Phylogenetic study on eimerian and capillariid parasites of farmed ring-necked pheasants (*Phasianus colchicus* L.)

（飼養コウライキジ(*Phasianus colchicus* L.）のアイメリア科ならびに毛細線虫科寄生虫の分子系統学的研究）

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// Abstract

My study consisted of two chapters. The first chapter, titled "Eimerian and capillariid infection in farmed ring-necked pheasants (*Phasianus colchicus karpowi*) in Ehime, Japan, with special reference to their phylogenetic relationships with congeners, focused on the ring-necked pheasant, *Phasianus colchicus* Linnaeus 1758, a bird species originally endemic to Asia and the eastern regions of Europe, including Caucasus and Balkans. It has been widely naturalized throughout Europe and North America as game birds. In certain places within Japan, this pheasant species is farmed for exotic meat markets, as is the case of the green pheasant, *Phasianus versicolor* Vieillot 1825, which is exclusively endemic to the Japanese archipelago. Owing to their importance as game birds in the northern hemisphere, ring-necked pheasants have been the subject of various parasitological surveys, and their infection by various nematodes, such as capillariids, heterakids, and *Syngamus trachea*, as well as intestinal coccidians has been recorded. The second chapter, titled "New coccidian *Eimeria iyoensis* n. sp. (Apicomplexa: Eucoccidiorida: Eimeriidae) in farmed ring-necked pheasants (*Phasianus colchicus* L.) in Ehime, Japan," focused on the identification of a new coccidian species.

We employed integrated taxonomic approaches to eimerian and capillariid species from farmed ring-necked pheasants in Japan. During this study, we performed a parasitological examination of the gastrointestinal tract of farmed ring-necked pheasants (*Phasianus colchicus karpowi*) on two farms in Ehime, Japan.

Fecal examination through flotation and sedimentation methods (43, 103, and 50 samples in three consecutive years from 2020, respectively) detected coccidian oocysts (5–58%), or capillarid (40–56%) and heterakid eggs (45–72%). Following artificial sporology, most sporulated coccidian oocysts were ellipsoidal without micropyle nor residuum, but with 1–3 polar refractile granules, morphologically reminiscent of *Eimeria phasiani* (Apicomplexa: Eucoccidiorida: Eimeriidae). Intensive sequencing of mitochondrial cytochrome *c* oxidase

subunit I gene (*cox-1*) using pan-eimerian primers and multiple oocyst samples from different pheasants indicated a single species. We characterized, for the first time, the complete *cox-1* sequence of *E. phasiani,* known to be prevalent in wild and captive ring-necked pheasants worldwide.

Worm recovery under a dissection microscope revealed two capillariid and one heterakid nematode species: *Eucoleus perforans* (Nematoda: Trichocephalida: Capillariidae) in the esophageal epithelium (prevalence, 8–73%), *Capillaria phasianina* (Capillariidae) in the cecal mucosa (10–87%), and *Heterakis gallinarum* (Nematoda: Ascaridida: Heterakidae) in the cecal lumen (69–88%). The small subunit ribosomal RNA gene (SSU rDNA) of *E. perforans* was perfectly identical to that in a previous isolate from farmed Japanese green pheasants (*P. colchicus versicolor*) at a distant locality in Japan. The SSU rDNA of *C. phasianina* was characterized, for the first time, demonstrating a sister relationship with *Capillaria anatis*, parasites found in the ceca of domestic ducks, geese, and various wild anatid birds.

In a subsequent study, 35 fecal samples were collected from two- to three-month-old ring-necked pheasants from four pheasant-rearing farms in Ehime Prefecture, Japan. Microscopic examination using a saturated sugar solution technique detected numerous subspherical oocysts from the samples of one farm and ellipsoidal *E. phasiani* oocysts from the three other farms. The subspherical oocysts were artificially sporulated and measured 18.6 µm by 15.7 µm with a 1.18 shape index (n = 150). Each oocyst contained four 10.7 µm × 5.8 µm sporocysts (n =30) and one coarse refractile polar granule; no micropyle or residua were detected. Each sporocyst contained two sporozoites with one large and one small refractile body and sparsely distributed residua. The complete, 1,443-bp cox-1 sequence of this isolate exhibited low sequence identity with published *Eimeria* spp. sequences including *E. phasiani* that was previously recorded in the same area. Meanwhile, the oocyst morphology most closely resembled that of *Eimeria tetartooimia*, but distinct in morphology of refractile polar granules and sporocyst residua. The

available GenBank *cox-1* sequence of *E. tetartooimia* exhibited a sequence identity of < 94.5% with the study isolate. Here, the coccidian isolate identified in this study represents a new *Eimeria iyoensis* n. sp. capable of infecting ring-necked pheasant.

These methods allowed the researchers to gain a comprehensive understanding of the eimerian and capillariid infections recorded in the ring-necked pheasants and their phylogenetic relationships with congeners. The findings from this study contribute to the broader understanding of these parasites and their impact on the health of farmed pheasants. The study also opens up avenues for further research into the molecular characterization of these parasites, which can contribute to the development of more accurate diagnostic tools.

General Introduction

The ring-necked pheasant (Phasianus colchicus Linnaeus, 1758) is a popular gamebird species in the pheasant family Phasianidae. (Hiatt & Fisher, 1947), while the green pheasant (*Phasianus versicolor*), also known as the Green Hen, is the national bird of Japan.

It is native to Asia and parts of Europe, like the northern foothills of the Caucasus and the Balkans. It has been widely introduced elsewhere as a gamebird. In parts of its range, namely in places where none of its relatives occur, such as in Europe, where it is naturalized, it is simply known as the "pheasant". Ring-necked pheasant is both the name used for the species as a whole in North America and also the collective name for a number of subspecies and their intergrades that have white neck rings.

In Asia, ring-necked pheasants are native to the Korean Peninsula and is widely distributed in Japan from Hokkaido to Okinawa, including the Sakishima Islands. In due course, these birds spread throughout most of Europe and North America, where they are now found in wild populations. The ring-necked pheasants and the endemic green pheasants are reared primarily by the exotic meat trade in Japan. This practice highlights their importance in the economy and the cultural relevance these birds hold all across the globe. Monitoring the health of these valuable birds and preventing parasitic diseases is economically important.

Several *eimeria* spp. can infect pheasants. of capillariids and heterakids such as pinworms, there by reducing their health and production. *Capillaria s*pp. (Leibowitz, 1962) especially in free-range poultry systems, where they may result to heavy diseases and production losses. Poultry factors such as production system, age and hygienic condition are the most relevant in explaining parasite prevalence (Lozano et al., 2019). Furthermore, the knowledge of phylogenetic relationships and diversity of these parasites is indispensable to ensure proper management and efficient treatment strategies. *Eimeria* spp. and *capillaria* spp. is responsible for significant economic losses in poultry farming, especially in free-range and organic production systems (Lozano et al., 2019).

The *eimeria* spp. represent a large group of single-cell parasites that are ubiquitous and parasitize broad range of vertebrate hosts. More than 1800 species of Eimeria have been described and it is estimated that most of the species (more than 98%) are still awaiting their discovery (Duszynski, 2011, Vrba, 2015).

Coccidiosis is caused by the *eimeria* spp. which is a major concern, causing significant morbidity and mortality in affected herds. Capillariasis is a type of infection that is due to *capillaria* spp. and can cause almost lethal intestinal inflammation with subsequent waves of mortality in pheasants.

These parasitic diseases are important for each of them individually, whereas the host-parasite association involving other eimerian and capillarid parasites that infect ring-necked pheasants is less clear. The relationships of these parasites have usefulness in elucidating their evolution and the biology (including the host-parasite relationships) of these parasites, and most importantly, it will help in controlling them.

The purpose of my study was to address this by determining the phylogenetic placement of eimerian and capillariid parasites infecting farmed ring-necked pheasants. This research will determine the genetic diversity and evolutionary relations of these parasites, therefore leading better management and control employing molecular approaches.

The study has several main objectives: In the beginning, we are collected and analyzed samples from farmed ring-necked pheasants, and identified existing eimerian and capillarid parasite species through a comprehensive morphological and molecular analysis. This research work is characterized by the integrated use of morphological and molecular identification methods to determine the taxonomic classification of these parasites.

Futhermore, the study reconstructed the phylogenetic relationships of the identified parasite species using genetic markers. It used methods of phylogenetic analysis such as maximum likelihood and Bayesian inference to determine the evolutionary relationships between parasites and their placement within the broader eimerian and capillarid lineages. In addition, the characteristics of localizers and geographic distribution of parasites identified in the study were

compared. It compared the parasites found in the ring-necked woodpecker with parasites recorded in other host species and geographic regions, providing insight into the evolutionary dynamics of host parasites and the potential for interspecific transmission.

The study aimed at providing an overview between the congruence of richness in hosts and parasite lineages and how this may impact pheasants with eimerian- and capillariid-type of parasites, in relation to its diversity, evolutionary relationships, and host-specificity. Such knowledge may be useful to enhance diagnostic procedures, treatment regimes and control measures leading to reduction of the consequences of these gastrointestinal parasitic infections. Researchers and veterinarians as well as bird breeders of ring-necked pheasants or other game birds are those to whom the results of this study will be relevant. The study based on previous research on the prevalence and impact of gastrointestinal parasites in free-range and farmed poultry production systems. (Lozano et al., 2019) This study gives a deeper insight into these two important parasite entities by providing in detail phylogenetic analysis of eimerian and capillarid parasites.

Chapter I

Eimerian and capillariid infection in farmed ring-necked pheasants (*Phasianus colchicus karpowi*) in Ehime, Japan, with special reference to their phylogenetic relationships with congeners

This work described in this chapter has been published as follows:
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1.1. Abstract

I performed a parasitological examination of the gastrointestinal tract of farmed ring-necked pheasants (*Phasianus colchicus karpowi*) on two farms in Ehime, Japan. Fecal examination through flotation and sedimentation methods (43, 103, and 50 samples in three consecutive years from 2020, respectively) detected coccidian oocysts (5–58%), or capillarid (40–56%) and heterakid eggs (45–72%). Following artificial sporology, most sporulated coccidian oocysts were ellipsoidal without micropyle nor residuum, but with 1–3 polar refractile granules, morphologically reminiscent of *Eimeria phasiani* (Apicomplexa: Eucoccidiorida: Eimeriidae). Intensive sequencing of mitochondrial cytochrome *c* oxidase subunit I gene (*cox-1*) using pan-eimerian primers and multiple oocyst samples from different

pheasants indicated a single species. We characterized, for the first time, the *cox-1* sequence of *E. phasiani*, known to be prevalent in wild and captive ring-necked pheasants worldwide. Worm recovery under a dissection microscope revealed two capillariid and one heterakid nematode species: *Eucoleus perforans* (Nematoda: Trichocephalida: Capillariidae) in the esophageal epithelium (prevalence, 8–73%), *Capillaria phasianina* (Capillariidae) in the cecal mucosa (10–87%), and *Heterakis gallinarum* (Nematoda: Ascaridida: Heterakidae) in the cecal lumen (69–88%). The small subunit ribosomal RNA gene (SSU rDNA) of *E. perforans* was perfectly identical to that in a previous isolate from farmed Japanese green pheasants (*Phasianus colchicus versicolor*) at a distant locality in Japan. The SSU rDNA of *C. phasianin*a was characterized, for the first time, demonstrating a sister relationship with *Capillaria anatis*, parasites found in the ceca of domestic ducks and geese and various wild anatid birds.

1.2. Introduction

The ring-necked pheasant, *Phasianus colchicus* Linnaeus, 1758, is a bird species originally endemic to Asia and the eastern regions of Europe, including Caucasus and Balkans. It has been widely naturalized throughout Europe and North America as game birds (Robertson 1997). In certain places within Japan, this pheasant species is farmed for exotic meat markets, as is the case of the green pheasant, *Phasianus versicolor* Vieillot, 1825, which is exclusively endemic to the Japanese archipelago. Owing to their importance as game birds in the northern hemisphere, ring-necked pheasants have been the subject of various parasitological surveys, and their infection by various nematodes, such as capillariids, heterakids, and *Syngamus trachea* Montagu, 1811, as well as intestinal coccidians has been recorded (Tyzzer 1929; Ormsbee 1939; Trigg 1967a; Norton 1976; Wacha 1973; Fisher and Wacha 1976; Draycott et al. 2000; Tampieri et al. 2005; Gassal and Schmäschke 2006; Atkinson et al. 2008; Omoto and Koresawa 2015; Rząd et al. 2021). However, accurate identification of some parasites in pheasants, particularly those causing coccidiosis, remains a challenge, as microscopic differentiation of one species from the congeners is often difficult for non-experts, while molecular taxonomic approaches for the characterization of parasites in pheasants are scarce (Sakaguchi et al. 2020; Matsubayashi et al. 2021).

More than eight nominal *Eimeria* spp. (Apicomplexa: Eucoccidiorida: Eimeriidae) have been recorded in pheasants, based on morphological and other biological criteria (Joyner and Long 1974; Norton 1976; Kinoshita et al. 2021). However, there have been limited attempts at the molecular characterization of coccidian oocysts for species differentiation (Miska et al. 2010; Vrba and Pakandl 2015). Notably, both approaches have not been combined to date. In other words, the molecular characterization of morphologically identified *Eimeria* spp. has not been performed, except for *Eimeria tetartooimia* Wacha, 1973 (Matsubayashi et al. 2021). Molecular analyses are invaluable for the differentiation and classification of *Eimeria* spp.,

enabling a more accurate understanding of the eimerian diversity in chickens and turkeys (Schwarz et al. 2009; Ogedengbe et al. 2011, 2014; El-Sherry et al. 2013, 2014, 2015, 2017; Vrba et al. 2011; Vrba and Pakandl 2014, 2015; Morgan and Godwin 2017).

Schwarz et al. (2009) amplified genomic DNA from apicomplexan oocysts, including various *Eimerian* spp. from chickens, using genus-specific primers targeting nuclear small subunit ribosomal RNA gene (SSU rDNA), the first and second internal transcribed spacer (ITS) regions, and the mitochondrial cytochrome *c* oxidase subunit I gene (*cox-1*). They obtained a spectrum of divergent SSU rDNA and ITS sequences, in addition to a consistent *cox-1* sequence for a single species. Vrba et al. (2011) obtained two types of divergent SSU rDNA sequences from a single-oocyst-driven line of *E. mitis* Tyzzer, 1929, confirming, for the first time, the presence of divergent, paralogous SSU rDNA copies within a single species of *Eimeria*, as previously reported in *Plasmodium* spp., *Cryptosporidium parvum*, and *Babesia bigemina* (McCutchan et al. 1988; Reddy et al. 1991; Corredor and Enea 1994; Qari et al. 1994; Le Blancq et al. 1997). El-Sherry et al. (2013) demonstrated similar results for multiple single-oocyst-driven lines of *E. meleagrimitis* Tzzer, 1929 in turkeys. They reported that two types of SSU rDNA sequences from each of the single-oocyst-derived lines of *E. meleagrimitis* had a mean sequence identity of 97.4%, whereas the mean sequence identity within types was 99.1–99.3%. These results indicate that, unlike mitochondrial *cox-1* sequences, SSU rDNA sequences alone are not reliable molecular markers for the species identification of coccidians, although they demonstrate a clear utility in the phylogenetic reconstruction of apicomplexan parasites at the genus and higher taxonomic ranks (Ogedengbe et al. 2011, 2014; Poplstein and Vrba 2011; Vrba et al. 2011; El-Sherry et al. 2013, 2014).

Currently in development are integrated taxonomic approaches for characterizing members of the family Capillariidae Railliet, 1915 (Nematoda: Trichocephalida: Trichinelloidea), the taxonomic identification of which is complicated by the need for careful

manipulation of fragile thin worms for subsequent morphological observation (Tamaru et al. 2015; Sakaguchi et al. 2020). For the diverse species of this family, which is classified into more than 27 genera (Moravec 1982, 2001; Gibbons 2010), and its closely related family Trichuridae, SSU rDNA sequences are reliable molecular markers for species identification, even when used alone (Tamaru et al. 2015; Phosuk et al. 2018; Sakaguchi et al. 2020; Carvalho et al. 2023). In our previous study (Sakaguchi et al. 2020), two capillariid species, *Eucoleus perforans* (Kotlan et Orosz, 1931) López-Neyra 1946 in the crop and *Capillaria phasianina* Kotlan, 1940 in the ceca, were collected from farmed Japanese green pheasants in Kumamoto, Japan, and the genetic distinctness of *E. perforans* from *E. contortus* (Creplin, 1939) López-Neyra 1946 collected from the crop of geese in Indonesia was successfully demonstrated based on sequencing of the SSU rDNA. Moreover, *E. perforans* and *E. contortus* are frequently isolated from the crop of domestic birds, including chicken, turkey, goose, grouse, guinea fowl, partridge, pheasant, pigeon, and quail, and exhibit highly similar morphology, except for different eggshell surface ornamentation, as previously reported (Madsen 1951; Baruš and Sergejeva 1989b; Sakaguchi et al. 2020). Therefore, the molecular characterization of *E. perforans* is crucial for its accurate differentiation from *E. contortus.*

In our previous study, we did not perform molecular characterization of *C. phasianina* due to the limited availability of worms collected from the farmed Japanese green pheasants (Sakaguchi et al. 2020). Therefore, the present study aimed to molecularly characterize *C. phasianina*. I employed integrated taxonomic approaches in eimerian and capillariid species from farmed ring-necked pheasants in Japan using mitochondrial *cox-1* or nuclear SSU rDNA sequencing.

1.3. Materials and methods

1.3.1. Parasite collection and laboratory examination

The gastrointestinal tracts of farmed ring-necked pheasants, *Phasianus colchicus karpowi* Buturlin, 1904, were collected on December 8, 2020 (43 samples from Pheasant Farm A), November 10, 2021 (103 samples from Pheasant Farm A), and November 1, 2022 (50 samples from Pheasant Farm B) at a poultry processing plant for pheasant meat in the southwestern region of Ehime Prefecture, Japan. Raw visceral samples were immediately sent to the parasitology laboratory at Yamaguchi University, being kept at 4˚C. After arrival, small (uncertain) volumes of cecal contents were individually transferred to 15-mL plastic tubes, suspended in 2.5% potassium bichromate solution (approximately 4 mL per tube) by approximately 24 h after sacrifice, and kept at room temperature (around 20˚C) until microscopic observation. Other parts of the viscera were individually packed in plastic bags and frozen at -20˚C until examination.

Fecal samples from the ceca were microscopically observed after standard processing for the oocyst/egg flotation technique using a saturated sugar solution and sedimentation technique (Garcia 2007). Frozen gastrointestinal tract samples from the proventriculus or gizzard to the rectum were individually thawed in running tap water~~,~~ and divided into four parts: 1) gizzard and, when available, proventriculus, 2) upper part of the small intestine, 3) lower part of the small intestine, and 4) ceca and colon. Each part was cut open longitudinally, scratched carefully by gloved fingers, and suspended in 1.5 L tap water for worm sedimentation. After repeated washing of sediments to obtain clear supernatant, gastrointestinal contents (sediments) were individually examined under a dissection microscope. Esophagi containing crops were separately collected from other viscera, and parasites were similarly examined under a dissection microscope.

Sporulated coccidian oocysts were observed using a microscope equipped with differential interference contrast imaging (Olympus BX60; Olympus Co., Shinjuku, Tokyo, Japan), and

photographed at a magnification of × 800, with the obtained images then transformed into photographs with Adobe® Photoshop® ver. 11.0 (Adobe Systems, San Jose, California, USA). Photographs were printed at high magnification. The collected helminth parasites were preserved in either 10% neutral-buffered formalin solution or 70% ethanol, and categorized by host organ, parasite sex, and certain morphological characteristics under a dissection microscope. Intensive morphological analysis of formalin-fixed specimens was performed under the conventional optic microscope mentioned above, at high magnifications (Tamaru et al. 2015; Sakaguchi et al. 2020). Figures were drawn under another microscope (Olympus CH2) equipped with a camera lucida. Measurements were performed on these drawn figures using a digital curvimeter type S (Uchida Yoko, Tokyo, Japan), when necessary, and are expressed in micrometers (oocysts) or millimeters (helminth) as the mean or means ± standard deviations (SD), with the range in parentheses. Collected specimens were deposited in the Meguro Parasitological Museum, Tokyo, Japan (specimen numbers 25017–25024).

1.3.2. DNA extraction, polymerase chain reaction (PCR), and sequencing

Coccidian oocysts preserved in 2.5% potassium bichromate solution were concentrated through a sedimentation technique in glass tubes, transferred to 2 mL plastic tubes, and washed three times via centrifugation with pure distilled water. The boiling (in a water bath) and freezing (-30˚C) of 30 μL of oocyst suspension (pure distilled water) in a plastic tube were repeated three times before DNA extraction. Nematode parasites preserved in 70% ethanol were washed three times with pure distilled water in 2 mL plastic tubes. The DNA of coccidian oocyst and helminth samples was extracted individually using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions. PCR amplification of overlapping fragments of *cox-1* of coccidian species and SSU rDNA of nematodes was performed using different sets of primers (Suppl. Table S1). Except for three coccidian-specific *cox-1* primers reported by previous studies

(Gjerde 2013; Imai and Barta 2019; Duff et al. 2022), COI_UNI_199F, COIRm, and mtRNA20_UNI_R, several new primers were designed in-house using the online software Primer3web ver. 4.0.0 (Untergasser et al. 2012) and referring to highly conserved *cox-1* regions of *Eimeria* spp. with published whole mitochondrial genomes (22 taxa from chickens, turkeys, rabbits, horses, skunks, ferrets, and mice): DDBJ/EMBL/GenBank accession nos. [chicken] KX094948 (*E. acervulina*), HQ702480 (*E. brunetti*), KX094954 (*E. necatrix*), KX094945 (*E. praecox*); HG994976 (*E. tenella*); [turkey] KJ608415 (*E. adenoeides*), KJ608416 (*E. dispersa*), KR108296 (*E. innocua*), KJ608418 (*E. meleagridis*); [rabbit] HQ173877 (*E. coecicola*), KP025693 (*E. flavescens*); KP009592 (*E. intestinalis*), KP025690 (*E. irresidua*), KF419217 (*E. magna*), KP025691 (*E. media*), HQ173887 (*E. perforans*), HQ173890 (*E. stiedai*), KP025692 (*E. vejdovskyi*); [horse] MW354691 (*E. leuckarti*); [skunk] KT203398 (*E. mephitidis*); [ferret] NC_039745 (*E. furonis*); and [mouse] KX495129 (*E. falciformis*). Finally, three new primers (Eim_cytb382F, Eim_cox1_658F, Eim_cox1_1356R) were used in addition to the aforementioned primers used in previous studies (see Table 1).

For coccidian *cox-1*, two semi-nested and one nested PCR systems, involving two first-round PCRs and one or two second-round PCRs, were performed, and nucleotide sequencing of PCR amplicons was conducted with the primers used for second-round PCRs, and additional primers for sequencing (Table 1). For capillariid SSU rDNA, three conventional PCR amplifications were performed as per our previous studies (Tamaru et al. 2015; Sakaguchi et al. 2020). For heterakid rDNA from the 3'-terminal part of the SSU rDNA to the large subunit rDNA, which included ITS regions and 5.8S rDNA, four conventional PCR amplifications were employed using primers previously used in our laboratory for PCR amplification of helminth rDNA fragments (Tran et al. 2016; Kamimura et al. 2018). The DNA polymerase used was Blend Taq -Plus- (TOYOBO, Kita-ku, Osaka, Japan), and PCRs were conducted using 20- or 25-μL reaction solutions in a thermal cycler, as per the following cycling protocol: 2 min at 94°C; followed by 35 cycles at 94°C for 30 s, 52˚C (*cox-1*) or 62°C (rDNA) for 30 s, and 72°C

for 90 s; and final extension at 72°C for 7 min. The PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly with the primers for amplification and sequencing (see Table 1). The nucleotide sequences reported in the current study are available from the DDBJ/EMBL/GenBank databases under the accession numbers LC777439–LC777442.

1.3.3. Phylogenetic analysis

Fragments of the newly obtained *cox-1* and rDNA sequences of parasites collected in this study were analyzed to identify highly similar nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information website (NCBI: https://www.ncbi. Nlm.nih.gov/). For phylogenetic analysis, the newly obtained *cox-1* and rDNA sequences of parasites in this study and related sequences retrieved from the GenBank database (NCBI) were aligned using the MEGA7 software (Kumar et al. 2016), with subsequent manual adjustments. Regions judged to be poorly aligned and base positions with ambiguous nucleotides in any sequence were excluded from subsequent analyses: 1,513 characters, of which 436 were variable, remained for subsequent analysis of capillariid SSU rDNA. For coccidian species, two sets of *cox-1* sequences of different lengths were prepared; (1) 1,257 characters, of which 455 were variable, and (2) 420 characters, of which 158 were variable, remained for subsequent analysis. Maximum likelihood (ML) analysis was performed using the program PhyML (Guindon and Gascuel 2003; Dereeper et al. 2008) provided on the "phylogeny.fr" website (http://www.phylogeny.fr/). This program is a built-in package of the substitutional model 'HKY85'. The probability of inferred branches was assessed by the approximate likelihood ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

1.4. Results

1.4.1. Parasitological examination

Microscopic examination of cecal feces from farmed pheasants detected coccidian oocysts (5–58%), and capillariid (40–56%) or heterakid eggs (45–72%), as shown in Table 2. Floatation and sedimentation techniques were used for the detection of coccidian oocysts and nematode eggs, respectively. In this study, the examined volume of cecal feces varied between individuals, from a considerably small amount to, in some cases, an amount as much as the size of a little fingertip. Thus, the exact numbers of oocysts and eggs per unit were unknown. Worm recovery from the esophagus and cecum under a dissection microscope was successful, and three nematode species were differentiated based on morphology. Capillariid worms from the esophagi and ceca were *E. perforans* and *C. phasianina*, respectively, consistent with morphological features of these species reported by Baruš and Sergejeva (1989a, b), as well as Sakaguchi et al. (2020). Another nematode obtained from the ceca was *Heterakis gallinarum* Schrank, 1788, consistent with the morphological features of the species (Yevstafyeva et al. 2018). Worm recovery was variable (Table 2), with 8–73% for *E. perforans* in the esophagus and crop, 10–87% for *C. phasianina* in the cecum, and 69–88% for *H. gallinarum* in the cecum. The parasite faunas prevalent in pheasants from Farms A and B were similar.

1.4.2. Morphological observation and molecular analysis

1.4.2.1. *Eimeria phasiani* Tyzzer, 1929

Coccidian oocysts that were preserved in a 2.5% potassium dichromate solution successfully developed into sporulated oocysts, with four sporocysts each containing two sporozoites (eight sporozoites per oocyst). Most oocysts were ellipsoidal but sometimes had a broadly or elongated ellipsoidal shape (Fig. 1), and their dimensions exhibited slight variation

(Fig. 2). The morphological characteristics of oocysts in the feces of different origins (e.g. different individuals, farms or year) were consistent.

Sporulated oocysts had double-layered oocyst walls, smooth in texture. Micropyle and oocystic or sporocystic residua were absent. Each sporulated oocyst had one to three refractile polar granules. Predominant and ellipsoidal oocysts (n = 42) measured 22.4 ± 4.8 (19.2–27.1) µm in length and 15.6 ± 3.2 (14.1–16.8) µm in width, with a shape index of 1.44 ± 0.17 (1.24–1.92). Fusiform sporocysts (n = 42) measured 11.6 ± 2.4 (9.6–13.1) µm in length and 5.8 ± 1.1 (4.9–6.65) µm in width, each possessing a Stieda body. Broadly ellipsoidal oocysts (Fig. 1c), showing a shape index of 1.28 (1.15–1.49; n=21), were similarly measured, and they were compared to those of *E. phasiani* and other *Eimeria* spp. from pheasants with morphometric similarity (Table 2). Apart from intentional measurements of oocysts based on spore shape, the dimensions of unintentionally chosen oocysts (n = 590 from Farms A and B) are plotted in Fig. 2; 23.1 ± 1.9 (17.7–29.8) µm in length and 17.0 ± 1.2 µm (13.3–22.3) µm in width, with a shape index of 1.36 ± 0.12 (1.00–1.82).

Nucleotide sequencing of partial *cox-1* fragments of coccidian oocysts (655 bp), amplified via semi-nested PCR using a primer pair of Eim_cox1_658F and Eim_cox1_1356R (Table 1), was attempted using 10 samples: four samples from Farm A and six samples from Farm B. All examined samples of coccidian oocysts exhibited an identical *cox-1* sequence, suggesting the presence of a single species in all these samples. For some of these samples (five samples), longer identical *cox-1* sequences (1,554bp) were obtained (DDBJ/EMBL/GenBank accession no. LC777442). A BLAST search using this newly obtained *cox-1* sequence revealed that it exhibited the highest similarity with that of *Eimeria* sp. ex *Perdix perdix* VV-2014 (accession no. KJ547709; 1,257bp) with 99.60% (1,252/1,257) identity, followed by *E. adenoeides* (KJ608415) with 95.88% (1,490/1,554) identity, *E. meleagridis* (KJ608418) with 95.75% (1,488/1,554) identity, *E. gallopavonis* (KJ608413) with 95.11% (1,478/1,554) identity, and

other *Eimeria* spp. Compared with deposited *cox-1* sequences of *Eimeria* spp. from pheasants (*Phasianus* spp.), those of *Eimeria* sp. *Phasianus colchicus* /24-6.10-s3 (HM117019) showed 96.79% (783/809) identity, *Eimeria* sp. ex *Phasianus colchicus* VV-2014 (KJ547708) showed 93.72% (1,178/1,257) identity, and *E. tetartooimia* TN4-21 (LC612541) from the captive green pheasant in Japan showed 90.24% (379/420) identity.

Phylogenetic relationships are shown in Fig. 3 (ML phylogenetic tree based on 1,513-bp long *cox-1* sequences) and Fig. 4 (ML phylogenetic tree based on 420-bp long *cox-1* sequences). *Eimeria* spp. from a variety of domestic poultry, including chicken, turkey, pheasant, partridge, guineafowl, duck, and peafowl, were segregated into two groups. The newly characterized *E. phasiani* isolate formed a group with avian *Eimeria* spp., such as *E. adenoeides*, *E. gallopavonis*, and *E. meleagridis* in turkeys, or *E. necatrix* and *E. tenella* in chickens (Figs. 3 and 4), with site preference for the lower small intestine and ceca.

Remarks: Measurements of ellipsoidal oocysts were consistent with those of *E. phasiani* (Table 3), showing a shape index (length/width of oocysts) of 1.44 (1.24–1.92; n=42). In addition, the presence of at least one refractile polar granule, but not micropyle, oocystic nor sporocystic residua, or any other morphological features were noted in the sporulated oocysts (Tyzzer 1929; Haase 1939; Ormsbee 1939; Trigg 1967a). Broadly ellipsoidal oocysts resembled those of *E. pacifica* Ormsbee, 1939, *E. duodenalis* Norton, 1967 or *E. dispersa* Tyzzer, 1929, when considering oocyst dimensions and a shape index of 1.28 (1.15–1.49). The selected broadly ellipsoidal oocysts had a homogeneous outer layer of the oocyst wall and a few refractile polar granules generally at a polar end. In contrast, the outer layer of *E. pacifica* oocyst walls is striated radially in cross-section, with one to four refractile polar granules scattered in oocyst fluid, while one polar granule is usually positioned at a polar end (Wacha 1973). Sporulated oocysts of *E. duodenalis* are more spherical, with a shape index of 1.1 (1.0– 1.2), when compared to the broadly ellipsoidal oocysts observed in this study, with a shape

index of 1.28 (1.15–1.49). Further, *E. duodenalis* sporulated oocysts have widely scattered, multiple pieces of refractile granules in the oocyst fluid (Wacha 1973). *Eimeria dispersa* is polyxenous and produces patent infection in turkeys, grey partridge *Perdix perdis* (Linnaeus, 1758), chukar *Alectoris chukar* (Gray, 1830), ring-necked pheasants, ruffed grouse *Bonasa umbellus* (Linnaeus, 1766), Japanese quail *Coturnix japonica* Temminck et Schlegel, 1849 and northern bobwhites (Tyzzer 1929; Doran 1978; Long and Millard 1979; El-Sherry et al. 2015, 2017). The dimensions and shape of *E. dispersa* oocysts excreted in the feces of different host poultry species vary and change over the course of oocyst shedding (Long and Millard 1979; El-Sherry et al. 2017). The broadly ellipsoidal oocysts observed in this study are morphologically differentiated from *E. dispersa* based on the following: double-layered s oocyst wall (a single layer in the latter species), and the presence of refractile polar granule (absent in the latter species). Moreover, sequencing of mitochondrial *cox-1* demonstrated that all isolates exhibiting morphological variation of oocysts are distinct from *E. dispersa*, showing only 90.24% (1,128/1,250) identity with *cox-1* sequences (LC777442 vs. HG793048, KJ608416) and a phylogenetic distance between these two species (Figs. 3 and 4). The *cox-1* sequences of the current isolates (LC777442) and an isolate from a grey partridge, "*Eimeria* sp. ex. *Perdix perdix* VV-2014" (KJ547709) are highly similar, sharing 99.60% (1,252/1,257) identity, but morphological identification of the latter isolate was not attempted. However, Vrba and Pakandl (2015) provided approximate dimensions of the oocysts as 25 μm by 18 μm (shape index = 1.39), slightly bigger than *E. phasiani* oocysts. Further, they simultaneously obtained another isolate from a pheasant, "*Eimeria* sp. ex Phasianus colchicus VV-2014" (KJ547708), which showed 93.87% (1,180/1,257) identity with the *cox-1* sequence of the current isolate from farmed pheasants, but had highly similar oocyst dimensions (22 μm by 15 μm; shape index = 1.47) to *E. phasiani*, as previously reported (Tyzzer 1929; Ormsbee 1939; Haase 1939; Trigg 1967a). The morphological and molecular characterization of these two isolates from a partridge and pheasant reported by Vrba and Pakandl (2015) is critical for advancing accurate

species identification based on the *cox-1* sequences given that such studies on pheasant *Eimeria* spp. are scarce.

1.4.2.2. *Eucoleus perforans* (Kotlan et Orosz 1931) López-Neyra 1946

A range of 1–39 worms were collected from the mucosa of the esophagus, including the crop. These worms were either entirely or mostly embedded in the esophageal epithelium (Fig. 5). The posterior ends of male worms were slightly tapered and bluntly terminated with two rounded dorsolateral lobes, on which a distinctive papilla was evident at the level of the cloacal opening (Fig. 6a-e). Spicules were indistinct due to insufficient sclerotization, except for one male worm shown in Fig. 6c and 65e. The spicular sheath was covered with cuticular spines, when visible (Fig. 6b, d). The posterior ends of female worms were abruptly tapered and rounded, and the anus was terminally situated (Fig. 6k). The vulva had no appendages (Fig. 6f, g). Eggs were barrel-shaped with a reticulated surface (Fig. 6h-j). All morphological features of the worms collected from the esophagus in the present study were consistent with those of *E. perforans* reported in a previous study from our laboratory (Sakaguchi et al. 2020), except for the egg-shell surface ornamentation (smooth vs. reticulated). Measurements are shown in Table 4. The SSU rDNA sequence of the current isolate (LC777439) showed 100% (1,758/1,758) identity with that of *E. perforans* isolated from farmed green pheasants in Japan (LC424997).

Remarks: The morphological features of *E. perforans* specimens collected in the present study were consistent with those of species reported by Sakaguchi et al. (2020), except for egg-shell ornamentation. Reticulated ornamentation of the egg-shell surface has been reported in all three *Eucoleus* spp., *E. annulatus* (Molin, 1858), *E. contortus*, and *E. perforans*, which are prevalent in domestic fowls (Carvalho et al. 2019; Borba et al. 2021); however, Baruš and Sergejeva (1989b) and Sakaguchi et al. (2020) observed a smooth egg-shell surface for *E.*

perforans. Irrespective of such morphological differences, the SSU rDNA sequences of two *E. perforans* isolates with reticulated and smooth egg surfaces were identical, as mentioned above. The prevalences of *E. perforans* in pheasants from Farm A through different years showed apparent differences (8% in 2020 vs. 73% in 2021), as determined by parasitological examination of the esophagi. It is uncertain whether these values reflected the real prevalence of the capillariid species or technical problems during the parasitological examination.

1.4.2.3. *Capillaria phasianina* Kotlán 1940

Capillariid worms in the ceca were highly prevalent in the ring-necked pheasants as mentioned above, and 1–20 worms were collected from parasitized birds. The male worm had an enlarged caudal end with two massive ventrolateral lobes (Fig. 7). A well-sclerotized spicule was covered with a sheath densely armed with large triangular spines. Female worms had a vulva with tubular appendages at approximately the anterior 2/7 of the body and a rounded posterior end with a subterminal anus. Eggs had a punctuated surface with protruded lids. Measurements are shown in Table 5. The BLAST search for the SSU rDNA sequence of the current isolate (LC777440) determined the highest identity with *Capillaria anatis* (LC052334) at 95.71% (1,740/1,818), with 9 insert/deletion (indel) sites. An ML phylogenetic tree based on the SSU rDNA nucleotide sequences of representative species of the family Capillariidae is shown in Fig. 8. *Capillaria phasianina* had a sister relationship with *C. anatis* (Schrank, 1790) Travassos, 1915 and formed a clade with other *Capillaria* spp. with site preference to the ceca and colon. These include *C. spinulosa* (Linstow, 1890) Travassos, 1915 and *C. pudendotecta* Lubimova, 1947, distant from *C. madseni* Wakelin, Schmidt et Kuntz, 1970 with site preference to the small intestine.

Remarks: The capillariid worms collected in this study were morphologically identified as *C. phasianina*, which has been recorded in the ceca of various phasianid birds, such as pheasants, peafowl *Pavo cristatus* Linnaeus, 1758, grey partridge *Perdix perdix* (Linnaeus,

1758), Himalayan snowcrock *Tetraogallus himalayensis* G. R. Gray, 1843, helmeted guineafowl *Numida meleagris* (Linnaeus, 1758), and wild turkey *Meleagris gallopavo* Linnaeus, 1758, according to Baruš and Sergejeva (1989a). This species is phylogenetically close to *C. anatis*, which is the type species of the genus *Capillaria sensu stricto*, as defined by Moravec (1982), and has been recorded in the ceca of a variety of waterfowls of the order Anseriformes, or chickens, turkeys, guineafowls, and other avians (Baruš and Sergejeva 1989a).

1.4.2.4. *Heterakis gallinarum* Schrank, 1788

A high prevalence of this heterakid species was noted throughout the present study (69–88%). Upon exclusive detection of larvae or young juvenile worms alone, the prevalence became higher (89–94%) than the aforementioned values, which only included adult worms and developed juveniles. Selected male worms (n = 11) and female (n =10) measured 11.9–16.7 (13.7) mm and 13.8–17.4 (15.4) mm in length, respectively, with males possessing two spicules of unequal lengths, 0.96–1.31 (1.12) mm and 2.93–3.93 (3.33) mm. Measurements of the collected worms in this study were compared to those previously reported for *H. gallinarum* (Table 6). Nucleotide sequences of the 5.8S rDNA and two ITS regions of the current isolate (LC777441; 974bp) were largely identical to multiple deposited sequences of Chinese isolates of *H. gallinarum* on the DDBJ/EMBL/GenBank (e.g. KT310099—KT310157 with 99.79% (969 / 971)–100% (971 / 971) similarity).

Remarks: *Heterakis gallinarum* is a transporter of pathogenic protozoan *Histomonas meleagridis* (Smith, 1895), which causes serious histomoniasis in galliform birds, particularly in chickens, wild turkeys, chukar, and peafowls (Atkinson et al. 2008). In ring-necked pheasants, the disease is mild, and the birds act as a superior reservoir, contaminating the soil with *Histomonas*-bearing heterakid eggs (Lund and Chute 1972; Atkinson et al. 2008). No apparent

pathogenic changes induced by *H. gallinarum* or *H. meleagridis* were noted in the ceca and liver of our pheasants upon gross examination. *Heterakis isolonche* Linstow, 1906, which causes nodular typhlitis, has been recorded in pheasants (Mendonça 1953; Callinan 1987; Menezes et al. 2003; Sepielak et al. 2019). *Heterakis dispar* (Schrank, 1790) is common in anatid birds (Atkinson et al. 2008). Molecular differentiation of these three species based on nucleotide sequences of the ITS region is ongoing (Bobrek et al. 2019, 2022; Biswas et al. 2021), although critical morphological differences, such as in spicule lengths (equal or unequal, or length) and the number of caudal papillae, were evident among them (see Table 6).

1.5. Discussion

In this study, the helminth parasites recovered from farmed ring-necked pheasants were limited to three nematode species,–*E. perforans*, *C. phasianina*, and *H. gallinarum*. Their predominance in feral ring-necked pheasants in Europe has been previously reported (Draycott et al. 2000; Gassal and Schmäschke 2006; Rząd et al. 2021). Additionally, a high prevalence of the gapeworms *Syngamus trachea* in the trachea was previously reported in pheasants from the same farm surveyed in this study (Farm A in spring – summer of 2013) (Omoto and Koresawa 2015), when approximately 40% of farmed pheasants showed typical clinical signs of gaping, weakness with no drinking, anorexia, and emaciation, leading to a higher number of fatalities. Currently, gapeworms are almost absent from the farm.

A variety of capillariid species have been reported in pheasants, including *E. annulatus*, *E. contortus*, *E. perforans*, *Aonchotheca bursata* (Freitas et Almeida, 1934), and *C. phasianina* (Wakelin 1967; Baruš and Sergejeva 1989a, b, 1990; Pinto et al. 2004; Gassal and Schmäschke 2006). The three *Eucoleus* spp. are frequently isolated from the crop of domestic birds, including chicken, turkey, goose, guineafowl, partridge, pheasant, pigeon, and quail (Saif 2008),

and show few minor morphological differences such as the presence/absence of cuticular swelling at the head or different ornamentation of egg-shell surface (Baruš and Sergejeva 1989b; Sakaguchi et al. 2020). Sakaguchi et al. (2020) attempted molecular differentiation between *E. contortus* isolated from the crop of domestic geese (*Anser anser domesticus* (Linnaeus 1758)) and *E. perforans* from the crop of Japanese green pheasants, demonstrating 97.27% (1,748/1,797) identity of the SSU rDNA with two indels (LC424996 vs. LC424997). In our study, the *E. perforans* isolate from the farmed ring-necked pheasant (LC777439) exhibited an identical SSU rDNA sequence with *E. perforans* previously isolated from Japanese green pheasants (LC424997), confirming morphological identification (see Fig. 4). Recently, Deng et al. (2021) obtained the complete mitochondrial genome (14,118 bp; MW999680) and a partial SSU rDNA sequence of *E. annulatus* (968bp; MZ048355) from the crop of domestic chickens in China, which showed the highest identity (97.51%, but with 5 indels) with *E. perforans* from pheasants (LC424997 and LC777439), followed by *E. contortus* from the domestic geese (LC424996) with 95.84% identity and 7 indels, as well as other *Eucoleus* spp. and *Capillaria* spp. Gassal and Schmäschke (2006) reported the prevalence of capillariid species in 151 pheasants (118 wild and 33 farmed birds) in Germany, with 3, 30, 30, 30, and 80% for *E. annulatus*, *E. contortus*, *E. perforans*, *A. bursata*, and *C. phasianina*, respectively. Although the small intestine of pheasants was carefully examined in the present study, no capillariid worms, including *A. bursata*, were detected, whereas the prevalence of *C. phasianina* was high in examined pheasants from Farm A (Table 1). In summary, the present study reports for the first time the molecular characterization of *C. phasianina*, providing insights into its phylogenetic relationships with other *Capillaria* spp. and various capillariid species. The newly obtained SSU rDNA sequence of *C. phasianina* (LC777440) indicated its close sister relationship with *C. anatis* parasitizing the ceca of various birds, mainly Anseriformes (Wakelin 1964, 1965; Baruš and Sergejeva 1989a). *Capillaria phasianina* also shows a cosmopolitan distribution and parasitizes the ceca of various avian hosts, mainly Galliformes, such as

pheasants, partridges, chickens, turkeys, Old World quail, and peafowl (Kellogg and Prestwood 1968; Baruš and Sergejeva 1989a; Pinto et al. 2004).

Fatal or severe capillariasis caused by *Eucoleus* spp. in the upper digestive tract (originally crops and extending to the upper esophagus and mouth) is a major problem in guineafowls, turkeys, partridges, pheasants, and quails (Bickford and Gaafar 1966; Itagaki et al. 1974; De Rosa and Shivapprasad 1999; Pinto et al. 2004; Cruz et al. 2016), clinically manifested as droopiness, weakness, anorexia, vomiting, and emaciation. *Eucoleus* spp. from the esophagus of phasianid birds, such as pheasants, partridges, and grouses, have been identified as *E. perforans* (Pinto et al. 2004; Gassal and Schmäschke 2006; Sakaguchi et al. 2020), *E. contortus* (Millán et al. 2002, 2004; Tampieri et al. 2005; Gassal and Schmäschke 2006), or *E. annulatus* (Pinto et al. 2004; Tampieri et al. 2005; Gassal and Schmäschke 2006). *Eucoleus annulatus* requires earthworms as an intermediate host, whereas *E. perforans* and *E. contortus* have a direct life cycle without intermediate hosts (Cram 1936; Wehr 1936). Therefore, the accurate identification of capillariids in captive and wild phasianid birds could improve our understanding of their host specificity and ecology, contributing to control measures and prevention of the anthropogenic introduction of pathogens from poultry farms to fields upon the field release of game birds.

At least eight *Eimeria* spp. have been recorded in pheasants (*Phasianus* spp.): *Eimeria colchici* Norton, 1967; *E. dispersa* Tyzzer, 1929; *E. duodenalis* Norton, 1967; *E. langeroni* Yakimof et Matschoulsky, 1937; *E. megalostomata* Ormsbee, 1939; *E. pacifica* Ormsbee, 1939; *E. phasiani*; and *E. tetartooimia* Wacha, 1973 (Tyzzer 1929; Ormsbee 1939; Yakimoff and Matschoulsky 1937; Trigg 1967a; Norton 1967a, b, 1976; Wacha 1973; Kinoshita et al. 2021). Typically, these could be differentiated based on oocyst morphology such as dimensions, shape index, texture of the oocyst wall, presence or absence of micropyle, refractile polar granules, and residuum. However morphometric values frequently fall within the range of

variation of another *Eimeria* species. Therefore, it is difficult to conduct epidemiological surveys of gastrointestinal parasites using oocysts/eggs shed in the feces or other excretes, particularly when specific identification is conducted solely based on morphological criteria. Furthermore, as mentioned above, the oocyst dimensions and shape of a single *Eimeria* species vary between different host poultry species or throughout oocyst shedding (Long and Millard 1979; El-Sherry et al. 2017). Long and Millard (1979) reported that oocyst dimensions change with the time of shedding during the patent period of *E. dispersa* infection in turkeys as follows: 20.5 (16.7–23.6) μm by 17.6 (15.3–19.5) μm (n=26) with shape index 1.17 on day 5 post-inoculation (PI); 23.7 (19.4–27.1) μm by 18.8 (16.7–20.8) μm (n=100) with shape index 1.26 on day 6 PI; and 25.9 (20.8–28.5) μm by 20.2 (15.3–20.8) μm (n=67) with shape index 1.29 on day 7 PI. In addition to *E. dispersa*, other *Eimeria* spp. from pheasants or *E. tenella* in chickens also change the predominant dimensions of oocysts during the patent period (Fish 1931; Joyner and Long 1974).

Molecular data have become invaluable for the differentiation and classification of *Eimeria* spp., as demonstrated in their role in achieving a precise understanding of eimerian diversity in chickens and turkeys (Schwarz et al. 2009; Ogedengbe et al. 2011, 2014; El-Sherry et al. 2014, 2015, 2017; Vrba and Pakandl 2014, 2015; Morgan and Godwin 2017). To clarify the possibility of mixed infection by different *Eimeria* spp., we conducted molecular analyses targeting mitochondrial *cox-1* fragments using pan-eimerian *cox-1* primers (based on 22 taxa from chickens, turkeys, and five mammalian hosts). Sequencing of multiple samples demonstrated that they all contained oocysts of a single species, morphologically identified as *E. phasiani*. A BLAST search using the newly obtained *E. phasiani cox-1* sequence (LC777442; 1,554bp) revealed the highest similarity with a sequence of "*Eimeria* sp. ex. Perdix perdix VV-2014" (KJ547709; 1,257bp) with 99.60% (1,252/1,257) identity. Among three deposited *cox-1* sequences of *Eimeria* spp. from pheasants, the *E. phasiani* sequence identified

in the present study was the most similar to that of an isolate of an undetermined species, "*Eimeria* sp. Phasianus colchicus /24-6.10-s3" (HM117019) at 96.79% (783/809) identity. As partly discussed in the remarks above, it is unclear why *E. phasiani* isolated from pheasants has an almost similar *cox-1* sequence with *Eimeria* sp. isolated from the grey partridge, which had oocyst dimensions of 25 μm by 18 μm (Vrba and Pakandl 2015). Therefore, more *cox-1* sequences of *Eimeria* spp. from pheasants should be collected and properly characterized through classical taxonomic approaches, such as morphological criteria, as the molecular taxonomy of pheasant *Eimeria* spp. has not yet been reported. For this purpose, the use of a single-oocyst-driven line of *Eimeria* spp. propagated in the laboratory is ideal to characterize different species, as previously conducted to understand the diversity of chicken and turkey *Eimeria* spp. (Ogedengbe et al. 2011, 2014; El-Sherry et al. 2014, 2015, 2017; Vrba and Pakandl 2014, 2015). Before the introduction of the molecular characterization of *Eimeria* spp. for taxonomic identification, multiple parameters, such as the morphology of the endogenous stages, their location in the host and the lesions they produce, the timing of the prepatent and patent periods, host and site specificity, and immunological specificity, should all be used to support the morphological characterization of the sporulated oocysts (Joyners and Long 1974; Norton 1976).

When isolates with almost identical *cox-1* sequences (e.g. KJ547709 and LC777442) originate from different avian hosts, the species could be determined as one of the rare euryxenous *Eimeria* spp., such as *E. dispersa*, *E. innocua*, and *E. meleagridis* KR, which can be transmitted from turkeys to partridges and/or bobwhite quails (Vrba and Pakandl 2015). However, given that no previous reports have suggested the possible euryxenous nature of *E. phasiani* and the limited information on "*Eimeria* sp. ex. Perdix perdix VV-2014" (Vrba and Pakandl 2015), we postulate that *E. phasiani* cannot parasitize partridges.

The wide spectrum of *E. phasiani* oocyst dimensions (Fig. 2) and morphology complicated our analyses, with the optional selection of oocysts for measurements producing different shape indices, with 1.45 (1.24–1.92) for ellipsoidal oocysts and 1.28 (1.15–1.49) for broadly ellipsoidal oocysts. El-Sherry et al. (2015) noted that single-oocyst-driven lines of *E. adnoeides* (strains KCH, RM, NR, and Guelph) propagated in different turkeys shed oocysts with variable shape indices of 1.22, 1.40, 1.43, and 1.31, respectively, regardless of their identical *cox-1* sequences (Vrba and Pakandl 2014; El-sherry et al. 2015). They strongly recommended the use of the *cox-1* locus for genotyping of *Eimeria* spp. based on the findings that intraspecific variation at the *cox-1* locus ranged from 0 to 0.2% (3 nucleotide substitutions) over 1,257bp, whereas interspecific variation ranged from 2.6% (33 nucleotide substitutions) to 15.2% (191 nucleotide substitutions). This contrasts with the presence of the two types of SSU rDNA sequences in a single *Eimeria* sp., e.g. *E. meleagridis*, which exhibited only 97% similarity (El-Sherry et al. 2013; Vrba and Pakandl 2014). For an accurate understanding of the epidemiology of different *Eimeria* spp. prevalent in farmed or wild pheasants at different localities or on different continents, studies that establish a reliable platform of molecular taxonomy based on the *cox-1* sequence of accurately specified taxa are pivotal.

1.6. Acknowledgment

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Chapter II

New coccidian *Eimeria iyoensis* n. sp. (Apicomplexa: Eucoccidiorida: Eimeriidae) in farmed ring-necked pheasants (*Phasianus colchicus* L.) in Ehime, Japan

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2-1. Abstract

Eight *Eimeria* spp. (Apicomplexa: Eimeriidae) have been isolated from the ring-necked pheasant (*Phasianus colchicus* Linnaeus), native to the temperate zone of Asia and eastern regions of Europe. Enteric coccidiosis has become a major issue associated with the breeding of farmed pheasants for game bird release or meat production. In this study, 35 fecal samples were collected from two-to-three-month-old ring-necked pheasants from four pheasant-rearing farms in Ehime Prefecture, Japan. Microscopic examination using a saturated sugar solution technique detected numerous subspherical oocysts from the samples of one farm and ellipsoidal *Eimeria phasiani* Tyzzer, 1929 oocysts from the three other farms. The subspherical oocysts were artificially sporulated and measured 18.6 μm by 15.7 μm with a 1.18 shape index (n = 150). Each oocyst contained four 10.7 μm × 5.8 μm sporocysts (n = 30) and one coarse refractile polar granule; no micropyle or residua were detected. Each sporocyst contained two sporozoites with one large and one small refractile body and sparsely distributed residua. The complete, 1,443-bp cytochrome *c* oxidase subunit I gene (*cox1*) of this isolate exhibited low sequence identity with published *Eimeria* spp. sequences including *E. phasiani* that was previously recorded in the same area. Meanwhile, the oocyst morphology most closely resembled that of *Eimeria tetartooimia* Wacha, 1973, but with distinct refractile polar granules and sporocyst residua. The available GenBank *cox1* sequence of *E. tetartooimia* exhibited a sequence identity of < 94.5% with the study isolate. Here, the coccidian isolate identified in this study represents a new *Eimeria iyoensis* n. sp. capable of infecting ring-necked pheasant.

2-2. Introduction

The ring-necked pheasant, *Phasianus colchicus* Linnaeus (Galliformes: Phasianidae), is native to the temperate zone of Asia and the eastern regions of Europe, including the Caucasus and Balkans (Robertson 1997). However, following their introduction as game birds, they have also become widely naturalized in other parts of Europe and North America. Exclusively endemic to the Japanese archipelago is the green pheasant, *Phasianus versicolor* Vieillot; meanwhile, in various regions of Japan, including Ehime Prefecture, the ring-necked pheasants are farmed for commercial production of game meats.

Due to their importance as game birds, ring-necked pheasants are annually maintained via the artificial release of farmed birds in non-endemic regions. However, this practice has increased the incidence of enteric coccidiosis in farmed poults, which has been an area of research focus since the pioneering study by Tyzzer (1929). To date, eight causative coccidian species of the genus *Eimeria* (Apicomplexa: Eucoccidiorida: Eimeriidae) have been described (Table 7) (Tyzzer 1929; Yakimoff and Matschoulsky 1937; Ormsbee 1939; Trigg 1967a, b; Norton 1967a, b, 1976; Wacha 1973). Traditionally, these species are differentiated based on oocyst morphology, including dimensions, shape index, wall texture, presence or absence of micropyle, refractile polar granules, and residuum (Joyner and Long 1974; Norton 1976). However, oocyst morphometric ranges often overlap between species. Furthermore, Long and Millard (1979) reported that oocyst dimensions become significantly altered upon shedding during the patent period of *E. dispersa* infection in turkeys. Similar findings have been reported for other *Eimeria* spp.; however, the oocyst shape index (length/width) typically remains constant (Fish 1931; Duszynski 1971; Joyner and Long 1974).

Molecular characterization has proven invaluable for differentiating and classifying *Eimeria* spp. in chickens, turkeys, and other animals (Schwarz et al. 2009; Ogedengbe et al. 2011, 2014; El-Sherry et al. 2014, 2015, 2017; Vrba and Pakandl 2014, 2015; Morgan and

Godwin, 2017). For example, Schwarz et al. (2009) amplified genomic DNA from *Eimeria* spp. oocysts using specific primers targeting the nuclear small subunit ribosomal RNA gene (SSU rDNA), first and second internal transcribed spacer (ITS) regions, and the mitochondrial cytochrome *c* oxidase subunit I gene (*cox-1*). Although they obtained a consistent *cox-1* sequence for a single species, divergent SSU rDNA and ITS sequences were generated for the same species. Similarly, Vrba et al. (2011) and El-Sherry et al. (2013) reported two types of divergent SSU rDNA sequences for single-oocyst-driven *E. mitis* Tyzzer, 1929 lines in chickens and *E. meleagrimitis* Tyzzer, 1929 in turkeys, respectively. Meanwhile, El-Sherry et al. (2013) reported a mean sequence identity of 97.4% between two types of SSU rDNA sequences and 99.1%–99.3% identities within the same type of single-oocyst-driven species lines. Together, these results demonstrate the reliability of mitochondrial *cox-1* sequencing for the species identification of coccidia. Meanwhile, limited molecular data are available for assessing the coccidian oocysts shed by pheasants (Miska et al. 2010; Vrba and Pakandl 2015; Matsubayashi et al. 2021; Chapter 1 in this thesis). Molecular morphological characterization has only been successfully reported for two *Eimeria* spp., namely, *E. tetartooimia* Wacha, 1973 and *E. phasiani* Tyzzer, 1929 (Matsubayashi et al. 2021; Chapter 1 in this thesis).

In a previous study, was isolated *E. phasiani* from >180-day-old farmed ring-necked pheasants sacrificed for game meats in late November to early December in Ehime Prefecture, Japan. In the current study, fecal samples collected from young ring-necked pheasants at the same location in the summer were evaluated for other *Eimeria* spp. Ultimately, another *Eimeria* sp. was isolated with subspherical oocysts and dimensions smaller than those of *E. phasiani*. The morphological and molecular analyses of the newly collected oocysts suggest the isolate to be a new species, distinct from all eight *Eimeria* spp. previously isolated from pheasants.

2.3. Materials and methods

2.3.1. Sample collection and laboratory examination

Thirty-five fecal samples were collected from 2–3-month-old ring-necked pheasants, *Phasianus colchicus karpowi* Buturlin, from four pheasant-rearing farms in the southwestern region of Ehime Prefecture, Japan, in the last week of July 2023. Additionally, the gastrointestinal tracts were collected on October 23, 2023 (n = 20) and November 28, 2023 (n = 30) from pheasants, which were reared in a farm where subspherical coccidian oocysts were prevalent in the summer, at a poultry processing plant for game meats in Ehime Prefecture. Additionally, the gastrointestinal tracts of 19 farmed pheasants were collected on November 7, 2023, from a farm where *E. phasiani* ellipsoidal coccidian oocysts were prevalent in the summer.

Raw visceral samples stored at 4 °C were sent to the parasitology laboratory at Yamaguchi University. Within 24 h of sacrifice, small volumes of cecal contents were individually weighted on an electric balance GX-600 with a 0.01g minimum scale (A & D Instruments Ltd., Abingdon, Oxford, UK), transferred to 15-mL plastic tubes, suspended in 2.5% potassium dichromate solution (approximately 12 mL per tube), and stored at room temperature (around 20˚C) on a shaker until microscopic examination. The remaining viscera were preserved at −30 °C until examination (10 samples) or fixed in 10% neutral-buffered formalin for histological examination (30 samples).

Fecal samples and cecal contents preserved in 2.5% potassium dichromate solution were microscopically examined after standard processing using the oocyst/egg flotation technique with a saturated sugar solution (Garcia, 2007). Sporulated coccidian oocysts were observed microscopically as described previously (see Chapter 1 in this thesis). All the measurements are expressed in micrometers. Mean values and standard deviations are presented with the range values in parentheses.

2.3.2. Speculation of the coccidian endogenous location

Frozen or formalin-fixed intestinal tracts of selected pheasants, from which certain numbers of oocysts were microscopically detected in the cecal contents, were divided into eight parts following Doran (1978): (1) the descending duodenum, (2) ascending duodenum, (3)

jejunum to the Meckel's diverticulum, (4) upper ileum, (5) lower ileum, (6) proximal part of the cecum, (7) distal part of the cecum, and (8) colon. Histological sections of the intestines, 6 µm thickness, were prepared using standard methods (Kamimura et al. 2018). Scratched pieces of mucosa at different locations of frozen intestines and histological sections stained with hematoxylin-eosin were microscopically examined.

2.3.3. DNA extraction, polymerase chain reaction (PCR), and sequencing

Molecular analyses were performed as described previously (Chapter 1 in this thesis) with slight modifications. Briefly, coccidian oocysts preserved in 2.5% potassium dichromate solution from individual fecal samples were concentrated through a flotation technique on coverslips, washed into plastic dishes with distilled water, and transferred to 2-mL plastic tubes. The collected oocysts were then washed thrice via centrifugation with pure distilled water and subjected to repeated freezing and thawing thrice to disrupt the oocyst walls. DNA was extracted using an Illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare UK, Buckinghamshire, UK) according to the manufacturer's instructions. Semi-nested or nested PCR amplification of overlapping *cox-1* fragments of coccidian species was performed using different sets of primers (Table 8) in 20-µL reaction solutions on a thermal cycler. The DNA polymerase used was Blend Taq -Plus- (TOYOBO, Kita-ku, Osaka, Japan), and 1-µL of DNA extraction solution was added to the reaction solution. The following cycling protocol was applied: 2 min at 94 °C; followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s; and final extension at 72 °C for 7 min. The PCR products were purified using a FastGene Gel/PCR Extraction Kit (NIPPON Genetics Co., Tokyo, Japan) and sequenced directly with the primers for amplification and sequencing (Table 2). When direct sequencing results were not satisfactory, purified PCR products were cloned into the pTA2 plasmid vector (TArget Clone™; TOYOBO, Dojima Hama, Osaka, Japan) according to the manufacturer's instructions. Following propagation, the plasmid DNA was extracted using a FastGene Plasmid Mini Kit

(NIPPON Genetics Co., Tokyo, Japan), and inserts from multiple independent clones, at least three, were sequenced using universal M13 forward and reverse primers.

2.3.4. Phylogenetic analysis

The newly obtained *cox-1* sequence in this study was analyzed to identify highly similar nucleotide sequences using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information website (NCBI: https://www.ncbi. Nlm.nih.gov/). For phylogenetic analysis, the newly obtained *cox-1* in this study and related sequences retrieved from the GenBank database (NCBI) were aligned using the MEGA7 software (Kumar et al. 2016); 1,257 characters, of which 407 were variable, remained for subsequent analysis. Maximum likelihood (ML) analysis was performed using the program PhyML (Guindon and Gascuel, 2003) provided on the "phylogeny.fr" website (http://www.phylogeny.fr/) (Dereeper et al., 2008) as described previously (Chapter 1 in this thesis). This program is a built-in package of the substitutional model "GTR." Nodal support was assessed by the approximate likelihood ratio test, an alternative to the non-parametric bootstrap estimation of branch support (Anisimova and Gascuel 2006).

2.4. Results

2.4.1. Parasitological examination

Microscopic examination of the 35 fecal samples collected from young pheasants at four farms (10 samples each from three farms and five samples from one farm) in the last week of July 2023 detected two types of coccidian oocysts; i.e., type A: ellipsoidal oocysts of *E. phasiani* in three farms, and type B: subspherical oocysts of the genus *Eimeria*, slightly smaller than type A. A few to several type A oocysts were detected per individual sample (uncertain small amounts of feces) in three farms: seven (70%), two (20%), and one (20%) pheasant(s)

were positive in each farm. In contrast, dozens to hundreds of type B oocysts were detected per individual sample in eight (80%) pheasants from the fourth farm.

Additional samples were collected on October 23 (20 samples) and November 28, 2023 (30 samples) from a farm where type B coccidian oocysts were prevalent in pheasants in the summer. Type B oocysts were detected in 14 (70%) and 17 (56.8%) samples, with geometric means (GMs) of 21.9 (3.9–99.6) oocysts/gram (OPG) and 56.8 (4.8–418.4) OPG, respectively. Additional samples from a different farm, where type A coccidian oocysts (*E. phasiani*) were detected in the summer, were collected on November 7, 2023 (19 samples). *Eimeria phasiani* oocysts were detected in five (26.3%) samples, with a GM of 13.0 (2.8–30.0) OPG.

2.4.2. Morphological observation and molecular analysis

Subspherical type B oocysts identified in young pheasants at a farm in Ehime, Japan, (Figs. 9 and 10) were distinct from the typical ellipsoidal oocysts of *E. phasiani* (type A) reported previously from different farms in the region (Chapter 1 in this thesis) although they showed partial morphometric overlaps (Fig. 11). The shape and dimensions of the subspherical oocysts resembled those of *E. tetartooimia* with minor apparent differences in the oocysts' morphology between the two species. That is, differences were observed in the shape of refractile polar granules (coarse vs. spherical), absence/presence of substiedal body, and presence/absence of sporocyst residua. The morphological and molecular features justify the erection of a novel coccidian of ring-necked pheasants, *Eimeria iyoensis* n. sp.

***Eimeria iyoensis* n. sp.**

(Figs. 9–11)

Description

Sporulated oocysts are subspherical in shape with four sporocysts containing two sporozoites each, measuring 18.6 ± 1.0 (16.4–20.6) by 15.7 ± 1.1 (13.3–18.6) with a shape index of 1.18 ± 0.06 (1.02–1.46) (n=150). The oocyst wall is smooth with two layers, approximately 1.4 in thickness. Micropyle and oocyst residuum absent. A coarse refractile polar granule present, usually near one pole of oocysts. Fusiform sporocysts with a Stieda body, measuring 10.7 ± 1.4 (9.0–12.8) by 5.8 ± 0.7 (4.8–7.0) (n = 30). Substiedal body absent. Sporozoites with two large refractile bodies of different sizes. Sporocyst residiuum particles are sparsely distributed.

Endogenous development

Since the specimens examined in this study were collected from pheasants lightly infected with the coccidium in late autumn, no endogeneous stages (schizogony and gametogony) were observed.

Molecular characterization

Nucleotide sequencing of partial *cox1* fragments of type B coccidian oocysts (655 bp), amplified via semi-nested PCR using the Eim_cox1_658F and Eim_cox1_1356R primer pair after the first-round PCR using the Eim_cox_658F and mtRNA20-UNI_R primer pair (Table 8), was performed using five samples collected in the summer. Identical *cox-1* sequences were obtained, suggesting the presence of a single species in the five samples.

Using additional primers (Table 8), a 1,554-bp identical sequence containing a full-length *cox-1* sequence (1,443 bp) was obtained (DDBJ/EMBL/GenBank accession no. LC806970). A

BLAST search using this new *cox1* sequence indicated that *Eimeria praecox* Johnson, 1930 (accession no. KX094944, OP800514) shared the highest identity (92.72%, 1,338/1,443), followed by *Eimeria acervulina* Tyzzer, 1929 (KX094948, OP800525) with 92.65% (1,337 / 1,443) identity, and other *Eimeria* spp. When compared with deposited *Eimeria* spp. *cox-1* sequences from pheasants (*Phasianus* spp.), those of *Eimeria* sp. ex Phasianus colchicus VV-2014 (KJ547708) showed 94.67% (1,190/1,257) identity, *E. tetartooimia* TN4-21 (LC612541) 94.29% (396 / 420), *E. phasiani* EHN_262 (LC777442) 92.10% (1,329/1,443) identity, and *Eimeria* sp. Phasianus colchicus/24-6.10-s3 (HM117019) 90.61% (733/809). The phylogenetic relationships among the *Eimeria* spp. from *Phasianus* spp. based on the partial *cox-1* sequence shown in Fig. 12. The newly obtained sequence of the novel coccidian isolate did not form a subclade with any known coccidian isolates.

Remarks

The oocyst dimensions of the new coccidian species, *E. iyoensis* n. sp., are smaller than other reported *Eimeria* spp. from ring-necked pheasants, except for *E. duodenalis*, *E. pacifica*, and *E. tetartooimia* (Table 7). The *E. duodenalis* oocysts are marginally larger than those of *E. iyoensis* n. sp. with a similar shape index (1.1–1.2); however, differences exist in the refractile polar granule in oocyst fluids (one large particle near an oocyst pole vs. 3–8 small, widely scattered fractures in the fluid), and Stieda body (small but conspicuous without substiedal body vs. small inconspicuous with relatively large substiedal body). Similarly, the oocysts of *E. pacifica* are marginally larger than those of *E. iyoensis* n. sp. with a similar shape index (1.1–1.2); however, the former has distinct oocyst walls, e.g., mammilated oocyst surface (striated outer layer in cross-section), 1–4 refractile polar granules, and conspicuous sporocyst residiuum composed of densely packed, spherical granules of uniform size.

The morphometrics of *E. tetartooimia* and *E. iyoensis* n. sp. oocysts and sporocysts are the most similar. Additionally, common morphological traits were observed, including subspherical oocysts, smooth oocyst wall, absence of micropyle and oocyst residuum, and a single large polar granule (Table 1). Meanwhile, *E. iyoensis* n. sp. differs from *E. tetartooimia* in shape of refractile polar granules (coarse vs. spherical), absence of substiedal body (present in the latter species), and presence of sporocyst residuum (absent in the latter species). Molecular analysis of *E. tetartooimia* and *E. iyoensis* n. sp. mitochondrial *cox-1* DNA indicates the distinctness of these two species, when referring to Ogedengbe et al. (2011), who reported that the intraspecific variation of the *Eimeria* spp. *cox-1* sequences in chickens was 0.00023–0.004, whereas interspecific genetic distances from an *Eimeria* sp. to its nearest neighbor were 0.020–0.098. Due to the lack of information regarding the endogenous stages of *E. tetartooimia* and *E. iyoensis* n. sp., additional comparisons between the two species are not currently possible.

Taxonomic summary

Type host: *Phasianus colchicus* Linnaeus, 1758 (Galliformes: Phasianidae), ring-necked pheasant

Type locality: Kihoku-cho, Nanyo, Ehime, Japan

Prevalence: 56.8 – 80.0 %

Type specimen: Collection no. 25023, Meguro Parasitological Museum, Tokyo, Japan

Nucleotide sequence deposited: Accession number LC806970 at GenBank (mitochondrial DNA containing the complete *cox1* sequence)

Etymology: The specific name "*iyoensis*" is derived from the historical name of the type locality.

2.5. Discussion

Coccidiosis has become a major concern in farmed pheasants, with significant morbidity and mortality reported worldwide, including in Japan (Norton 1976; Liou et al. 2001; Gerhold et al. 2010; Tomoyoshi et al. 2014; Omoto and Koresawa 2015). Considering the current global distribution of ring-necked pheasants in the temperate zone of the northern hemisphere (Pupins and Pupina 2015) and the seasonal breeding of pheasants, clinical coccidiosis might be most prevalent in poults from May to July (2–7 weeks of age) and ending by August's end. Meanwhile, coccidiosis is uniformly light in grown yearlings and matured pheasants (Ormsbee 1939; Norton 1967a, b, 1976). Three pheasant coccidian species, *E. phasiani*, *E. duodenalis* and *E. colchici*, reportedly exhibit high pathogenicity: the former two species cause catarrhal enteritis in the small intestine and the proximal part of the ceca, while *E. colchici* causes severe cecal infection with the formation of white caseous cylindrical plugs in the cecal lumen (Tyzzer 1929; Norton 1967a, b, 1976; Trigg 1967b; McQuistion 1987; Goldová et al. 1998; Liou et al. 2001; Gerhold et al. 2010). McQuistion (1987) reported that oral inoculation of 50,000 mixed sporulated oocysts of four *Eimeria* spp. (*E. phasiani*, *E. pacifica*, *E. duodenalis*, and *E. tetartooimia*) to 3-week-old poults produced active oocyst expulsion 5 days post-inoculation (DPI), peaking at 5–7 DPI, markedly decreasing by 9 DPI, and persisting to at least 15 DPI (experiments endpoint). Meanwhile, oral inoculation of sporulated oocysts of a single species (*E. duodenalis*, *E. phasiani*, or *E. colchici*) resulted in similar temporal changes in oocyst production, while certain modifications in the peak time and end time of active oocyst shedding were observed (Norton 1967a, b; Trigg 1967; Liou et al. 2001).

Tomoyoshi et al. (2014) reported an outbreak of moribund and mortal coccidiosis in one of six breeding rooms of Japanese green pheasants near the end of June in a pheasant-rearing farm in western Japan. This farm transiently retained approximately 100–150 poults (from 19 days to 39 days of age before outdoor breeding) (from 19 to 39 days of age) within each breeding

room before outdoor breeding. Fatal coccidiosis with necrotic catarrhal enteritis in the small intestine and formation of white caseous cylindrical plugs in the cecal lumen was observed six days of transfer to the breeding room. The fecal examination revealed small subspherical oocysts measuring 18.9 µm by 15.0 µm with 1.97×10^6 OPG (Tomoyoshi et al. 2014). Considering the prominence of 18.9 µm × 15.0 µm subspherical coccidian oocysts, it is plausible that either *E. tetartooimia* or *E. iyoensis* n. sp. was responsible for this outbreak of coccidiosis in farmed green pheasants. However, detailed reports of *E. tetartooimia* are limited to fecal detection of oocysts in the ring-necked and Japanese green pheasants without clinical manifestation of coccidiosis (Wacha 1973; Matsubayashi et al. 2021). Similarly, the current understanding of the biology of *E. iyoensis* n. sp. is limited as described in this study. Hence, an in-depth exploration of the life cycles, particularly the endogenous stages in the hosts, of these two species is necessary to better understand their pathogenicity.

The differentiation of two coccidian species with morphologically similar oocysts, e.g., *E. tetartooimia* and *E. iyoensis* n. sp., is challenging in pheasants. Nevertheless, molecular analysis of the *cox1* fragments successfully differentiated these species. Traditionally, eight *Eimeria* spp. could be differentiated based on oocyst morphology, such as dimensions, shape index, texture of the oocyst wall, presence or absence of micropyle, refractile polar granules, and residua. However, morphometric values of coccidian oocysts vary and overlap between different species (Chapman 2008). Furthermore, different single-oocyst-driven lines of a single species with an identical *cox-1* sequence can exhibit variable oocyst dimensions and variable shape indices (e.g., 1.22, 1.40, 1.43, and 1.31 by KOH, RM, NR, and Guelph strains of *E. adnoeides*, respectively) (Vrba and Pakandl 2014; El-sherry et al. 2015). To clarify the taxonomic relationships among the described *Eimeria* spp. from pheasants (Table 7) and to elucidate the coccidian diversity in the host, additional efforts toward achieving successful molecular characterization are necessary. This has proven fruitful for coccidia in chickens and turkeys (Schwarz et al. 2009;

Ogedengbe et al. 2011, 2014; El-Sherry et al. 2014, 2015, 2017; Vrba and Pakandl 2014, 2015; Morgan and Godwin 2017). Before the introduction of molecular tools for assessing the taxonomy of *Eimeria* spp., multiple morphological traits were evaluated, including the morphology of sporulated oocysts and the endogenous stages, their location in the host, and the lesions they produce, the timing of the prepatent and patent periods, host and site specificity, and immunological specificity (Joyner and Long 1974; Norton 1976). Certainly, these parameters are critical to understanding diseases caused by different *Eimeria* spp., warranting further attention.

Regarding the age-dependent significance of coccidiosis in pheasants, Norton (1976) reported that the disease causes moribund signs like diarrhea, hematochezia, lethargy, weight loss, pallor, and anorexia, in poults 2–7 weeks of age, while the incidence of clinical coccidiosis in the growing poults between seven and 16 weeks of age dropped to approximately 10%. Similar findings were reported in turkeys experimentally infected with *E. adenoeides* (Moore and Brown 1951; Clarkson 1958). That is, the mortality rate in poults over 7 weeks of age was very low even after administering oocysts at a dose that was 10 to 20 times greater than that required to kill poults younger than 5 weeks of age. Meanwhile, in the current study, young pheasants in the last weeks of July (suspected age of two to three months) continued to shed high numbers of oocysts (suspected to exceed 1,000 OPG). Although the geomeans decreased to 21.9 and 56.8 OPG in October and November, the rate of oocyst shedding remained high (70% or 56.8%). Host resistance related to age resistance (innate immunity, not dependent on previous exposure to the coccidium) and imperfect acquired immunity (Chapman 2008) might contribute to the high prevalence and low oocyst shedding levels in grown pheasants.

Currently, the impact of coccidiosis caused by *E. iyoensis* n. sp. is unknown. Hence, exploration of its epidemiology and the life cycle, including the location of the endogenous stages and prepatent and patent periods, is necessary. In addition, molecular characterization of

the *Eimeria* spp. that parasitize pheasants should be conducted to establish the taxonomic relationships and reliable epidemiology of different species responsible for outbreaks of pheasant coccidiosis.

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General Discussion

My thesis title is "Phylogenetic study on eimerian and capillariid parasites of farmed ring-necked pheasants (*Phasianus colchicus* L.)," which encompasses two published study sections: "Eimerian and capillariid infection in farmed ring-necked pheasants in Ehime, Japan, with special reference to their phylogenetic relationships with congeners" and "New coccidian *E. iyoensis* ***n. sp.*** (Apicomplexa: Eucoccidiorida: *Eimeriidae)* in farmed ring-necked pheasants in Ehime, Japan". In my study, I examined parasitic infections in farmed ring-necked pheasants in Ehime, Japan, comprehensively and phylogenetic analyses of recovered *eimeria* and *capillarii* species. The investigation was organized into two major chapters, each looking at the different dimensional level of parasitic infection.

As in Chapter 1, the emphasis was on eimerian and capillariid infections in farmed as ring-necked pheasants, together with the phylogenetic relationships of these parasites to congeners. Regarding the helminth fauna of farmed ring-necked pheasants, three specific nematodes (*E. perforans, C. phasianina and H. gallinarum*) were detected as well.

Other surveys have noted this species as being common among feral ring-necked pheasants in Europe (Hiatt and Fisher, 1947). The co-infections of *eimeria* with capillariid has been diagnosed in the tested pheasant populations, it is a general prevalence in avian hosts emphasized by this study. (Fatoba & Adeleke, 2018). In the case of *eimeria* taxonomy, a number of alternative genetic markers have been investigated to circumvent the shortcomings associated with SSU rDNA and ITS sequences. It is also useful because the *cox-1* gene has a moderately high evolutionary rate, and it can be used to distinguish closely related species with relatively good resolution. (Hiatt & Fisher 1947, Goldráb et al. 1998).

Similarly, the *cox-1* gene analyses of the *eimeria* species observed in farm ring-necked pheasants had greater phylogenetic accuracy and provided insight into associations between previously identified include was also confirmed (Goldová et al. 1998, Fatoba & Adeleke, 2018).

Chapter 2. I identified this new species based on the morphology of its oocysts and the sequence of the cytochrome c oxidase subunit I gene, also known as *cox1*. Coccidiosis has become a major concern in farmed pheasants, with significant morbidity and mortality reported worldwide, including in Japan (Norton 1976; Liou et al. 2001; Gerhold et al. 2010; Tomoyoshi et al. 2014; Omoto and Koresawa 2015). The prepatent period of the most pathogenic species, *E. colchici*, was six days with patent from seven to 11 days post infection (dpi) and the maximum oocyst production on eight-nine dpi (Goldová et al., 1998). By contrast, the prepatent period of E. duodenalis was 4 days with a patent period length of 3-4 days but there was no significant increase in oocyst production. Conclusively, this study adds to the knowledge of coccidia and capillariid parasites taxa, phylogeny, and genetic diversity in farmed ring-necked pheasants. These results indicate that an appropriate and nexus conservative rather than antagonistic strategy is necessary in the context of coccidian taxonomy, because of the conspicious but complicated discrimination among morphologically distinct species by our classical approaches, together with the highly divergent SSU rDNA and ITS sequences and limited value of *cox-1* sequence information; hence there may be cryptic speciation or genetic variation throughout *eimeria* genus research focused on farmed ring-necked pheasants in Ehime, Japan. I discovered a variety of parasites, including a new coccidian species we've named *eimeria iyoensis* **n. sp.** This discovery broadens our understanding of the *eimeria s*pecies that infect these birds. In addition, I characterized the *cox-1* sequence of another species, *Eimeria phasiani*, and identified two species of capillariid nematodes and one species of heterakid nematodes. The high prevalence of these parasites underscores the health challenges faced by farmed pheasants.

In conclusion, this study contributes valuable insights into the parasitic infections in farmed ring-necked pheasants and their phylogenetic relationships. The findings underscore the need for ongoing surveillance and research to manage these infections effectively and safeguard the health and productivity of farmed pheasant populations.

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Table 1. Primers used for PCR and nucleotide sequencing		
Coccidian *cox-1*		
1-1) Primer pair 1-1 for the first-round amplification of semi-nested PCR:		
	F:	Eim_cox1_658F (5'-CAATTCTACGATGCYKCWTT-3')
	R:	mtRNA20_UNI_R (5'-GTATGGATTTCACGGTCAA-3')
1-2) Primer pair 1-2 for the second-round amplification of semi-nested PCR:		
	F:	Eim_cox1_658F (see above)
	R:	Eim_cox1_1356R (5'-ATCAGGAATTCTACGTGGCATAAC-3')
2-1) Primer pair 2-1 for the first-round amplification of semi-nested PCR:		
	F:	Eim_cytb382F (5'-CAAATGAGCTTCTGGGGTGC-3')
	R:	mtRNA20_UNI_R (see above)
2-2A) Primer pair 2-2A for the second-round amplification of semi-nested PCR:		
	F:	Eim_cox1_199F (5'-ATGATTTTCTTTGTAGTTATGCC-3')[a]
	R:	mtRNA20_UNI_R (see above)
	Primers for sequencing only	
	F:	Eim_cox1_658F (see above)
	R:	Eim_cox1_1356R (see above)
2-2B) Primer pair 2-2B for the second-round amplification of nested PCR:		
	F:	Eim_cox1_199F (see above)[a]
	R:	COIRm (5'-CCCAGAGATAATACAAAATGGAA-3')[b]
Capillariid SSU rDNA		
1) Primer pair 1 for PCR:		
	F:	Eurib1 (5'-ACCTGGTTGATCCTGCCAG-3')
	R:	Eurib2 (5'-CTTCCGCTGGTTCACCTACGG-3')
	Primers for sequencing only	
	F:	NSF573/19 (5′-CGCGGTAATTCCAGCTCCA-3′)
	F:	NSF1419/20 (5′-ATAACAGGTCTGTGATGCCC-3′)
	R:	NSR581/18 (5′-TCTCAGGCTCCCTCTCCGG-3′)
	R:	NSR1438/20 (5′-GGGCATCACAGACCTGTTAT-3′)
2) Primer pair 2 for PCR:		
	F:	NSF4/18 (5′-CTGGTTGATCCTGCCAGT-3′)
	R:	NSR1438/20 (see above)
	Primers for sequencing only	
	F:	NSF573/19 (see above)
	R:	NSR581/18 (see above)
3) Primer pair 3 for PCR:		
	F:	NSF573/19 (see above)
	R:	S.r.18S-SSU18R (5′-TGATCCTTCYGCAGGTTCAC-3′)
	Primers for sequencing only	
	F:	NSF1419/20 (see abpve)
	R:	NSR1438/20 (see above)
Heterakis gallinarum rDNA		
1) Primer pair 1 for PCR:		

	F:	NSF1419/20 (see above)
	R:	NC13(ITS1)/R (5′-GCTGCGTTCTTCATCGAT-3′
2) Primer pair 2 for PCR:		
	F:	NSF1419/20 (see above)
	R:	NC2(ITS2)/R (5′-TTAGTTTCTTTTCCTCCGCT-3′)
	Primers for sequencing only	
	F:	NC13(ITS2)/F (5′-ATCGATGAAGAACGCAGC-3′)
	R:	NC13(ITS1)/R (see above)
	R:	S.r.18S-SSU18R (see above)
3) Primer pair 3 for PCR:		
	F:	NSF1419/20 (see above)
	R:	28S-408R/20 (5'-TTCACGCCCTCTTGAACTCT-3')
	Primers for sequencing only	
	F:	NC13(ITS2)/F (see above)
	R:	S.r.18S-SSU18R (see above)
	R:	NC13(ITS1)/R (see above)
4) Primer pair 4 for PCR:		
	F:	B.p.28S/F (5′-AGCGGAGGAAAAGAAACTAA-3′)
	R:	NLR1432-1/22 (5'-GTTGTTACACACTCCTTAGCGG-3')
	Primers for sequencing only	
	F:	28S-839F/20 (5'-TATCCGACCCGTCTTGAAAC-3')
	R:	28S-408R/20 (see above)

[a] Modified from COI_UNI_199F (5'-ATGATYTTCTTTGTAGTTATGCC-3') reported by Imai and Barta (2019)

[b] Reported by Gjerde (2013)

Table 2. Microscopic detection of oocysts/eggs in the feces, and parasite recovery from the gastrointestinal tracts of farmed pheasants[a]

Fecal examination	Coccidian oocysts	Capillariid egg	*Heterakis* eggs
1) Dec. 8, 2020 (Farm A)	5% (2 / 43): 98–818	56% (24 / 43): 1–476 (10.6)	63% (27 / 43): 1–553 (13.7)
2) Nov. 10, 2021 (Farm A)	25% (26 / 103): 1–1,847 (11.9)	40% (41 / 103): 1–701 (8.5)	45% (46 / 103): 1–246 (4.5)
3) Nov. 1, 2022 (Farm B)	58% (29 / 50): 1–2,532 (24.8)	42% (21 / 50): 1–137 (8.0)	72% (36 / 50): 1–501 (17.4)
Worm recovery	*Eucoleus perforans*	*Capillaria phasianina*	*Heterakis gallinarum*
1) Dec. 8, 2020 (Farm A)	8% (2 / 26): 1	87% (53 / 61): 1–20 (4.3)	75% (46 / 61)[b]: 1–150 (7.3)
2) Nov. 10, 2021 (Farm A)	73% (93 / 128): 1–39 (3.8)	82% (89 / 108): 1–17 (3.2)	69% (74 / 108)[c]: 1–91 (4.7)
3) Nov. 1, 2022 (Farm B)	44% (22 / 50): 1–9 (2.3)	10% (5 / 50): 1	88% (44 / 50)[d]: 1–213 (8.0)

[a] Prevalence expressed by percentage (number of positive samples/number of examined samples): intensity expressed by range (geomean)

[b] Prevalence when including larvae and juvenile worms: 90% (55 / 61)

[c] Prevalence when including larvae and juvenile worms: 89% (96 / 108)

[d] Prevalence when including larvae and juvenile worms: 94% (47 / 50)

Table 3. Comparison of measurements of *Eimeria phasiani* isolates in this study with morphologically similar *Eimeria* spp. from phasianid birds

	Eimeria phasiani		*E. phasiani*	*E. phasiani*	*E. phasiani*	*E. phasiani*[b]
	Morphotype A	Morphotype B				
	Ehime, Japan	Ehime, Japan	USA	Washington, USA	Germany	UK
	Present study	Present study	Tyzzer (1929)	Ormsbee (1939)	Hasse (1939)	Trigg (1967)
Oocyst	(n=42)	(n=22)	(n=100)	(n=50)	—	—
Length	22.4 (19.2–27.1)	23.7 (19.0–26.9)	23.0 (19.8–26.4)	25 (21–27)	23.2 (19.2–26.4)	24.7 (20.1–30.9)
Width	15.6 (14.1–16.8)	18.5 (15.5–20.9)	15.9 (13.2–17.8)	17 (15–19)	16.2 or 15.7 (13.2–18.0)	17.1 (14.1–20.5)
Shape index[a]	1.44 (1.24–1.92)	1.28 (1.15–1.49)	1.41	1.41	1.43–1.47	1.44
Sporocyst	(n=42)	(n=22)	—	—	—	—
Length	11.6 (9.6–13.1)	12.4 (9.7–14.7)	—	—	—	14.3 (12.9–15.9)
Width	5.8 (4.9–6.6)	6.3 (5.6–7.7)	—	—	—	6.7 (5.6–7.4)

Table 2 (continued)

	E. pacifica	*E. pacifica*	*E. dispersa* ex. pheasant	*E. dispersa* ex. quail	*E. dispersa* ex. turkey	*E. dispersa*[c]
	Washington, USA	Montana, USA	USA	USA	Briston, UK	Briston, UK
	Ormsbee (1939)	Wacha (2011)	Tyzzer (1929)	Tyzzer (1929)	Long and Millard (1979)	El-Sherry et al. (2017)
Oocyst	(n=30)	(n=50)	—	—	(n=385)	(n=30)
Length	22 (17–26)	21.1 (19.0–23.1)	19.8 (15.3–22.8)	22.8 (17.2–26.4)	24.2 (16.7–28.5)	26 (24–28)
Width	18 (14–20)	17.5 (15.6–19.0)	17.7 (13.9–19.7)	18.8 (15.4–22.4)	19.3 (14.6–23.6)	21 (19–23)
Shape index[a]	1.23	1.2 (1.1–1.4)	1.12	1.21	1.26	1.21
Sporocyst	—	(n=50)	—	—	(n=74)	(n=30)
Length	—	10.9 (10.2–12.2)	—	—	13.1 (11.8–13.9)	14 (12–16)
Width	—	6.1 (4.8–6.8)	—	—	7.4 (6.3–8.3)	8 (7–9)

[a] Shape index = Length / Width of oocysts

[b] A single-oocyst-driven line of *E. phasiani* propagated in pheasants, designated 'Silwood BI' strain

[c] A single-oocyst-driven line of *E. dispersa* propagated in turkeys, designated 'Briston' strain

Table 4. Measurements of *Eucoleus perforans* and *E. contortus* dwelling in the esophageal mucosa of poultry[a]

Parasite species	*E. perforans*	*E. perforans*	*E. perforans*	*E. perforans*	*E. contortus*	*E. contortus*	*E. contortus*
Host	*Phasianus colchicus karpowi*	*Phasianus colchicus karpowi*	*Phasianus versicolor*	*Meleagris gallopavo, Numida meleagris, Perdix perdix, Phasianus colchicus*	Anser cygnoides domesticus	*Larus argentatus, Philomachus pugnax, Charadrius hiaticula, Anas crecca, Anas acuta, Anas falcata*	*Cairina moschata domestica*
Locality	Farm A, Ehime, Japan	Farm B, Ehime, Japan	Kumamoto, Japan	Europe, Asia	Surabaya, Indonesia	Europe, Asia	Soure, Pará, Brazil
Reference	Present study	Present study	Sakaguchi et al. (2020)	Baruš and Sergejeva (1989b)	Sakaguchi et al. (2020)	Baruš and Sergejeva (1989b)	Carvalho et al. (2019)
Male	(n=8)	(n=11)	(n=9)	(n=?)	(n=9)	(n=41)	(n=10)
Body length	21.7–41.6 (27.9)	30.5–48.7 (38.2)	22.8–28.4 (26.0)	32.5–60.0	15.9–19.4 (18.4)	6.8–11.6	11–16
Max. body width	0.05–0.10 (0.07)	0.07–0.13 (0.10)	0.04–0.07 (0.06)	0.09–0.12	0.05–0.08 (0.07)	0.06–0.07	0.04–0.05
Muscular esophagus length	0.39–0.55 (0.45)	0.54–0.89 (0.69)	—	—	—	—	0.22–0.31
Total esophagus length	4.29–7.41 (5.87)	7.09–10.96 (8.88)	5.10–11.87 (6.47)	>7.5–10.7	4.97–6.02 (5.60)	> 2.96–4.50	4–6
Length ratio of posterior body to anterior body	3.10–4.62 (3.72)	2.81–4.41 (3.51)	1.26–3.97 (3.22)	—	2.12–2.50 (2.28)	—	—
Spicule length	—[b]	—	—	—	—	0.36–0.47	0.65–0.98
Female	(n=6)	(n=9)	(n=37)	(n=?)	(n=3)	(n=29)	(n=10)
Body length	22.9–29.5 (25.9)	43.1–84.2 (62.9)	14.7–40.8 (297)	57–82	28.2–35.0 (31.5)	10.8–20.0	21–29
Max. body width	0.10–0.12 (0.11)	0.11–0.23 (0.18)	0.07–0.15 (0.11)	0.15–0.27	0.11–0.16 (0.13)	0.08–0.12	0.04–0.08
Muscular esophagus length	0.26–0.51 (0.45)	0.67–0.97 (0.77)	—	—	—	—	0.29–0.45
Total esophagus length	5.25–7.03 (6.12)	7.0–13.5 (10.8)	4.4–7.7 (6.5)	9.5–14.5	7.1–8.0 (7.6)	> 2.8–4.5	5–8
Length ratio of posterior body to anterior body	2.57–4.04 (3.26)	4.16–5.76 (4.84)	2.31–4.59 (3.52)	—	2.48–3.68 (3.06)	—	—
Distance from esophageal end to vulva	0.12–0.20 (0.16)	0.05–0.28 (0.17)	0.02–0.18 (0.09)	—	0.05–0.36 (0.19)	—	—
Vulva from anterior end	5.4–7.2 (6.3)	7.0–13.7 (11.0)	—	—	—	—	6–8
Egg length	0.053–0.061 (0.056)	0.048–0.054 (0.051)	0.043–0.053 (0.049)	0.041–0.056	0.051–0.058 (0.055)	0.055–0.067	0.040–0.050
Egg width	0.023–0.028 (0.026)	0.022–0.026 (0.024)	0.018–0.026 (0.023)	0.021–0.028	0.023–0.024 (0.024)	0.022–0.027	0.020–0.040

[a] Measurements are expressed in millimeter by range, when possible, followed by mean in parentheses

[b] No available data

Table 5. Measurements of *Capillaria phasianina* and *C. anatis* dwelling in the ceca of poultry[a]

Parasite species		*C. phasianina*	*C. phasianina*	*C. phasianina*	*C. anatis*	*C. anatis*	*C. anatis*
Host		*Phasianus colchicus karpowi*	*Phasianus colchicus versicolor*	*Phasianus colchicus, Perdix perdix, Pavo cristatis*	Chicken	Chicken	Chicken
Locality		Farm A, Ehime, Japan	Kumamoto, Japan	Russia	Surabaya, Indonesia	Kagoshima, Japan	Davao Oriental, Philippines
Reference		Present study	Sakaguchi et al. (2020)	Baruš and Sergejeva (1989a)	Sakaguchi et al. (2020)	Tamaru et al. (2015)	Tamaru et al. (2015)
Male		(n=14)	(n=1)	(n=7)	(n=3)	(n=28)	(n=21)
	Body length	12.7—25.1 (19.5)	19.3	14.7—19.0	11.0—14.0 (12.2)	6.4—10.0 (8.6)	7.7—14.1 (12.4)
	Max. body width	0.051—0.071 (0.062)	0.065	0.045—0.050	0.053—0.065 (0.059)	0.036—0.064 (0.049)	0.050—0.072 (0.064)
	Muscular esophagus length	0.23—0.56 (0.45)	—[b]	—	—	—	—
	Total esophagus length	5.33—8.74 (7.23)	5.48	>5.6—6.7	4.76—5.77 (5.29)	3.44—5.06 (4.30)	3.50—6.69 (5.69)
	Length ratio of posterior body to anterior body	1.38—1.93 (1.63)	2.52	—	1.06—1.42 (1.30)	0.82—1.29 (1.01)	1.02—1.41 (1.17)
	Spicule length	1.50—2.9 (2.60)	2.55	1.55—2.40	0.94—1.15 (1.05)	0.73—1.21 (1.00)	0.89—1.12 (1.01)
Female		(n=6)	(n=1)	(n=8)	(n=2)	(n=29)	(n=18)
	Body length	26.33—40.23 (30.24)	19.77	22—28	13.56—14.23 (13.90)	7.25—16.58 (11.98)	12.61—20.83 (17.39)
	Max. body width	0.068—0.082 (0.075)	0.065	0.065—0.084	0.055—0.058 (0.056)	0.050—0.080 (0.065)	0.060—0.106 (0.086)
	Muscular esophagus length	0.48—0.59 (0.52)	—	—	—	—	—
	Total esophagus length	8.10—9.56 (8.65)	5.44	>6.6—8.7	5.24—5.48 (5.36)	3.00—6.56 (4.83)	4.97—6.94 (6.23)
	Length ratio of posterior body to anterior body	2.13—3.21 (2.48)	2.64	—	1.59	1.09—1.88 (1.48)	1.37—2.13 (1.78)
	Distance from esophageal end to vulva	0.051—0.103 (0.078)	—	—	0.023—0.068 (0.045)	0.011—0.083 (0.042)	0.011—0.117 (0.040)
	Vulva from anterior end	8.15—9.67 (8.73)	—	—	—	—	—
	Egg length	0.050—0.060 (0.054)	0.041—0.049 (0.046)	0.052—0.057	0.054—0.059 (0.057)	0.053—0.063 (0.059)	0.049—0.066 (0.059)
	Egg width	0.024—0.029 (0.026)	0.024—0.025 (0.025)	0.025—0.027	0.024—0.029 (0.027)	0.024—0.034 (0.029)	0.026—0.037 (0.029)

[a] Measurements are expressed in millimeter by range, when possible, followed by mean in parentheses.

[b] No available data

Table 6. Morphometric comparison of *Heterakis* spp. known from phasianid birds[a]

Parasite species	*H. gallinarum*	*H. gallinarum*	*H. gallinarum*	*H. isolonche*	*H. dispar*
Host species	*Phasianus colchicus karpowi*	*Anser anser*	*Chrysolophus pictus*; *Phasianus colchicus torquatus;*	*Chrysolophus pictus*; *Phasianus colchicus torquatus;*	*Anser anser*
Locality	Ehime, Japan	Ukraine	Brazil	Brazil	Ukraine
Reference	Present study	Yevstafyeva et al. (2018)	Mendonça (1953)	Mendonça (1953)	Yevstafyeva et al. (2018)
Male	(n=10)	(n=20)	(n=?)	(n=?)	(n=20)
Body length	6.71—9.48 (7.74)	6.12—10.25 (8.26)	6.87—8.74	9.61—12.49	11.58—15.67 (13.36)
Max. body width	0.22—0.36 (0.27)	0.27—0.38 (0.31)	0.27—0.34	0.50—0.54	0.36—0.42 (0.39)
Esophagus length	0.79—0.99 (0.86)	—[b]	0.97—1.13	1.38—1.63	—
Length of corpus	0.56—0.76 (0.64)	—	—	—	—
Length of bulb	0.16—0.24 (0.19)	—	0.30—0.32	0.43—0.47	—
Width of bulb	0.12—0.17 (0.14)	—	0.18—0.24	0.30—0.35	—
Length of left spicule	0.59—0.73 (0.64)	0.55—0.77 (0.66)	0.54—0.80	1.53—1.83	0.38—0.42 (0.40)
Length of right spicule	1.56—2.11 (1.75)	1.92—2.85 (2.27)	1.51—2.18	1.54—1.94	0.37—0.42 (0.39)
Length ratio (R/L)	2.52—3.10 (2.75)	2.82—4.75 (3.47)	ca. 3.1	1.00—1.09	0.88—1.11 (0.99)
Length of precloacal sucker	0.056—0.068 (0.063)	0.070—0.082 (0.076)	ca. 0.083	0.18—0.27	0.141—0.159 (0.150)
Distance between precloacal sucker and cloaca[c]	0.11—0.18 (0.14)	—	0.10—0.13	0.10—0.18	—
Tail length	0.34—0.48 (0.42)	—	0.40—0.45	0.58—0.64	—
Female	(n=10)	(n=20)	(n=?)	(n=?)	(n=20)
Body length	8.63—10.87 (9.59)	7.60—11.08 (9.41)	9.45—11.05	13.50—15.41	13.18—19.25 (16.43)
Max. body width	0.25—0.31 (0.28)	0.32—0.42 (0.37)	0.38—0.42	0.57—0.60	0.52—0.64 (0.58)
Esophagus length	0.84—1.10 (0.93)	—	1.11—1.17	1.61—1.81	—
Length of corpus	0.60—0.82 (0.69)	—	—	—	—
Length of bulb	0.25—0.32 (0.30)	—	0.28—0.33	0.40—0.50	—
Width of bulb	0.16—0.23 (0.19)	—	0.20—0.23	0.26—0.30	—
Vulva from the posterior end	4.06—5.27 (4.72)	3.19—4.98 (4.35)	4.22	7.07—7.13	8.19—11.12 (9.50)
Tail length	0.83—1.12 (0.98)	1.09—1.28 (1.17)	1.21	1.14—1.61	0.078—0.092 (0.087)
Egg length	0.066—0.080 (0.073)	0.064—0.069 (0.067)	0.061—0.078	0.071—0.083	0.066—0.071 (0.068)
Egg width	0.039—0.046 (0.043)	0.038—0.040 (0.039)	0.041—0.043	0.042—0.049	0.044—0.048 (0.046)

Table 7 Morphological comparison of *Eimeria* spp. oocysts isolated from ring-necked pheasants (measurements in µm)

Species	Oocyst					Sporocyst			Reference
	Length	Width	Shape Index	Micropyle	Polar granules	Length	Width	Residiuum	
E. colchici Norton, 1967	27.4 (19.0 – 33.5)	16.7 (13.0 – 21.0)	1.64	inconspicuous	1 – 3	14.6 (11.5 –16.5)	6.6 (6.0 – 7.5)	+	Norton (1967b)
E. dispersa Tyzzer, 1929	19.8 (15.3 – 22.8)	17.7 (13.9 – 19.7)	1.12	–	–	n.d.	n.d.	n.d.	Norton (1967b)
	24.2 (16.7 – 28.5)	19.3 (14.6 – 23.6)	1.26	–	–	13.1 (11.8 –13.9)	7.4 (6.3 – 8.3)	+	Long & Millard (1979)
	26 (24 – 28)	21 (19 – 23)	1.2	–	–	14 (12 –16)	8 (7 – 9)	+	El-Sherry et al. (2017)
E. duodenalis Norton, 1967	21.2 (18.0 – 24.0)	18.6 (15.4 – 21.4)	1.14	–	–	12.8 (9.9 –15.4)	7.3 (6.0 – 8.6)	+	Norton (1967a)
	20.3 (18.4 – 21.8)	18.1 (16.3 – 19.7)	1.1 (1.0 – 1.2)	–	3 – 8 fractured	12.6 (11.6 –13.6)	6.7 (6.1 – 6.8)	+	Wacha (1973)
E. langeroni Yakimof & Matschoulsky, 1937	32.5 (30.0 – 36.0)	18.4 (16.0 – 20.0)	1.77	–	–	n.d.	n.d.	n.d.	Yakimoff & Matschoulsky (1937)
E. megalostomata Ormsbee, 1939	24 (21 – 29)	19 (16 – 22)	1.27	Present	1	n.d.	n.d.	n.d.	Ormsbee (1939)
E. pacifica Ormsbee, 1939	22 (17 – 26)	18 (14 – 20)	1.23	–	2 – 3	n.d.	n.d.	n.d.	Ormsbee (1939)
	21.1 (19.0 – 23.1)	17.5 (15.6 – 19.0)	1.2 (1.1 – 1.4)	–	1 – 4	10.9 (10.2 –12.2)	6.1 (4.8 – 6.8)	+	Wacha (1973)
E. phasiani Tyzzer, 1929	23.0 (19.8 – 26.4)	15.9 (13.2 – 17.8)	1.45	–	1 – 3	n.d.	n.d.	n.d.	Tyzzer (1929) after Norton (1967b)
	23.2 (19.2 – 26.4)	16.2 (13.2 – 18.0)	1.43 – 1.47	–	n.d.	n.d.	n.d.	–	Haase (1939) after Trigg (1967)
	24.7 (20.1 – 30.9)	17.1 (14.1 – 20.5)	1.44	–	1 – 3	14.3 (12.9 –15.9)	6.7 (5.6 – 7.4)	–	Trigg (1967)
	22.4 (19.2 – 27.1)	15.6 (14.1 – 16.8)	1.44 (1.24 – 1.92)	–	1 – 3	11.6 (9.6 –13.1)	5.8 (4.9 – 6.6)	+	Argamjav et al. (2023)
E. tetartooimia Wacha, 1973	18.6 (17.0 – 20.4)	16.5 (15.0 – 18.4)	1.1 (1.0 – 1.2)	–	1 (occasionally 2)	10.8 (9.5 –11.6)	5.6 (4.8 – 6.1)	–	Wacha (1973)
	17.2 (14.7 – 20.0)	14.8 (13.3 – 16.7)	1.2 (1.0 – 1.4)	–	1	9.8 (6.7 – 13.3)	5.9 (4.7 – 7.3)	–	Matsubayashi et al. (2021)
Eimeria iyoensis **n. sp.**	18.6 (16.4 – 20.6)	15.7 (13.3 – 18.6)	1.18 (1.02 – 1.46)	–	1	10.5 (6.8 – 12.8)	5.8 (4.8 – 7.5)	+	Present study

Table 8. Primers used for PCR amplification of coccidian *cox1*

	Primer	Position[a]
1-1) First-round amplification:		
F:	Eim_cox1_658F (5'-CAATTCTACGATGCYKCWTT-3')[b]	761
R:	mtRNA20_UNI_R (5'-GTATGGATTTCACGGTCAA-3')[c]	1573
1-2) Second-round amplification of semi-nested PCR:		
F:	Eim_cox1_658F (see above)	761
R:	Eim_cox1_1356R (5'-ATCAGGAATTCTACGTGGCATAAC-3')[b]	1459
2-1) First-round PCR amplification:		
F:	Eim_cytb382F (5'-CAAATGAGCTTCTGGGGTGC-3')[b]	-628
R:	mtRNA20_UNI_R (see above)	1573
2-2A) Second-round PCR amplification of nested PCR:		
F:	Eim_cytb986F (5'-TTGGAGCTCAATTACCTCAAGAAG-3')[b]	-24
R:	Inv_COI_262R (5'-AAWGCGGCATCRTAGAATTG-3')[d]	780
2-2B) Second-round PCR amplification of nested PCR:		
F:	COI_UNI_199F (5'-ATGATYTTCTTTGTAGTTATGCC-3')[c]	302
R:	Eim_cox1_1356R (see above)	1459
Primer for sequencing only		
R:	COIRm (5'-CCCAGAGATAATACAAAATGGAA-3')[e]	1260
2-2C) Second-round PCR amplification for semi-nested PCR:		
F:	Eim_cox1_658F (see above)	761
R:	mtRNA20_UNI_R (see above)	1573

[a] Position of the 5' terminal nucleotide of primer, relative to the 5' terminal nucleotide of the *cox1* sequence of *E. phasiani* (DDBJ/EMBL/GenBank accession no. LC777440)

[b] Argamjav et al. (2023)

[c] Imai & Barta (2019)

[d] Ogedengbe et al. (2014)

[e] Gjerde (2013)

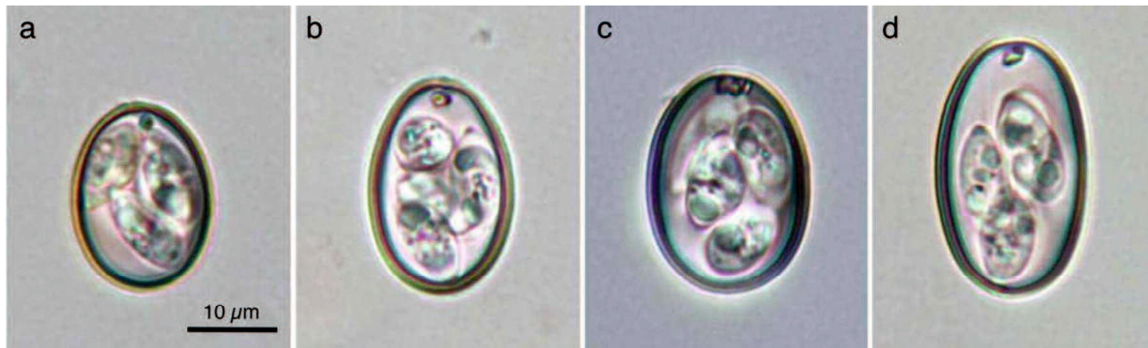

Fig. 1. Sporulated oocysts of *Eimeria phasiani* from farmed ring-necked pheasants in Ehime, Japan. Oocysts shows variation in shape, e.g. ellipsoidal, broadly ellipsoidal, and long ellipsoidal, with an average shape index (SI = oocyst length/width) of approximately 1.36. Refractile polar granules, one to three in number, are visible inside the oocyst fluid near the apical pole here, but no oocyst nor sporocyst residua are found. All photographs (a–d) are at the same magnification, and a scale bar is shown in a. Each oocyst is measured as follows: a, 22.3 μm by 15.8 μm with 1.41 SI; b, 24.7 μm by 16.7 μm with 1.47 SI; c, 25.1 μm by 18.3 μm with 1.37 SI; and d, 29.3 μm by 17.2 μm with 1.70 SI. Oocysts resembling those shown in a and b are predominant.

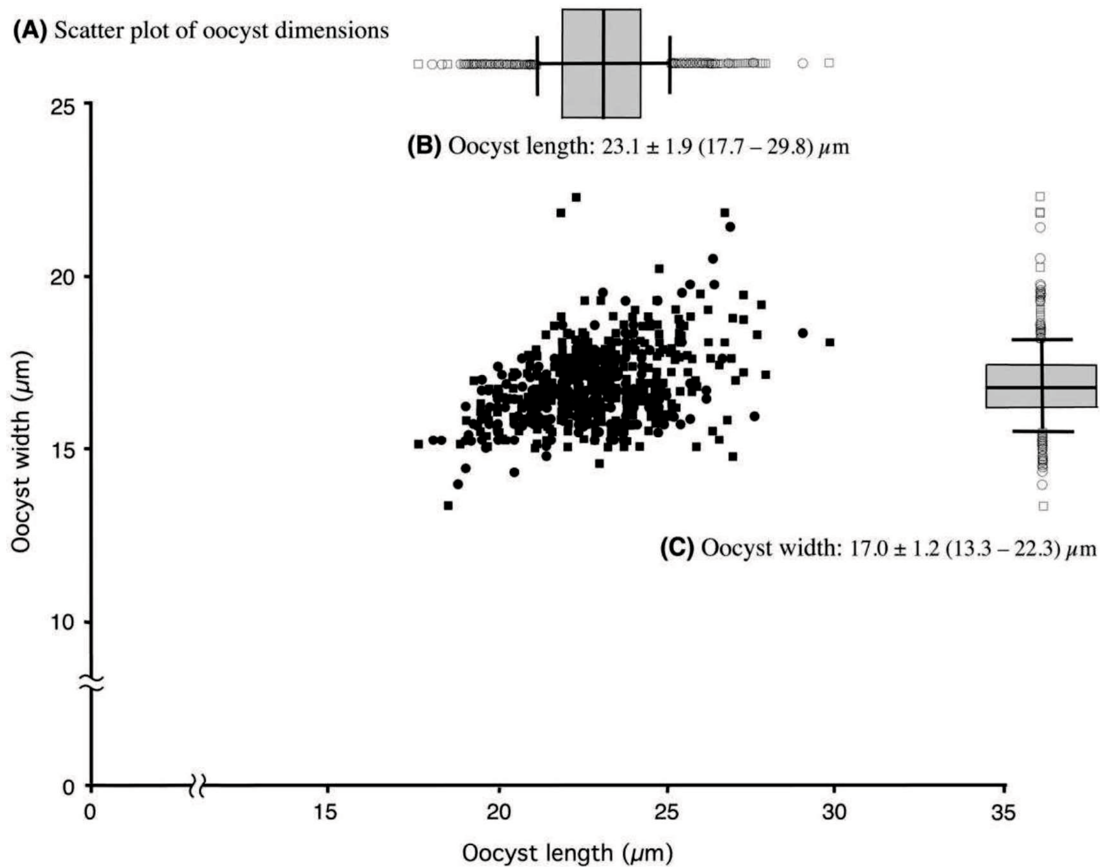

Fig. 2. Morphometry of *Eimeria phasiani* oocysts collected from farmed ring-necked pheasants examined in this study. (**A**) Scatter plot of the dimensions of *E. phasiani* oocysts collected from pheasants in Farm A (closed circles; n=263) and Farm B (closed squares; n=327); (**B**) Modified box-and whisker plot of oocyst lengths of *E. phasiani* (n=590); and (**C**) Modified box and whisker plot of oocyst widths of *E. phasiani* (n=590). The boxes show values for 50% of oocysts, and the medium line indicates the average value. The bar around the box indicates one standard deviation (SD) value, and outlier data points shown by open circles and squares indicates the origin of oocysts (Farms A and B, respectively). Shape indices of oocysts from Farms A and B were 1.35 (1.15–1.73; n=263) and 1.36 (1.00–1.82; n=327), respectively.

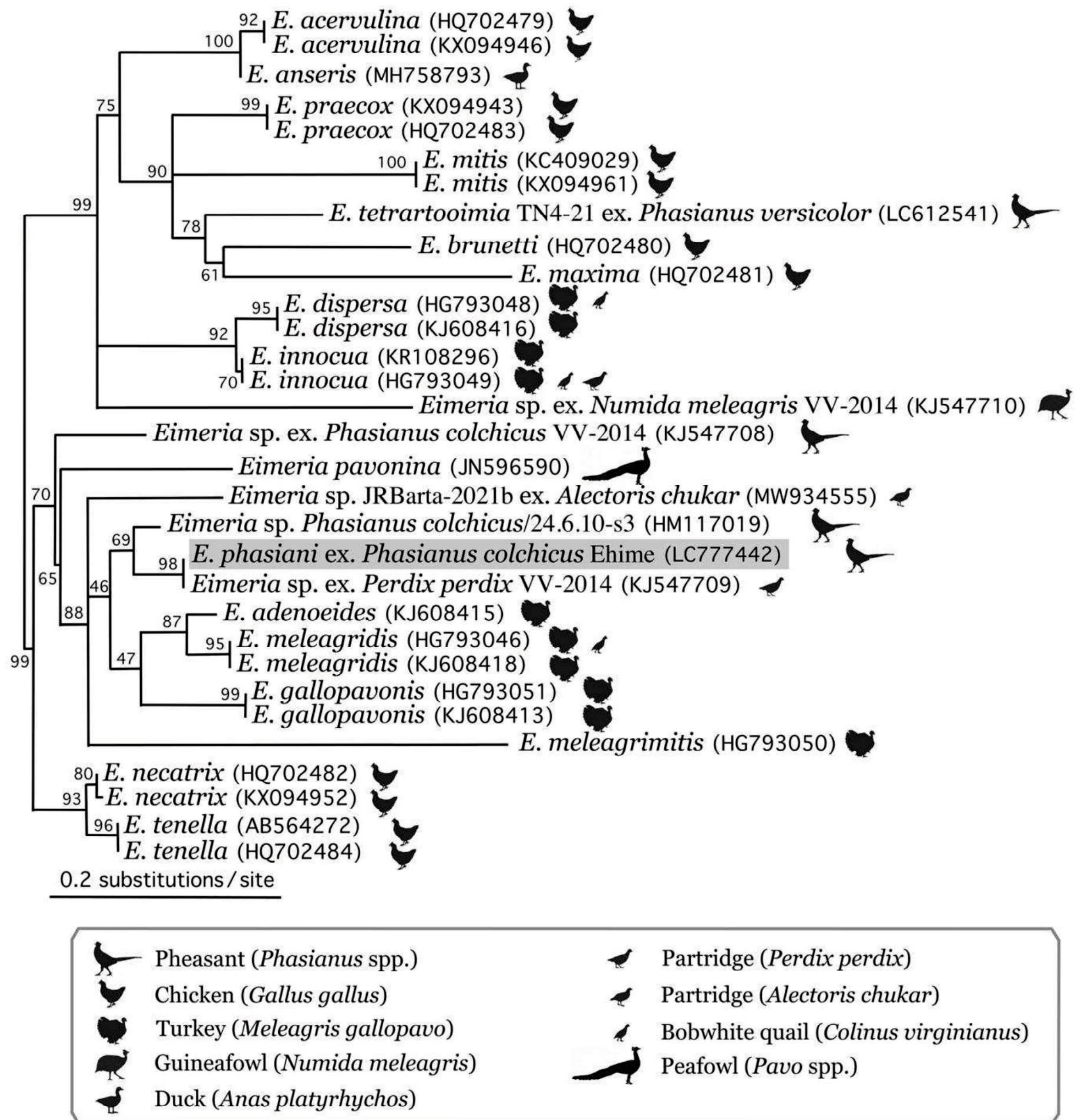

Fig. 3. ML phylogenetic tree based on the 1,513-bp long mitochondrial *cox-1* sequences of representative *Eimeria* spp. from avian hosts. The species name is followed by the GenBank accession number in parentheses for most cases. For several species with particular interest, the species name is followed by the isolate name and/or hostname and the GenBank accession number in parentheses. The newly obtained *E. phasiani cox-1* sequence in the present study is marked with a grey background.

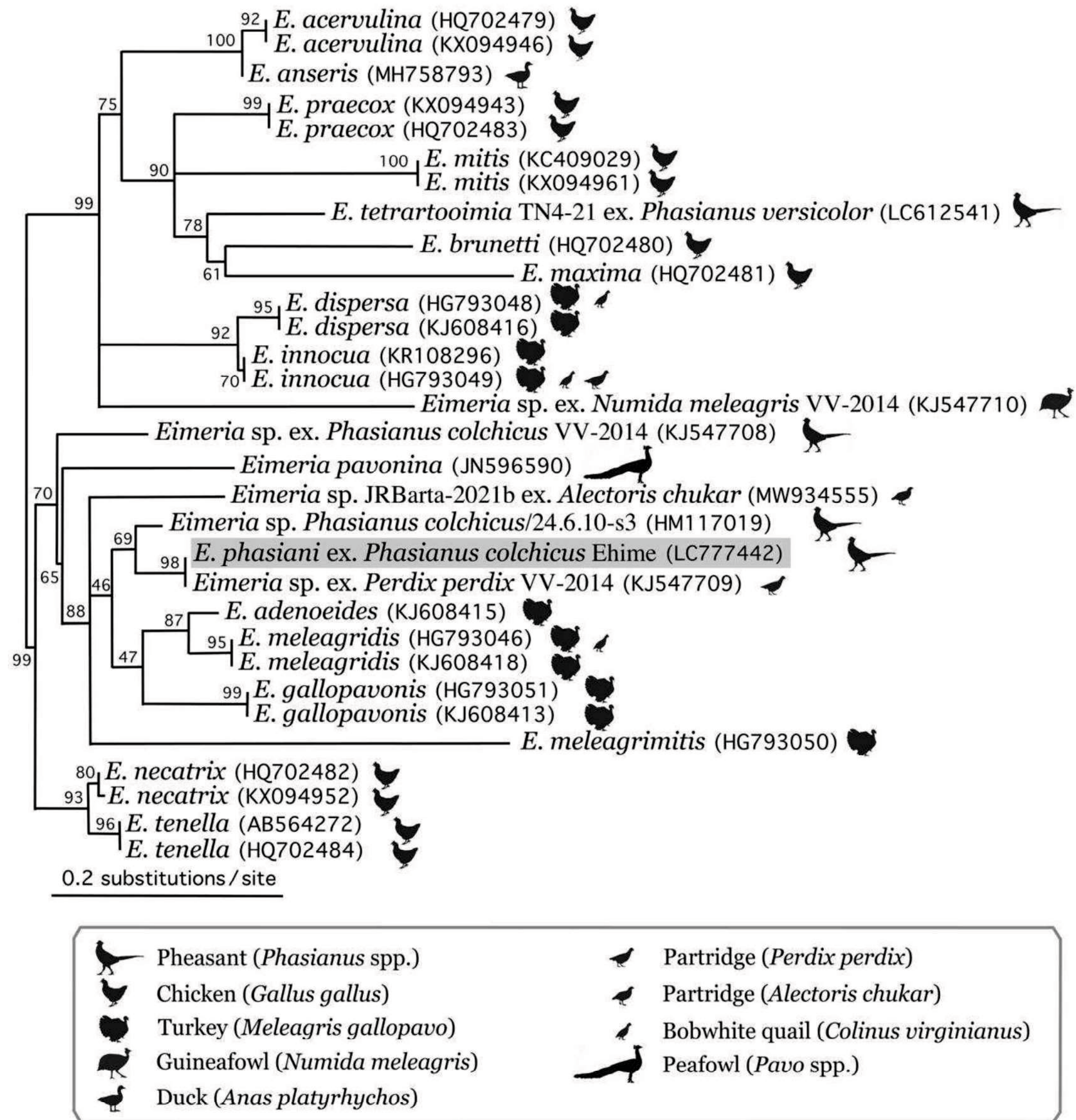

Fig. 4 Maximum likelihood (ML) phylogenetic tree based on the 420-bp long mitochondrial *cox-1* sequences of representative *Eimeria* spp. from avian hosts. Species names are followed by the GenBank accession number in parentheses for most cases. For several species of particular interest, the species name is followed by the isolate name and/or hostname, with the GenBank accession number in parentheses. The newly obtained *E. phasiani cox-1* sequence in the present study is marked with a grey background. ML phylogenetic tree based on the 1,513-bp long *cox-1* sequences of representative *Eimeria* spp. from avian hosts is provided as Suppl. Fig. 3.

Fig. 5. Epithelial sheet of the esophagus showing parasitism of a female *Eucoleus phasianina* worm in the tissue. A fixed epithelial sheet was immersed in 30% glycerin solution to achieve transparency of the tissue and observed under dissection microscopy.

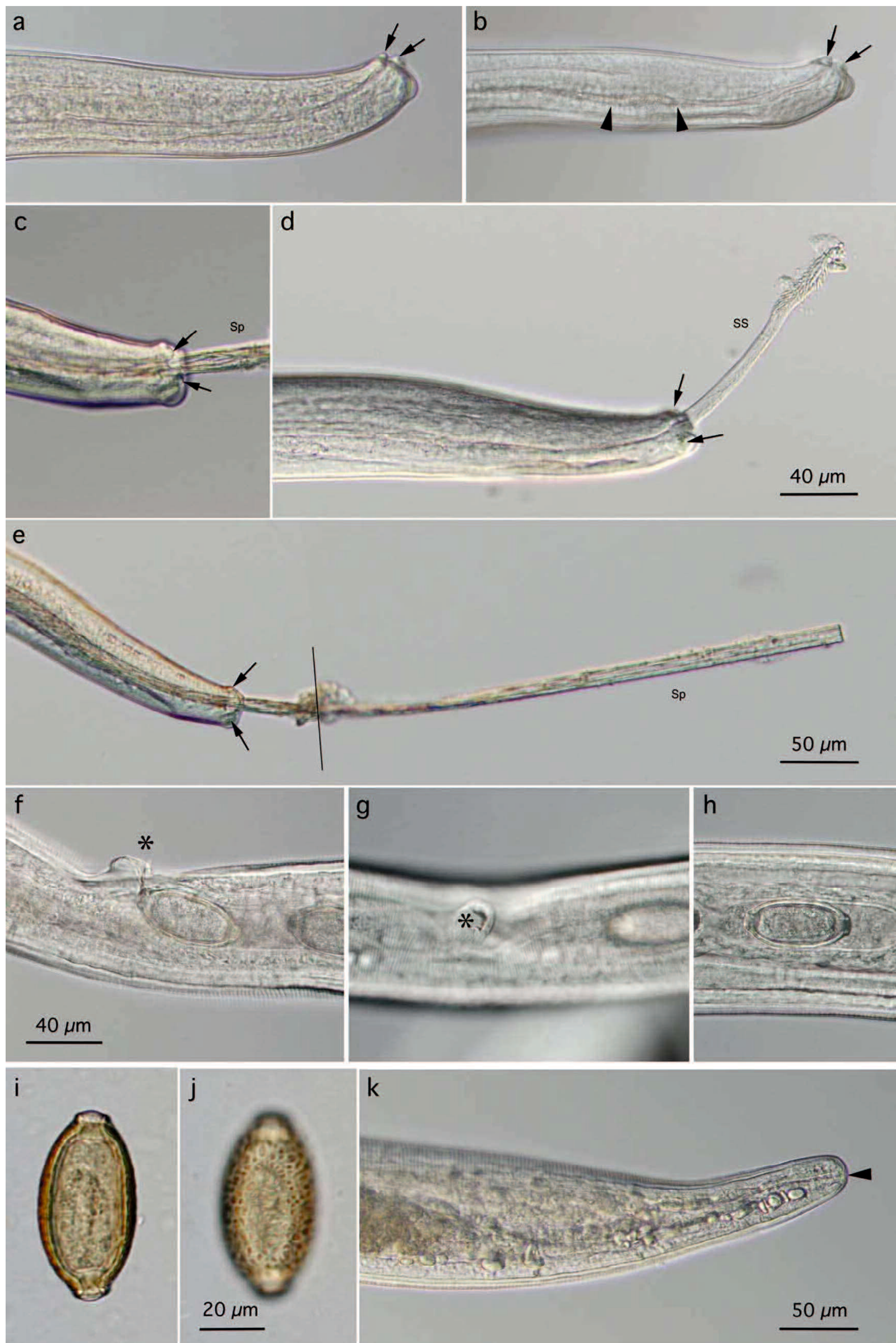

Fig. 6. Morphology of *Eucoleus perforans* from farmed ring-necked pheasants. (**a**) Lateral view of the caudal end of a male worm with two dorsal papilla-like protrusions (arrows). (**b**)

Lateral view of the caudal end of a male worm with two dorsal papilla-like protrusions (arrows). The spiny region of the housed specular sheath is visible between arrowheads. (**c**) Dorsal view of the caudal end of a male worm with two dorsal papilla-like protrusions (arrows). The spicule (Sp) is protruded through the cloaca. (**d**) Dorsal view of the caudal end of a male worm with two dorsal papilla-like protrusions (arrows). The spicular sheath (SS) protruded from the cloaca. (**e**) Dorsal view of the caudal end of a male worm with two dorsal papilla-like protrusions (arrows). The spicule (Sp) is protruded through the cloaca. Two photographs are combined at the position indicated by a line. (**f**) Lateral view of a female worm, focusing on the vulva (asterisk). The uterus (two intrauterine eggs are visible) extends to the right side. (**g**) Ventral view of a female worm, focusing on the vulva (asterisk). The uterus (a single intrauterine egg is visible) extends to the right side. (**h**) Intra-uterine egg with two plugs and a thick egg shell. The surface is not smooth. (**i**, **j**) Sagittal and superficial views of an embryonated egg with a reticulated egg-shell surface. This is one of the eggs originating from cecal contents and preserved in a 2.5% potassium bichromate solution for approximately two years. (**k**) The caudal end of a female worm, showing the terminal anus (arrowhead). Photographs (**a** – **d**) are at the same magnification, and the scale bar is shown in (**d**). Similarly, photographs (**f** – **h**) and photographs (**i** and **j**), respectively, are at the same magnification, and the scale bar is shown in either photograph of each set (**f** and **j**).

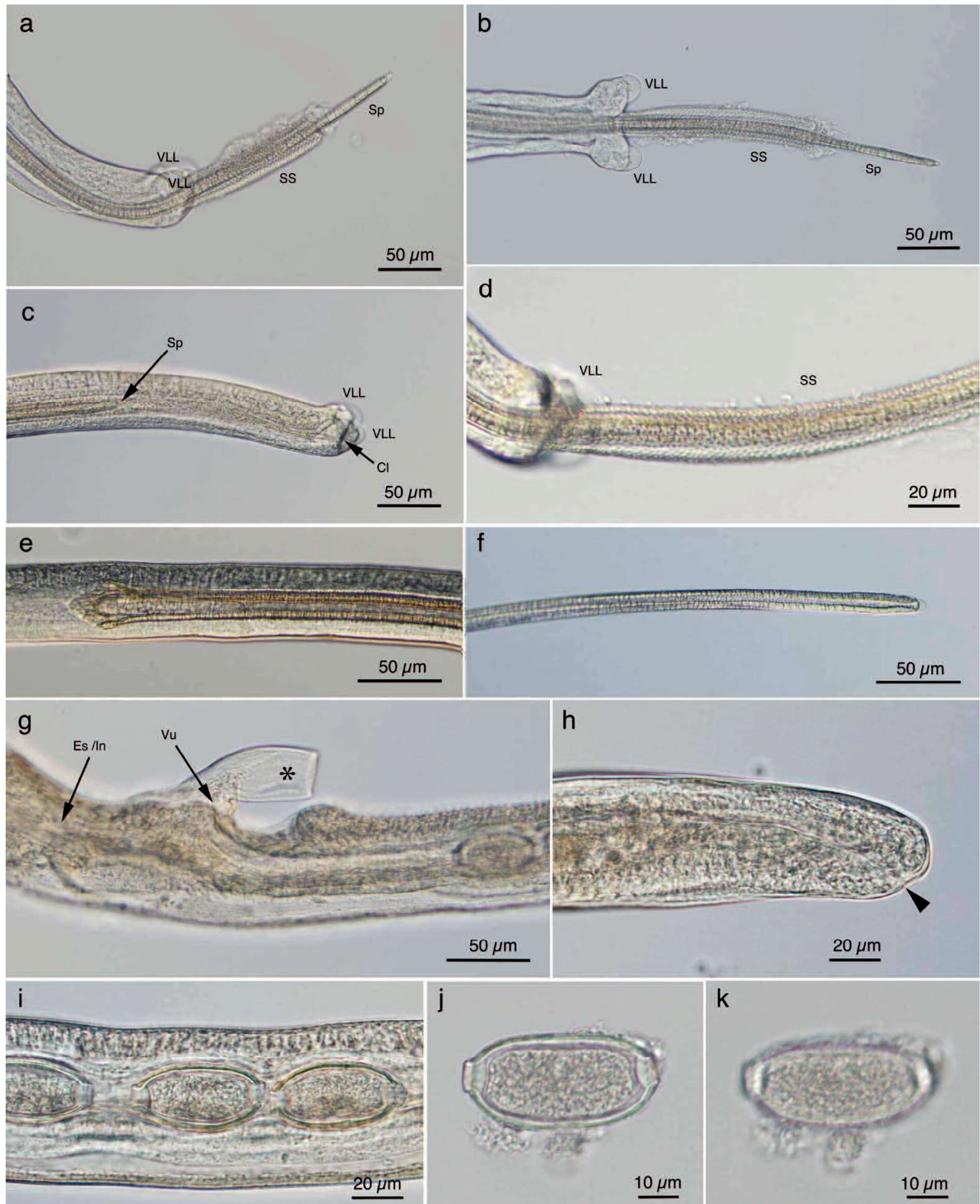

Fig. 7. Morphology of *Capillaria phasianina* from farmed ring-necked pheasants. (**a**) Lateral view of the caudal end of a male worm with two massive ventrolateral lobes (VLL). The spicule (SS), partly covered by the spicular sheath (SS), protruds from the cloaca. (**b**) Dorsal view of the caudal end of a male worm with the protruded spicule (Sp). Two massive ventrolateral lobes

(VLL), and spicular sheath (SS). (**c**) Lateral view of the caudal end of a male worm with two massive ventrolateral lobes (VLL). The distal end of the spicule (Sp) and cloaca (Cl) are indicated by arrows. (**d**) High magnification of the caudal end of a male worm with a protruded spicular sheath (SS) armed by distinct spines. (**e**) The proximal end of the spicule is in the body of a male worm. (**f**) The distal end of the spicule protruded from the cloaca of a male worm. (**g**) Lateral view of a female worm at the mid-body, showing a vulva (Vu) with tubular appendage (asterisk). Esophagus/intestine junction (Es/In). (**h**) The caudal end of a female worm with a subterminal anus (arrowhead). (**i**) Intra-uterine eggs with two plugs and a thick egg-shell. The surface is not smooth. (**j**, **k**) Sagittal and superficial views of an intrauterine egg with a punctuated egg-shell surface. Photographs (**a**) to (**c**) are at the same magnification, and the scale bar is shown in (**c**). Similarly, different sets of photographs (**e–g**), (**h** and **i**), and (**j** and **k**) are, respectively, at the same magnification, and the scale bar is individually shown.

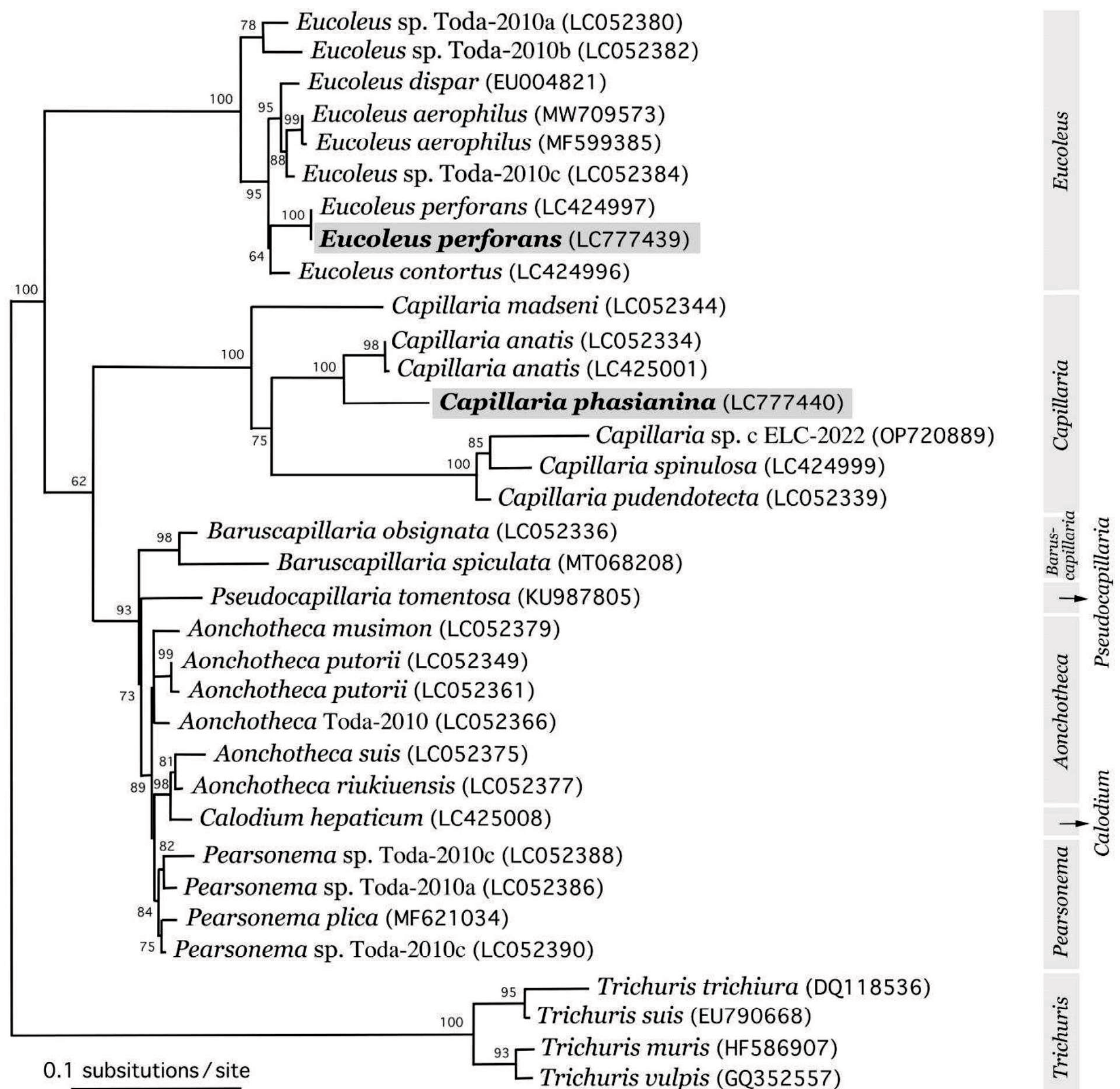

Fig. 8. ML phylogenetic tree based on the SSU rDNA sequences of representative capillariids. Species names are followed by GenBank accession numbers in parentheses. The newly obtained SSU rDNA sequence in the present study is marked with a grey background.

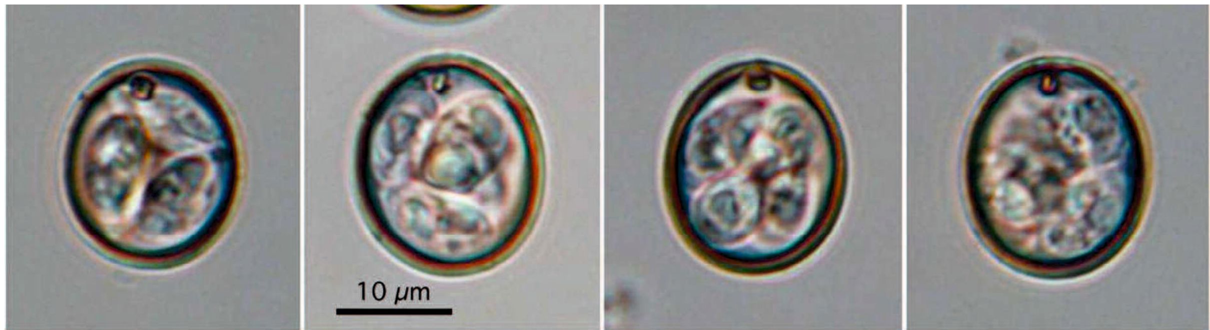

Fig. 9. Sporulated oocysts of *Eimeria iyoensis* **n. sp.** obtained from farmed ring-necked pheasants. Refractile polar granules are located inside the oocyst fluid near the apical pole; no oocyst residuum is observed. All photographs are at the same magnification.

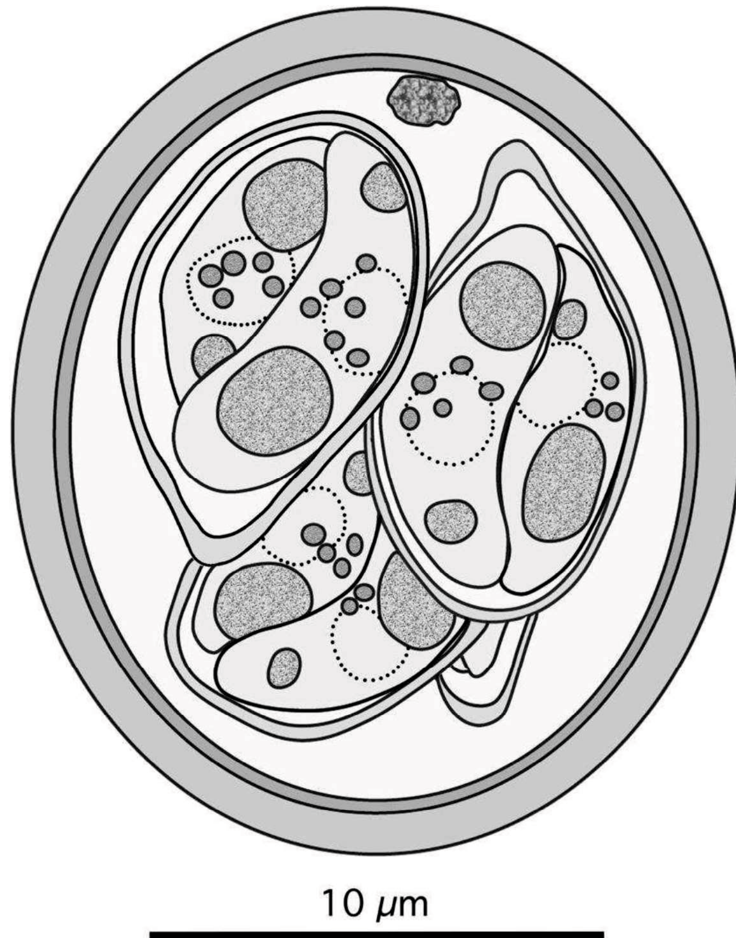

Fig. 10. Schematic of an *Eimeria iyoensis* **n. sp.** oocyst collected from farmed ring-necked pheasants.

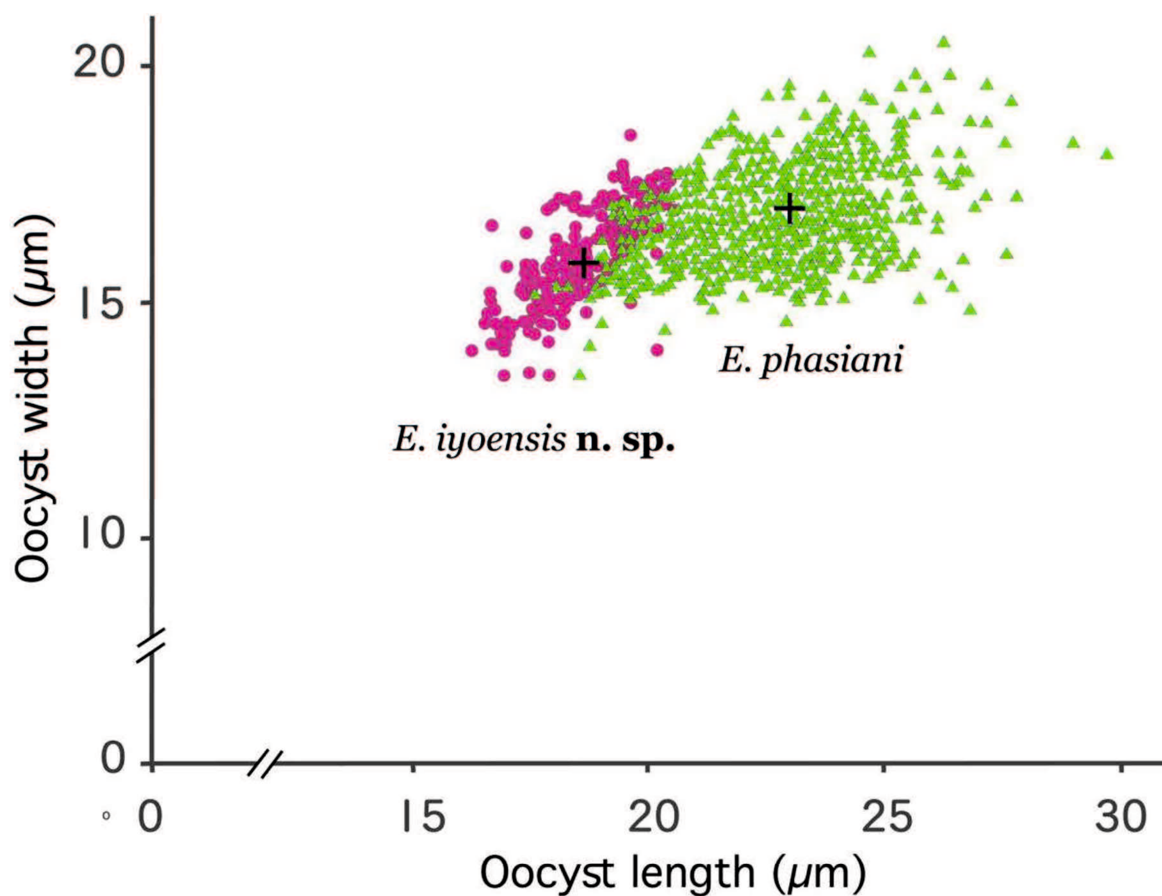

Fig.11. Scatter plot of the *Eimeria iyoensis* **n. sp.** and *E. phasiani* oocyst dimensions collected from farmed ring-necked pheasants. (**Red round dots**) Oocysts of *E. iyoensis* **n. sp.** (n = 150), showing 18.6 (16.4–20.6) µm by 15.7 (13.3–18.6) µm with a shape index of 1.18 (1.02–1.46). (**Green triangle dots**) Oocysts of *E. phasiani* (n = 590), showing 23.1 (17.7–29.8) µm by 17.0 (13.3–22.3) µm with a shape index of 1.36 (1.00–1.82). Data on *E. phasiani* oocysts was obtained from our previous study (Chapter I of this thesis).

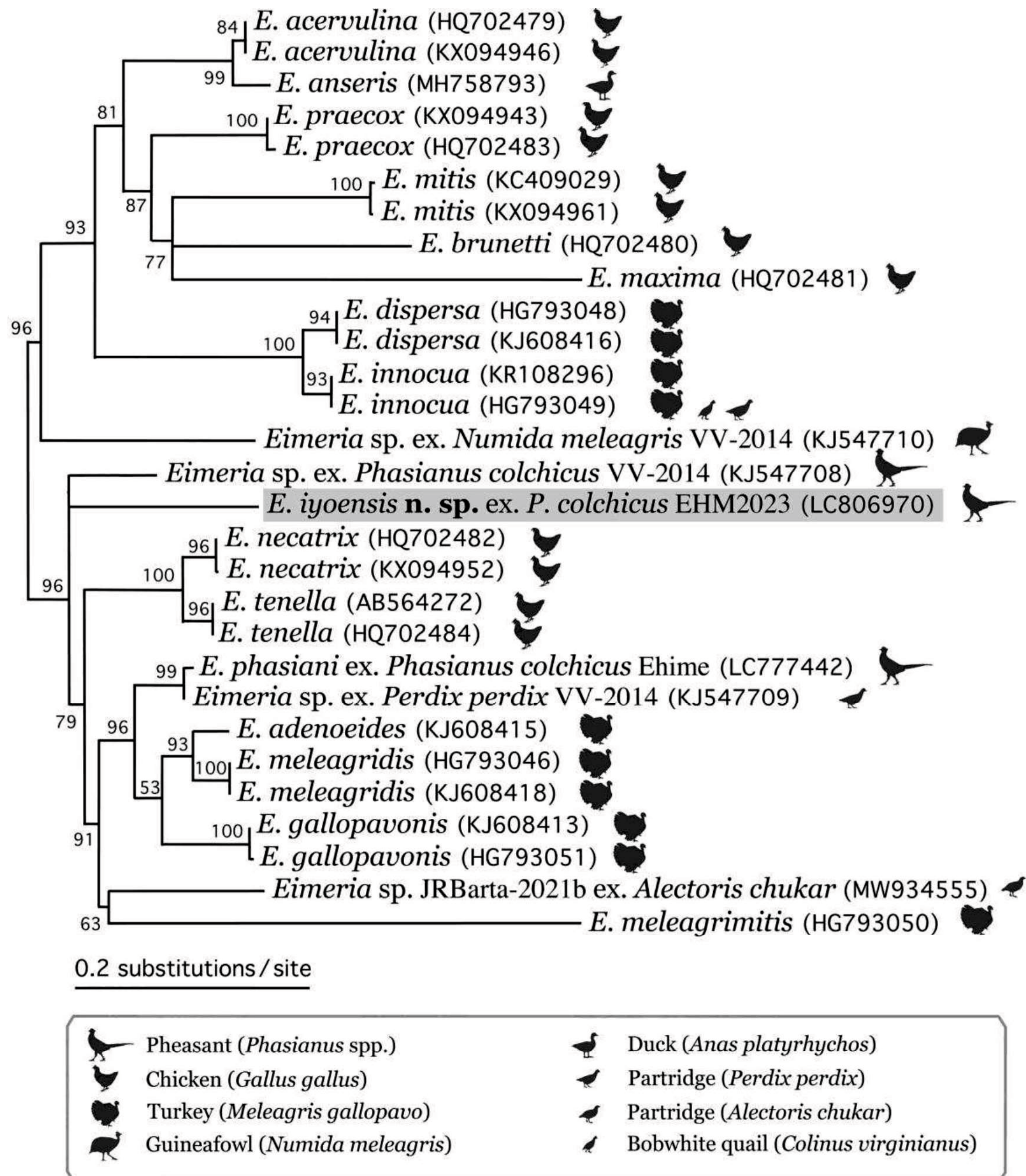

Fig. 12. Maximum likelihood (ML) phylogenetic tree based on 1,257-bp long mitochondrial *cox1* sequences of representative *Eimeria* spp. from avian hosts. The species name is followed by the GenBank accession number in parentheses for most cases. For several species of particular interest, the species name is followed by the isolate name and/or hostname and the GenBank accession number in parentheses. The newly obtained *cox1* sequence of *E. iyoensis* n. sp. is marked with a grey background.