kriegsmanni* was originally isolated from a single oocyst, and thus the result show that the two types might have allelic sequence heterogeneity in the 18S rDNA. Based on phylogenetic analyses, the two types of *E. kriegsmanni* 18S rDNA formed one of two clades among murine *Eimeria* spp.; these *Eimeria* clades reflected morphological similarity among the *Eimeria* spp. This is the third molecular phylogenetic characterization of a murine *Eimeria* spp. in addition to *E. falciformis* and *E. papillata*.

Key words: *Eimeria kriegsmanni*, 18S ribosomal RNA gene, mouse, phylogenetic analysis

Eimeria species (referred to as coccidia) belong to the phylum Apicomplexan, which includes obligate intracellular protozoa such as *Toxoplasma* or *Cryptosporidium*. Parasites in the *Eimeria* genus represent more than 1,100 described species isolated from vertebrates including livestock and birds, and invertebrates [1]. Classification of these parasites has been largely based on host specificities, parasite life cycles, and morphological features of sporulated oocysts, or some combination thereof. However, it is often difficult to distinguish among species based on morphology because many similarities exist; consequently, molecular techniques have proven very useful for identification and classification of these parasites [2, 3].

Among mice as one group of vertebrate hosts, 14 species of *Eimeria* have been described [1, 4]; however, only two species, *E. falciformis* and *E. papillata*, have been characterized genetically [5, 6]. These two species can serve as stout tools in the laboratory for the study of mucosal immunology, the development (susceptibility/effectiveness) of new drugs or novel vaccine antigens against coccidiosis, and as mice models for other apicomplexans, which have difficulties to be analyzed [7-9]. The pathogenicity or life cycle of *Eimeria* species are known to vary even within one host genus, especially as seen with avian or bovine *Eimeria* spp. [10-12]. Thus, it is necessary to clarify the biological and genetical differences among the species and approach the research in many aspects by multiple species or strains.

Previously, we examined an *Eimeria* spp. strain that was originally isolated from naturally infected commercial mice. This parasite, designated *E. krijgsmani*, is more virulent than *E. falciformis* in mice, and is morphologically and biologically different from other murine *Eimeria* spp. [4]. However, *E. krijgsmani* has not been characterized using molecular approaches. Here, we attempted to genetically characterize this strain so that it can be used as one of laboratory models to contribute on mammalian enteric eimeriosis.

The strain *E. krijgsmani* was obtained from the Division of Tropical Diseases and Parasitology, Department of Infectious Diseases, Kyorin University School of Medicine, and

maintained by passage through coccidia-free mice in the Laboratory of Parasitology, Joint Faculty of Veterinary Medicine, Kagoshima University. The animal experiments described here were performed with the approval of the committee at Kagoshima University (No. A10061 and No. VM13006). Mice (3 week-old ICR mice, CREA Japan, Tokyo) were inoculated with 10^2 - 10^3 sporulated *E. kriegsmanni* oocysts; 7-10 days later, oocysts were collected from feces, and purified via the sugar flotation method [13]. These oocysts were allowed to sporulate in a 2% potassium dichromate solution at 25°C for 2-3 days and then stored at 4°C until use.

Extraction buffer (100 mM Tris/HCl, 100 mM NaCl, 0.5% sodium dodecyl sulfate and 10 mM EDTA) and 50 µg of Proteinase K were used to extract DNA from oocysts; a phenol-chloroform-isoamyl alcohol solution (phenol: chloroform: isoamyl alcohol = 25 : 24 : 1) and subsequent ethanol treatment were used to further purify the DNA. Partial 18S ribosomal RNA gene (rDNA) sequences were amplified via polymerase chain reaction (PCR) with CYC1FE (5'-TAC CCA ATG AAA ACA GTT T-3') and CYC4RB (5'-CGT CTT CAA ACC CCC TAC TG-3') primers [14]. This primer pair is suitable for amplification of partial 18S rDNA in *Eimeria* spp. [15]. Each 50-µl PCR amplification mixture comprised 1 µl of each primer (50 µM), 2 µl of template DNA, 25 µl of AmpliTaq Gold 360 Master Mix (Invitrogen, USA), and 21 µl of Milli-Q. Each mixture was heated at 95°C for 10 min and then subjected to 35 cycles of 94°C for 30 sec, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were separated via electrophoresis in 1.5 % agarose gels, stained with ethidium bromide, and visualized with a Gel Doc (Bio-Rad, Japan) apparatus. Each amplicon of the expected size, 562 bp, was inserted into a pGEM-T Easy Vector (Promega, USA), and each recombinant plasmid was transformed into *Escherichia coli* (DH5α strain) and isolated via the alkali SDS-lysis method. Each plasmid insert was confirmed via *EcoRI* (Roche Diagnostics, Japan) digestion. For each product, 10 colonies were selected, and respective plasmids were purified (Qiagen Plasmid mini Kit, Qiagen science, USA) and then sequenced (Applied Biosystems 3730xl DNA analyzer,

Applied Biosystems, USA). The sequences were aligned with representative nucleotide sequences from *Eimeria* spp. using ClustalW with initial fixed parameter values at the DNA Data Bank of Japan (DDBJ, <http://clustalw.ddbj.nig.ac.jp/top-j.html>). The FASTA program at the DDBJ (<http://www.ddbj.nig.ac.jp/search/fasta-j.html>) was used to assess homology among the obtained partial gene sequences. Finally, a neighbor joining algorithm was used to construct a phylogenetic tree; the Tamura-Nei model and 1,000 bootstrap replicates were used to calculate evolutionary distances. The MEGA software package (version 5) was used to draw the resulting tree [16].

As a result, partial sequences of *E. krijgsmanni* were successfully determined, and two types of sequences having 1 bp differences (Fig. 1) were observed, and thus were designated Type A (AB849922) and Type B (AB849923) of *E. krijgsmanni*. The 18S rDNA nucleotide sequence of *E. krijgsmanni* Type A shared 99.7 %, 99.3 %, or 97.7 % identity with *E. krijgsmanni* Type B, *E. falciformis*, or *E. papillata*, respectively. *E. krijgsmanni* was originally isolated from a single oocyst, and thus the result show that the two types might have allelic sequence heterogeneity in the 18S rDNA although detailed information is not available. A phylogram was constructed that included these sequences with those from other *Eimeria* spp. (Fig. 2). Both types of *E. krijgsmanni* rDNA were located within the clade of rodents that included *E. falciformis*. Reportedly, phylogenetic analyses indicate that rodent *Eimeria* species form two distinct clades, and these molecular identified clades reflect morphological similarity among species (e.g. groups with/without oocyst residuum) [6]. It was recently suggested that each clade, designated rodents 1st and rodents 2nd, arose by adaptive rather than cophylogenetic processes and was confirmed as showing morphological features [17]. Previously, sporulated oocysts of *E. krijgsmanni* were determined to be morphologically more similar to those of *E. falciformis* and *E. schuffneri* than to those of other murine *Eimeria* spp.; therefore, these three species were distinguished only by merozoites or microgametes in their life cycle [1, 4]. Although genetic analysis of *E. schuffneri* has not been reported yet, results of the present study may support these previous findings.

115 We clarified the sequences of the *E. krijgsmani* 18S rDNA and determined the
116 phylogenetic position of *E. krijgsmani*. This is the third characterization of murine *Eimeria* spp.
117 following to *E. falciformis* and *E. papillata* [5, 6]. In conclusion, establishment of a genetically
118 and biologically characterized *E. krijgsmani* strain could provide a useful tool for future
119 understanding of intestinal parasites.

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References

- [1] Levine ND. The protozoan phylum Apicomplexa, vol I. Boca Raton: CRC; 1988.
- [2] Cere N, Licois D, Humbert JF. Study of the inter- and intraspecific variation of *Eimeria* spp. from the rabbit using random amplified polymorphic DNA. Parasitol Res 1995;81:324-8.
- [3] Johnston DA, Fernando MA. *Eimeria* spp. of the domestic fowl: analysis of genetic variability between species and strains using DNA polymorphisms amplified by arbitrary primers and denaturing gradient-gel electrophoresis. Parasitol Res 1995;81:91-7.
- [4] Matsui T, Fujino T, Kobayashi F, Morita T, Imai S. Life cycle of *Eimeria kriegsmanni*-like coccidium in the mouse (*Mus musculus*). J Vet Med Sci 2006;68:331-6.
- [5] Barta JR, Martin DS, Carreno RA, Siddall ME, Profous-Juchelkat H, Hozza M, Powles MA, Sundermann C. Molecular phylogeny of the other tissue coccidia: *Lankesterella* and *Caryospora*. J Parasitol 2001;87:121-7.
- [6] Zhao X, Duszynski DW. Phylogenetic relationships among rodent *Eimeria* species determined by plastid ORF470 and nuclear 18S rDNA sequences. Int J Parasitol. 2001;31:715-9.
- [7] Kazanji M, Laurent F, Péry P. Immune responses and protective effect in mice vaccinated orally with surface sporozoite protein of *Eimeria falciformis* in ISCOMs. Vaccine 1994;12:798-804.
- [8] Stange J, Hepworth MR, Rausch S, Zajic L, Kühl AA, Uyttenhove C, Renauld JC, Hartmann S, Lucius R. IL-22 mediates host defense against an intestinal intracellular parasite in the absence of IFN- γ at the cost of Th17-driven immunopathology. J Immunol 2012;188:2410-8.
- [9] Dkhil MA. Anti-coccidial, anthelmintic and antioxidant activities of pomegranate (*Punica granatum*) peel extract. Parasitol Res 2013;112:2639-46.
- [10] Dauschies A, Najdrowski M. Eimeriosis in cattle: current understanding. J Vet Med B Infect Dis Vet Public Health 2005;52:417-27.
- [11] Sharman PA, Smith NC, Wallach MG, Katrib M. Chasing the golden egg: vaccination against

poultry coccidiosis. *Parasite Immunol.* 2010;32:590-8.

[12] Dkhil MA, Abdel-Baki AA, Wunderlich F, Sies H, Al-Quraishy S. Dietary selenium affects intestinal development of *Eimeria papillata* in mice. *Parasitol Res* 2014;113:267-74.

[13] Matsubayashi M, Kimata I, Iseki M, Hairi T, Tani N, Sasai K, Baba E. Infectivity of a novel type of *Cryptosporidium andersoni* to laboratory mice. *Vet Parasitol* 2005;129:165-8.

[14] Jinneman KC, Wetherington JH, Hill WE, Omiescinski CJ, Adams AM, Johnson JM, Tenge BJ, Dang NL, Wekell MM. An oligonucleotide-ligation assay for the differentiation between *Cyclospora* and *Eimeria* spp. polymerase chain reaction amplification products. *J Food Prot* 1999;62:682-5.

[15] Abe N, Tanoue T, Ohta G, Iseki M. First record of *Eimeria furonis* infection in a ferret, Japan, with notes on the usefulness of partial small subunit ribosomal RNA gene sequencing analysis for discriminating among *Eimeria* species. *Parasitol Res* 2008;103:967-70.

[16] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28: 2731-9.

[17] Kvičerová J, Hypša V. Host-parasite incongruences in rodent *Eimeria* suggest significant role of adaptation rather than cophylogeny in maintenance of host specificity. *PLoS One* 2013;8:e63601.

Captions to Figures

Fig. 1. Alignment of the two types of *E. kriegsmanni* 18S rDNA partial sequence with those of *E. falciformis* and *E. papillata*. Dashes indicate gaps, dots indicate bases that are identical to the *E. kriegsmanni* Type A bases, and the asterisk indicates the base that differs between *E. kriegsmanni* Type A and Type B. E. k Type A=*E. kriegsmanni* Type A, E. k Type B=*E. kriegsmanni* Type B, E. falci=*E. falciformis* and E. papill=*E. papillata*.

Fig. 2. Phylogram of *Eimeria kriegsmanni* and other *Eimeria* spp. inferred by neighbor-joining analysis of a partial sequence from 18S rDNA. Accession numbers are shown in parentheses. Specific hosts of each *Eimeria* species are indicated on the right side. Two sequence types of *E. kriegsmanni* are bold.

Figure 1

	10	20	30	40	50
E. k Type A	TACCCAATGA	AAACAGTTTC	GAGGTAGTGA	CGAGAAATAA	CAATACAGGG
E. k Type B					
E. falci		C...			
E. papill	-	..-.....			
	60	70	80	90	100
E. k Type A	CATTTTTTGT	TCTGTAATTG	GAATGATGGG	AATGTAAAC	CCTTTCAGAT
E. k Type B					
E. falci					
E. papill	A..C				
	110	120	130	140	150
E. k Type A	TAACAATTGG	AGGGCAAGTC	TGGTGCCAGC	AGCCGCGGTA	ATTCCAGCTC
E. k Type B					
E. falci					
E. papill					
	160	170	180	190	200
E. k Type A	CAATAGTGTA	TATTAGAGTT	GTTGCAGTTA	AAAAGCTCGT	AGTTGGATTT
E. k Type B					
E. falci					
E. papill					
	<div>*</div> 210	220	230	240	250
E. k Type A	CTGCCGTGGT	CATCCGGTTC	CGCCTGTATG	GGTGTGCGCC	TGGTTTGCCC
E. k Type B	...T.....				
E. falci	...T.....				
E. papill	...T.....	C..	C.....		
	260	270	280	290	300
E. k Type A	TCGGCTTTCT	TCCGTTAGCC	TTCGGCGCTT	CACTGCGTCT	GTTGGTGTTT
E. k Type B					
E. falci					
E. papill		G.....	...C.....		

Figure 2

Graphical Abstract