



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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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 included 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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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 phylogenetic 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

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

Sampling date	No. of positive samples/ examined samples	<i>Eimeria</i> spp.		Average OPG (No. of samples) ^{a3}	Location
		<i>E. uekii</i>	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).

^{a3} OPG was calculated only in birds that shed countable numbers of oocysts in feces.

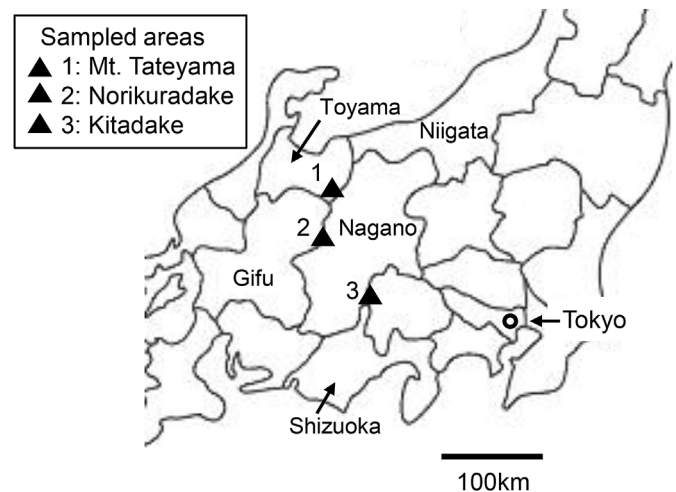


Fig. 1. Location of three sampled areas in Japan, Mt. Tateyama (36°35'N, 137°36'E), Norikuradake (36°6'N, 137°33'E), and Kitadake (35°40'N, 138°14'E) (triangle boxes 1–3).

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.

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 μ l. 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 μ l 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 targeting mitochondrial cytochrome



Fig. 2. *Eimeria* oocysts detected in the feces of Japanese rock ptarmigans. (A) *E. uekii* and (B) type B. Scale bars indicate 10 μ m.

c oxidase subunit I (COI) gene (approximately 780 bp) (Ogedengbe et al., 2011; Yang et al., 2013) and *28S rRNA* gene (approximately 1420 bp) (Schrenzel et al., 2005; Yang et al., 2014) were conducted. PCR products were sequenced, and phylogenetic trees were constructed as described previously (Matsubayashi et al., 2018a).

2.5. Histopathological examinations

We obtained five chicks (Nos. a–e), which were protected in the cage at Kitadake and unintentionally dead in 2016. They were preserved at -20°C before necropsy; the heart, liver, kidneys, stomach, duodenum, jejunum, ileum, cecum, and colon were collected, fixed in 10% neutral-buffered formalin, and processed for routine histological examinations. Tissue sections were stained with hematoxylin and eosin (HE) and Periodic acid-Schiff. Stained histological sections were

examined under a light microscope ($400\times$). Histological scores were determined based on the number of parasites (+, a few parasites in some fields; ++, 1–5 parasites per field; +++, > 5 parasites per field).

3. Results

We detected oocysts of *Eimeria* spp. in 22 (36.1%) of 61 examined samples at three areas of the Japanese Alps (Table 1) and two types of the oocysts were morphologically found (Fig. 2) (Table 2). No other parasites were found. *E. uekii*-type oocysts were ellipsoidal in shape, had no oocyst residuum, and contained 1–2 ovoid polar granules. The micropyle was indistinct or absent. Sporulated oocysts ($n = 50$; length \times width) measured $23.7 \pm 2.0 \mu\text{m}$ ($22.0\text{--}27.1 \mu\text{m}$) \times $15.3 \pm 1.2 \mu\text{m}$ ($13.3\text{--}18.1 \mu\text{m}$) and had a shape index (L/W) of 1.56 ± 0.18 ($1.26\text{--}1.89$). Sporocysts ($n = 50$) measured $11.7 \pm 1.3 \mu\text{m}$ ($9.1\text{--}14.3 \mu\text{m}$) \times $6.9 \pm 0.7 \mu\text{m}$ ($5.7\text{--}8.1 \mu\text{m}$) and had a shape index (L/W) of 1.71 ± 0.22 ($1.29\text{--}2.25$). Stieda body and sporocyst residuum were present and observed in sporozoites.

Type B-like oocysts were subspherical, had no oocyst residuum or micropyle, and contained one ovoid polar granule. Sporulated oocysts ($n = 50$; length \times width) measured $20.6 \pm 0.9 \mu\text{m}$ ($18.6\text{--}21.8 \mu\text{m}$) \times $17.5 \pm 0.7 \mu\text{m}$ ($14.6\text{--}19.1 \mu\text{m}$) and had a shape index (L/W) of 1.18 ± 0.05 ($1.08\text{--}1.26$). Sporocysts ($n = 50$) measured $11.8 \pm 0.7 \mu\text{m}$ ($10.0\text{--}13.6 \mu\text{m}$) \times $7.1 \pm 0.5 \mu\text{m}$ ($5.9\text{--}8.2 \mu\text{m}$) and had a shape index (L/W) of 1.65 ± 0.12 ($1.38\text{--}2.00$). Stieda body and sporocyst residuum were present. After incubation at 25°C , *E. uekii* and type B were sporulated for 24 h and 24–48 h, respectively. After comparisons with previous studies (Kamimura and Kodama, 1981; Ishihara et al., 2006), we identified the oocysts isolated in this study as *E. uekii* and type B oocysts. Regarding experimental infections using quails, we could not confirm any clinical symptoms or detect any oocysts of *Eimeria* spp. during 20 dpi.

Specific fragments of the *18S rRNA* gene using DNA prepared from one oocyst were successfully amplified by PCR analyses with primer pairs 1FE-4RB. Partial sequences of the products for *E. uekii* (Accession Nos. LC380046–LC380054) and type B (Accession Nos. LC380055–LC380068) were determined, and we constructed a phylogenetic tree of the gene (Fig. 3). *E. uekii* and type B isolates formed a sister group to chicken *Eimeria* spp. and turkey *Eimeria* spp., respectively, and separated from other *Eimeria* spp. from mammals such as rodents and cattle. Although the *28S rRNA* gene region was not amplified, *COI* gene sequences from some isolates (Accession Nos. LC380069–LC380076) could be analyzed (Fig. 4). Phylogenetic analyses of *COI* gene showed that both *Eimeria* isolates formed their own clusters; *E. uekii* was included in chicken *Eimeria* spp. and type B was included in turkey *Eimeria* spp. Based on these findings and those from previous studies (Ishihara et al., 2006; Matsubayashi et al., 2018b), we propose type B of *Eimeria* from Japanese rock ptarmigans as *E. raichoi*, as described below.

3.1. Descriptions

E. raichoi n. sp. (Fig. 5)

Host: Japanese rock ptarmigans (*Lagopus muta japonica*).

Locality: Hida Mountains of the Northern Japanese Alps and Akaishi Mountains of the Southern Japanese Alps, Japan.

Prevalence: Seasonally variable.

Other hosts: Unknown.

Prepatent period: Unknown.

Patent period: Unknown.

Site of infection: Unknown.

Sporulation time: 24–48 h.

Table 2
Comparative morphologies of *Eimeria* spp. from wild Japanese rock ptarmigans and rock ptarmigans from other countries.

Hosts (countries)	Oocysts					Sporocysts					ST		Reference
	length × width (range)	L/W	Mi	OR	No. of PG (range)	length × width (range)	L/W	SB	RB	SR	SR	SR	
<i>L. m. japonica</i> (Japan)													
This study	23.7 (20.0–27.1) × 15.3 (13.3–18.1)	1.56 (1.26–1.89)	±	–	1–2	11.7 (9.1–14.3) × 6.7 (5.7–8.1)	1.71 (1.29–2.25)	+	ND	ND	ND	24–48 h	
<i>E. uekii</i>	24 (19–29) × 16 (13–21)	1.5 (1.2–1.7)	+	–	1–2 (2 × 1)	12 (11–15) × 6 (5–7)	ND	+	2.2–3	+	2.2–3	24 h	Kamimura and Kodama, 1981
<i>E. uekii</i>	23.7 (20.2–28.8) × 15.6 (13.7–18.8)	1.5 (1.2–1.7)	±	–	(1.5–3.1)	12.4 (9.7–14.4) × 6.7 (5.8–7.8)	1.8 (1.4–2.3)	+	1.3–4	+	1.3–4	< 24 h	Ishihara et al., 2006
This study (type B)	20.6 (18.6–21.8) × 17.5 (14.6–19.1)	1.18 (1.08–1.26)	–	–	1	11.8 (10.0–13.6) × 7.1 (5.9–8.2)	1.65 (1.38–2.00)	+	ND	ND	ND	24 h	
Type B of <i>Eimeria</i>	21.3 (13.6–25.9) × 19.1 (13.1–24.5)	1.1 (1.0–1.4)	–	–	(1.5–2.8)	12.1 (9.6–13.7) × 7.4 (5.8–9.2)	1.6 (1.2–2.0)	+	1.4–4	+	1.4–4	< 48 h	Ishihara et al., 2006
<i>L. m. islandorum</i> (Iceland)													
<i>E. muta</i>	24.9 (19.5–30) × 16.6 (14.5–19)	1.5 (1.2–1.8)	+	–	1–3	14.3 (12–16.5) × 6.3 (5.5–7)	2.3 (1.9–2.7)	+	3.9–2.9	+	3.9–2.9	ND	Skirnisson and Th Thorarinsdottir, 2007
<i>E. rjupa</i>	24.7 (20–28) × 22.2 (18–24.5)	1.1 (1.0–1.3)	–	–	1–2	14.4 (12–15.5) × 8.0 (6.5–9)	1.8 (1.6–2.2)	+	3.9–2.9	+	3.9–2.9	ND	Skirnisson and Th Thorarinsdottir, 2007
<i>L. m. rupestris</i> (Canadian arctic)													
<i>E. brinkmanni</i>	28.6 (26.0–29.7) × 18.8 (18.0–19.6)	1.52 (1.4–1.6)	–	–	1–2	13 × 7	ND	+	ND	–	ND	ND	Levine, 1953
<i>E. fanthami</i>	28.3 (27.0–29.2) × 18.8 (18.0–20.1)	1.52 (1.4–1.6)	–	–	1–3	ND	ND	+	ND	–	ND	ND	Levine, 1953
<i>L. m. hebeiticus</i> (Switzerland)													
<i>E. lagopodi</i>	24 × 15	ND	+	ND	ND	12	ND	ND	ND	ND	ND	ND	Galli-Valerio, 1929

Mi, micropyle; OR, oocyst residuum; PG, polar granules; SB, stieda body; RB, refractile body; SR, sporocyst residuum; ST, sporulation time. Sizes are in µm.

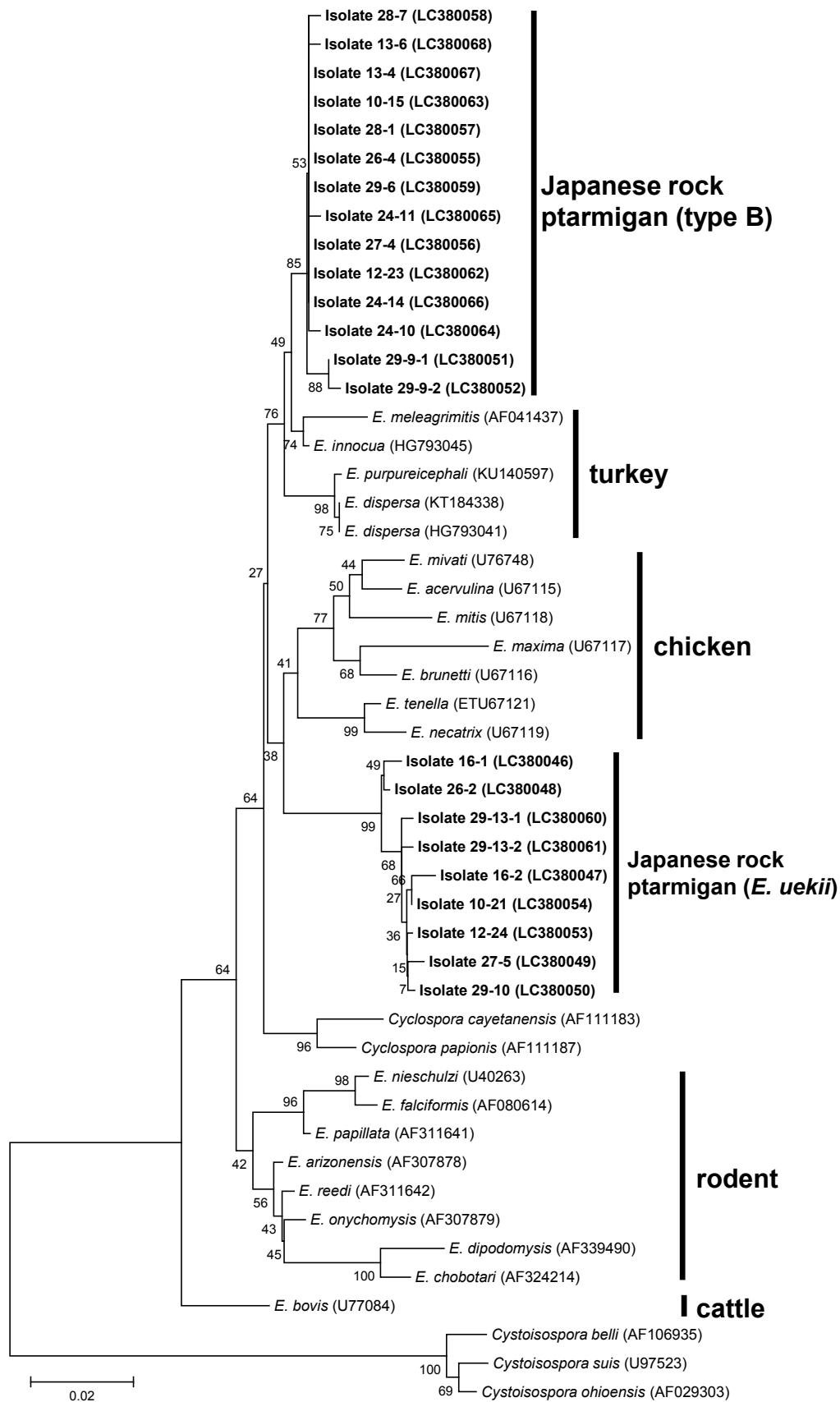


Fig. 3. Phylogram of *E. uekii*, type B, other *Eimeria* spp., and related parasites (*Cyclospora* spp.) inferred by the neighbor-joining method using partial *18S rRNA* gene sequences. Accession numbers and derived hosts are shown in parentheses. Scale bar represents substitutions per nucleotide, and bootstrap values are indicated (> 1000). *Cystoisospora* spp. are used as an outgroup taxon.

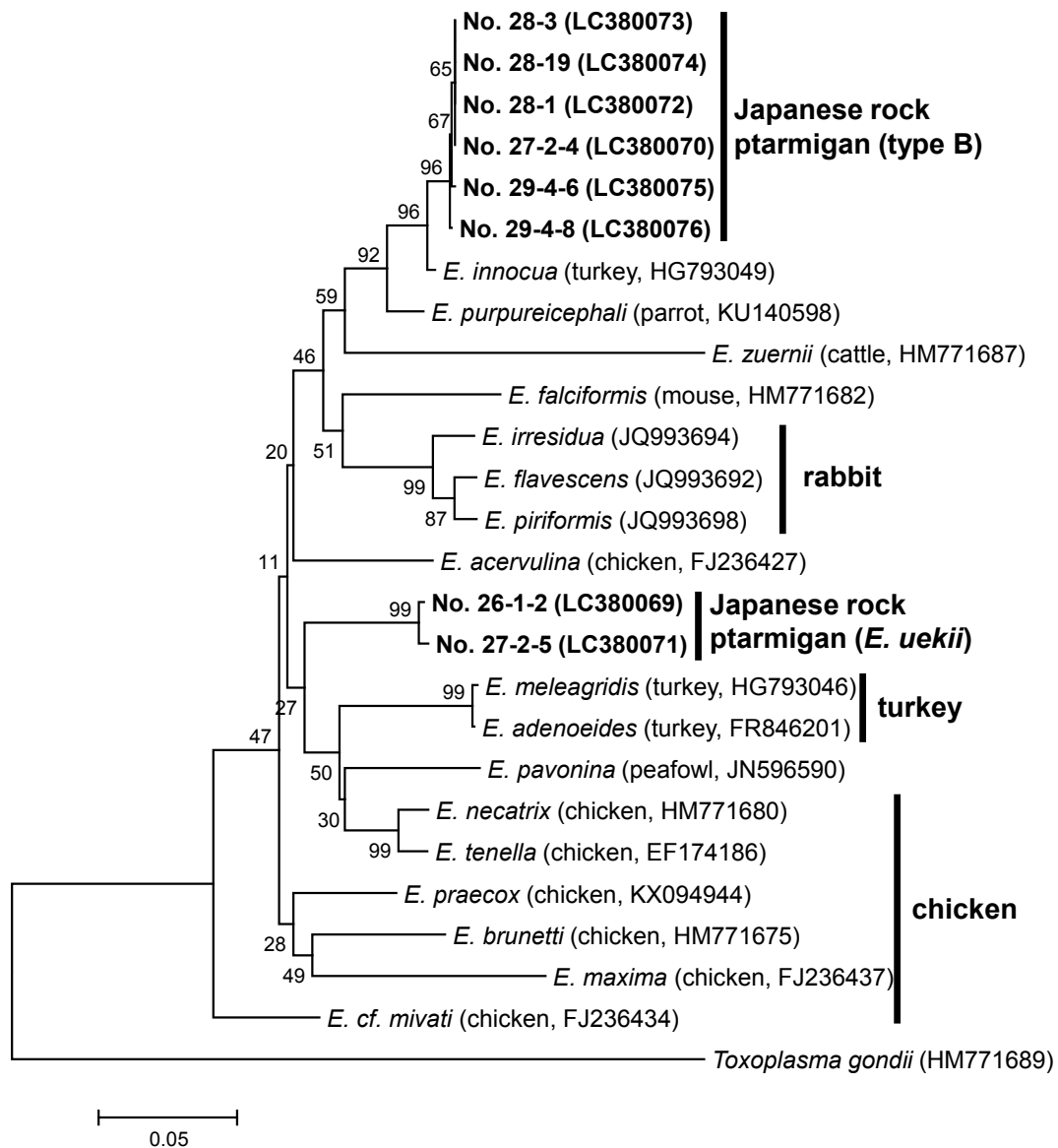


Fig. 4. Phylogram of *E. uekii*, type B, and other related *Eimeria* spp. inferred by the neighbor-joining method using partial mitochondrial cytochrome *c oxidase subunit I* gene sequences. Accession numbers and derived hosts are shown in parentheses. Scale bar represents substitutions per nucleotide, and bootstrap values are indicated (> 1000). *Toxoplasma gondii* is used as an outgroup taxon.

Note: Representative purified oocysts from infected birds have been deposited as syntype in the Meguro Parasitological Museum (4-1-1 Shimomeguro, Meguro, Tokyo 153-006, Japan; accession numbers MPM Col. 21449-a).

In histopathological examinations, all organs were severely degraded after death. However, we could observe some parasites in the small and large intestines of two chicks (Nos. a and b), namely, the duodenum (+ and +), jejunum (+++ and +), ileum (+++ and ++), and cecum (+++ and +++), respectively. There were no parasites in the stomach and colon. The parasites were mainly gamete, zygote, or oocysts and a few schizonts (Fig. 6) and were estimated as *E. uekii* and type B based on the morphologies of ellipsoidal and sub-spherical shapes, respectively. However, we could not determine the species and the infected location, such as epithelium cells or lamina propria, or the cause of death.

4. Discussion

Thus far, two types of *Eimeria* have been reported in wild Japanese rock ptarmigans. Their prevalence increases up until August (summer) and then decreases as the temperature decreases until November (winter) (Ishihara et al., 2006; Matsubayashi et al., 2018b). In the present study, infection rates were in accordance with previous findings although examined numbers were small (Table 2). Morphologies of the two type oocysts in our study were almost identical to those of *E. uekii* and type B as described previously (Kamimura and Kodama, 1981; Ishihara et al., 2006; Matsubayashi et al., 2018b). Although we could determine the phylogenetic positions of these isolates and type B were characterized as *E. raichoi*, sporulated oocysts of *E. uekii* and type B were similar to those of *E. muta* and *E. rjupa* from Iceland. Japanese rock ptarmigans are not migratory birds and inhabit only the timberline regions of the Japanese alpine zone at approximately 3000 m above sea

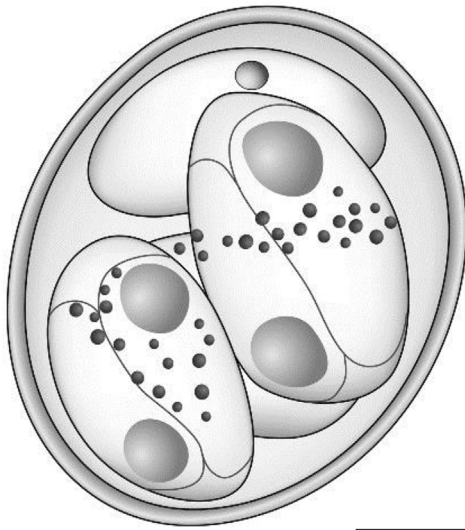


Fig. 5. Composite line drawing of oocyst of *Eimeria raichoi* n. sp (previously referred as type B). Scale bars indicate 10 μ m.

level. Thus, genetical analyses of *Eimeria* spp. from other rock ptarmigans are needed to clarify the classification and assess the evolution of *Eimeria* spp. according to adapted progression of rock ptarmigans.

The pathogenicity of *Eimeria* spp. infecting Japanese rock ptarmigans remains unknown. The developmental stages of parasites including gametes were detected in the intestines although detailed histopathological findings could not be determined. Therefore, there is a possibility that these parasites can affect the health status of rock ptarmigans. Previously, it has been reported that *E. uekii* may cause lesions in the host (Kamimura and Kodama, 1981). Furthermore, this species appeared to be phylogenetically closely related to chicken *Eimeria* spp., which contain highly virulent or chronically pathogenic species. Thus, further study is necessary to clarify the pathogenicity and assess these parasites as the key factor for conservation of Japanese rock ptarmigans as endangered species.

Compliance with ethical standards

Ethics statement

All experiments in the wild were carried out without using live animals. Thus, ethical approval of animal experimentation was not necessary. All examinations in the field study were permitted by the Ministry of the Environment Government of Japan. Fecal collection was performed in a non-invasive manner. No animals were sacrificed for the purpose of the field study. In the experimental infection study, the animals were treated in accordance with the protocols approved by the Animal Care and Use Committee in accordance with the Animal Experimentation Guidelines of Osaka City University (approval No. 15003). Human participants were not involved in this study.

Conflicts of interest

The authors declare that they have no conflict of interest.

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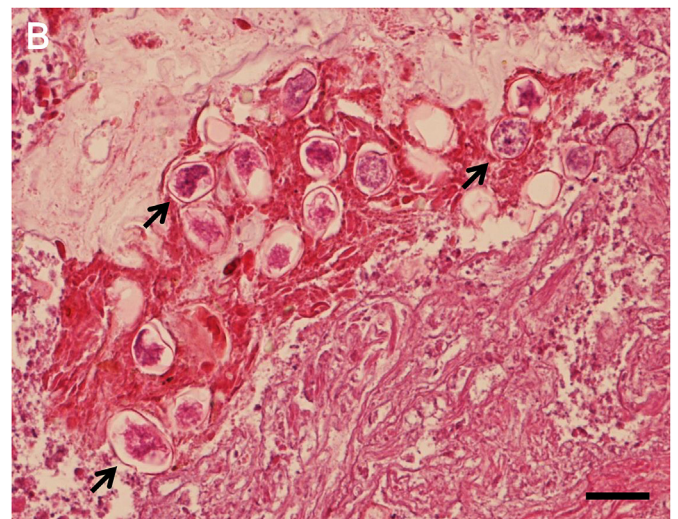
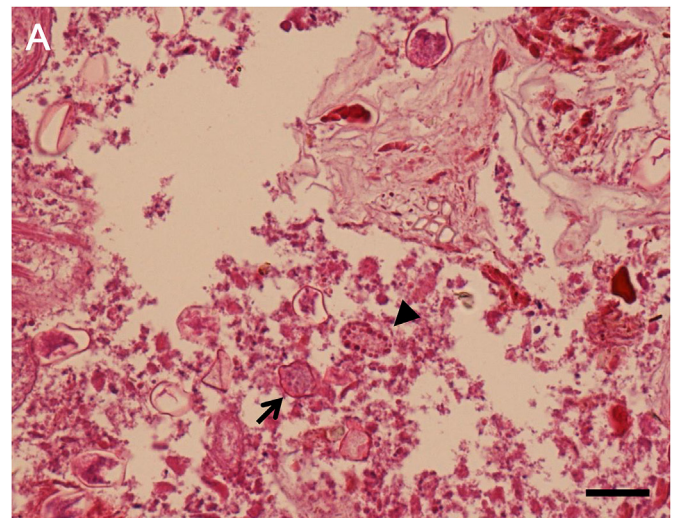


Fig. 6. Histopathologic sections of ceca which were obtained from dead chicks infected with *Eimeria* spp. Arrowhead indicates macrogametocytes with a prominent wall-forming body (A), and arrows indicate zygotes or early oocysts, which are surrounded by an oocyst wall (A and B). Pathological lesions could not be observed because of severe degradation after death. Scale bars indicate 20 μ m.

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