IJP: Parasites and Wildlife 7 (2018) 243–250

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Molecular identification of two *Eimeria* species, *E. uekii* and *E. raichoi* as type B, in wild Japanese rock ptarmigans, *Lagopus muta japonica*

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ARTICLE INFO

Keywords:
Japanese rock ptarmigan
Eimeria raichoi
Eimeria uekii
Lagopus muta japonica

ABSTRACT

Thus far, two types of *Eimeria* parasites (*E. uekii* and type B) have been morphologically identified in wild Japanese rock ptarmigans, *Lagopus muta japonica*. Although high prevalences were reported for these parasites, genetic analyses have not been conducted. We first clarified the phylogenetic positions of two eimerian isolates using genetic analyses of *18S rRNA* and *mitochondrial cytochrome c oxidase subunit I* gene regions. Consequently, of 61 samples examined, 21 and 11 samples were positive for *E. uekii* and type B, respectively. Additionally, the infection rate increased in the summer. Molecular analyses revealed both *Eimeria* isolates formed their own clusters; *E. uekii* was included in clades of chicken *Eimeria* and type B was include in clades of turkey *Eimeria*. Based on our findings in this study and previous data, we herein propose type B as *E. raichoi*. These genetic data will be helpful to conduct detailed classification and understand the impact of these parasites for conservation of endangered Japanese rock ptarmigans.

1. Introduction

Species in the genus *Eimeria* are protozoan parasites belonging to the phylum Apicomplexa. More than 4000 species of the parasites have been described, and they infect a wide range of vertebrate and invertebrate hosts (Levine, 1982), and are considered to be highly host-specific. Infection by eimerian parasites causes coccidiosis, which is mainly characterized as watery or bloody diarrhea, weight loss, and increased mortality rates of infected hosts (Fitzgerald, 1980; McDonald and Shirley, 2009). Although *Eimeria* spp. are the most important and prevalent parasites due to their economic impact, especially in livestock, *e.g.*, chicken and cattle, most *Eimeria* spp. causing disease in wild animals remain largely unknown and poorly characterized.

The study of *Eimeria* spp. from the rock ptarmigan (*Lagopus muta*) (Montin, 1781), in the order Galliformes, is important because of specificity of the host. The rock ptarmigan is a cold-adapted bird that inhabits alpine areas of the Northern Hemisphere. To date, seven *Eimeria* spp. have been identified in the rock ptarmigan: *E. lagopodi* from Switzerland (Galli-Valerio, 1929), *E. brinkmanni* and *E. fanthami* from Canada (Levine, 1953), *E. uekii* and type B from Japan (Kamimura and Kodama, 1981; Ishihara et al., 2006), and *E. muta* and *E. rjupa* from Iceland (Skirnisson and Th Thorarinsdottir, 2007). Descriptions of these species were primarily based on morphological (sporulated oocysts) and biological characteristics. However, these parameters can be insufficient for reliable differentiation among species owing to overlapping morphometric and biological features (Long et al., 1977). Therefore, molecular analyses are necessary to classify and precisely identify the species and to assess phylogenetical developments of the parasites as well as the hosts.

In terms of taxonomy, rock ptarmigans are currently divided into

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https://doi.org/10.1016/j.ijppaw.2018.06.005
Received 28 April 2018; Received in revised form 15 June 2018; Accepted 19 June 2018

23–30 subspecies, *e.g.*, *L. m. evermanni*, *L. m. kurilensis*, and *L. m. pleskei* (Johnsgard, 1983; del Hoyo et al., 1994; Holder et al., 2000). The Japanese rock ptarmigan (*L. m. japonica*) inhabits only the timberline regions of the Japanese alpine zone at approximately 3000 m above sea level. This subspecies is endemic to Japan and is thought to be endangered due to a decline in the overall population (estimated population: ≤2000 individuals). The Japanese rock ptarmigan was designated as a special natural monument of Japan in 1955 and is listed as vulnerable in the Japanese Red Data Book (Murata et al., 2007; Wildlife Division of the Ministry of the Environment, 2017). Recently, *Eimeria* infections were reported to be associated with a decrease in overall body condition and an increase in mortality in rock ptarmigans (*L. m. islandorum*) in Iceland (Stenkewitz et al., 2016). Although two types of *Eimeria* parasites, namely *E. uekii* and type B, highly infect the Japanese rock ptarmigan (Kamimura and Kodama, 1981; Ishihara et al., 2006; Matsubayashi et al., 2018b), molecular analyses of these parasites have not been conducted. In the present study, we genetically examined *E. uekii* and type B to determine their precise phylogenic positions and classifications.

2. Materials and methods

2.1. Examined areas and birds

In the present study, we collected fecal samples at Hida Mountains of the Northern Japanese Alps and Akaishi Mountains of the Southern Japanese Alps from May 2016 to August 2017; the areas extend over Toyama, Gifu, Nagano, and Niigata prefectures and Nagano, Yamanashi, and Shizuoka prefectures, respectively. We collected a total of 61 fresh Japanese rock ptarmigan fecal samples, including 6 chick samples as described in Table 1. Samples were collected from 3 sites: Mt. Tateyama (36°35′N, 137°36′E), Norikuradake (36°6′N, 137°33′E), and Kitadake (35°40′N, 138°14′E) (Fig. 1). In Kitadake, a temporary cage protection procedure was conducted for three families for 3 weeks starting at the end of June 2016. Three families (hen and her chicks) were individually housed during nighttime to avoid eventual predation by predators and sometimes during daytime when severe weather (strong wind and rain) occurred. Otherwise, the families were allowed to graze the natural food outside of the cage. In cages, food such as *Vaccinium ovalifolium*, *Oxytropis japonica* var. *japonica*, *Polygonum viviparum*, and *Stellaria nipponica* was collected from surrounding areas to offer to the birds. Previously collected fruits of *Vaccinium vitis-idaea* and commercially available worms (*Tenebrio* spp.) were also supplied as supplemental food. Fecal samples were placed in a cooler box, transported to our laboratory, and stored at 4 °C until analyses.

Table 1
Summary of examined Japanese ptarmigans and infection of *Eimeria* spp.

Sampling date		No. of positive samples/ examined samples	*Eimeria* spp.		Average OPG (No. of samples)[a3]	Location
			E. uekii	type B		
2016	May	0/9	–	–		
	June	6/10	6	2	1133.6 (1)	Tateyama
	July	2/6[a1]	2	–	240.0 (2)	Norikuradake
	August	4/5	3	4	2883.1 (3)	Kitadake
	November	0/5	–	–		Tateyama
2017	May	3/15	3	1		Tateyama and Norikuradake
	June	1/5	1	–		Tateyama
	August	6/6[a2]	6	4	7244.7 (6)	Kitadake
Total		22/61 (36.1%)	21	11		

Three chicks were included: two chicks in [a1] (positive for *E. uekii*) and three chicks in [a2] (positive for *E. uekii* and type B).

[a 3], OPG was calculated only in birds that shed countable numbers of oocysts in feces.

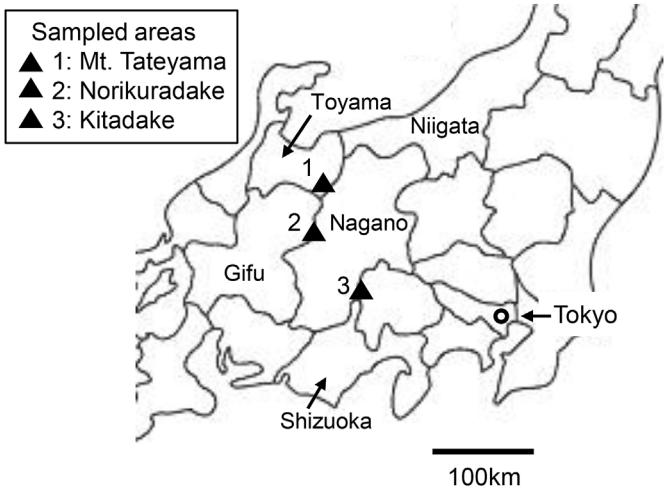

Fig. 1. Location of three sampled areas in Japan, Mt. Tateyama (36°35′N, 137°36′E), Norikuradake (36°6′N, 137°33′E), and Kitadake (35°40′N, 138°14′E) (triangle boxes 1–3).

2.2. Fecal examinations

The parasites were examined by sucrose centrifugal flotation methods (Uga et al., 2000). The number of oocysts per gram (OPG) was determined by counting after purification of the parasites. Several *Eimeria*-positive samples were incubated in a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution at 25 °C to allow the oocysts to sporulate. Sporulated oocysts were observed under a differential interference contrast microscope under oil immersion at 1000 × magnification. Internal structures of 50 oocysts were then analyzed.

2.3. Experimental infections

Purified sporulated oocysts from sample No. 60 were used for experimental infection. Four conventional Japanese quails (*Coturnix japonica*) (Delight Base, Aichi, Japan) were housed in wire-floored cages in coccidia-free rooms with free access to feed and water, which contained no anticoccidial drugs or antibiotics. Then, chicks (34-days-old) were orally inoculated with 2×10^4 of *E. uekii* and 0.5×10^4 of type B oocysts in 500 µl. Their feces were collected from 3 days post-inoculation (dpi) to 20 dpi and examined by sugar floatation methods.

2.4. Molecular identification

For genetic analysis of one *Eimeria* species, one oocyst of *E. uekii* or type B was isolated using a disposable glass capillary micropipette as previously described (Matsubayashi et al., 2005) and transferred to 8 µl of phosphate-buffered saline (pH 7.4) in a PCR tube. Isolated oocysts were treated by five freeze-thaw cycles, heated at 99 °C for 8–10 min, and then used as DNA template.

Molecular identification of *Eimeria* spp. was performed by PCR using the primer pair 1FE-4RB (approximately 560 bp), which targets the *18S ribosomal RNA* (*rRNA*) gene (Jinneman et al., 1999; Matsubayashi et al., 2005). Additionally, nested PCRs the targeting *mitochondrial cytochrome*