Detection of *Eumonospora henryae* (Apicomplexa: Sarcocystidae) from *Falco columbarius* (Falconiformes: Aves): Comparison of host–parasite phylogram and comments on the family Sarcocystidae Poche, 1913

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**ABSTRACT**

The genus *Eumonospora* Allen, 1933 (Apicomplexa: Sarcocystidae), an avian coccidia, is characterized by monosporocystic and octasporozoic oocysts without Stieda and substieda bodies. Some members of *Eumonospora*, which infect several raptor species, exhibit high levels of pathogenicity, making eumonosporiosis the leading cause of death in captive-bred raptors. The host specificity of these species appears to be mesostenoxenous, as evidenced by unsuccessful transmission between different orders of avian hosts. However, several studies have detected *Eumonospora* spp. in taxonomically distant avian hosts, indicating that some of these species may be euryxenous. In the current study, diarrheic fecal examination of a captive-bred juvenile Merlin (Falconiformes: Aves) in Tokyo, Japan, was conducted, and a large number of oocysts were morphologically and molecularly identified as *E. henryae* (Yakimoff and Matschulsky, 1932), a coccidia species reported only in Strigiformes. This is a new recorded host for this coccidia. Phylogenetic analyses via Bayesian inference and maximum likelihood methods using concatenated genomic datasets consisting of nuclear 18S rDNA, nuclear 28S rDNA and mitochondrial cytochrome C oxidase subunit 1 gene, revealed a well-supported monophyletic clade of *Eumonospora* spp. belonging to the family Sarcocystidae Poche 1913, which largely corresponded to the avian host phylogram. Therefore, based on distinguishable oocyst morphology, a new subfamily, Eumonosporinae, within the family Sarcocystidae, is proposed, and a reconsideration of the definition of Sarcocystidae is suggested. Further molecular characterization of this emerging pathogen, as well as clarification of its complete life cycle, including cyst-forming ability, is required for more appropriate generic assessment.

1. Introduction

An avian protozoan, genus *Eumonospora* Allen, 1933 (Apicomplexa: Sarcocystidae), which belongs to family Sarcocystidae Poche, 1913 according to molecular evidence, is characterized morphologically by monosporocystic and octasporozoic oocysts without Stieda and substieda bodies in their sporocysts (Allen, 1933; Chou et al., 2020). The pathogenicity and host specificity of this genus has been questioned. Nevertheless, *E. neofalconis* (Böer, 1982) and *E. kutzeri* (Böer, 1982) infections, which produce clinical signs such as regurgitation, hemorrhagic diarrhea, anorexia, and acute death without symptoms, have been reported in Falconiformes (Böer, 1982; Forbes and Simpson, 1997; Krone, 2002). On the other hand, an *Eumonospora* sp., was reportedly responsible for the deaths of three juvenile snowy owls (Strigiformes: Aves), as shown by a post mortem examination that found pathological lesions in the small intestine, the large intestine and caeca (Papazahariadou et al., 2001). Thus, eumonosporiosis is considered a disease associated with high mortality in captive-bred (CB) raptors in Europe, the Middle East, and North America (Forbes and Simpson, 1997; Krone, 2002; Mateuta and Samour, 2017; Pavlík et al., 1998; Upton et al., 1990). Attempts made by several studies to experimentally transmit *Eumonospora* spp. between various avian hosts were unsuccessful (Allen, 1933; Böer, 1982; Cawthorn and Stockdale, 1982), revealing that some members of the genus *Eumonospora* were unable to infect across families or orders. However, successful transmission of *E. falconis* (Wetzel and Enigk, 1937) across generic boundaries was reported, although this

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conclusion has been questioned and is considered to be erroneous (Upton et al., 1996; Wetzel and Enigk, 1939). Nevertheless, detection of *E. megafaedon* (Klüh, 1994) in hawks (Otididae: Aves) and *E. henryae* (Yakimoff and Matschulsky, 1932) in various genera of owls, indicates that some of these organisms may display a wide host spectrum (Chou et al., 2020; Schuster et al., 2016).

Merlin (*Falco columbarius* Linnaeus, 1758; Falconiformes: Aves), a medium-sized falcon with a worldwide distribution, is a migratory bird species that migrates to Japan and Korea during the winter season (Hoyo et al., 1992). The fecal specimen of an imported CB merlin found in Tokyo, Japan, was examined due to a watery diarrheic condition, and showed a high quantity of oocysts with a high degree of morphological similarity with those of *Eumonospora* spp., following sporulation. This was the first detection of this genus in Falconiformes in Japan. Therefore, a molecular analysis of this emerging pathogen was conducted to obtain further information, while the family definition of Sarcocystidae was reconfirmed via the classification of this unique genus.

2. Material and methods

2.1. Specimens

In November 2019, a fecal specimen collected from a female juvenile merlin (obtained from Belgium and kept in Tokyo, Japan), which was suffering from severe watery diarrhea from September 2019, was sent to the laboratory for examination. The fecal sample was placed in a vial and sent to the laboratory for examination. The fecal sample was placed in a vial and transported under a refrigerated condition. The bird was treated with orally administered toltrazuril which effectively reduced clinical symptoms. Examination by direct microscopy without flotation revealed a large quantity of unsporulated oocysts. A fecal suspension was filtered using a 180 μm stainless steel sieve (Tokyo Screen, Japan) to remove large particles, and the remaining suspension was centrifuged at 300 g for 10 min to sediment oocysts. After removing the supernatant, saturated sodium chloride solution was added to the sediment and centrifuged at 100 g for 5 min. Oocysts recovered from the supernatant were washed thrice with distilled water. Recovered oocysts were stored in 2.5% aqueous potassium dichromate (K₂Cr₂O₇) at room temperature for sporulation.

2.2. Morphological analysis

Most oocysts in the 2.5% K₂Cr₂O₇ solution sporulated by day 4, and observed under a BX41 microscope (Olympus, Japan) with differential interference contrast. Photomicrographs were obtained using a DP71 photomicroscope (Olympus, Japan). Measurements, made with ImageJ. (Schneider et al., 2012) using pictures taken under an oil immersion objective, were expressed as mean ± SD (μm) with the range in parentheses. Guidelines used for describing oocysts and sporocysts were as follows (Berto et al., 2014; Wilber et al., 1998): oocyst length (L) and width (W), their ranges and ratios (L/W), micropyle cap (MC), oocyst residuum (OR), polar granule (PG), sporocyst (SP), Stieda body (SB), substieda body (SSB), parastieda body (PSB), sporocyst residuum (SR), sporozoite (SZ).

2.3. Molecular analyses

Oocysts which sporulated in the 2.5% K₂Cr₂O₇ solution were washed thrice with double distilled water. Genomic DNA was extracted using a QIAGEN Power Soil DNA isolation kit (QIAGEN, Germany) according to the manufacturer’s protocol with a prolonged vortexing time of 20 min. The following primer pairs were used for PCR amplification: of the nuclear small subunit ribosomal DNA (18S) sequence, primers: CRYPTO, 5'-AACCTGGTATCGATCCGACTAG-3' and CRYPTO, 5'-GCCGTGATCCTGAGTCCACTAC-3' (Herwaldt et al., 2003); the nuclear large subunit ribosomal DNA (28S) sequence, primers: KL1, 5'-TACCCGGGTAACTTACG-3' and KL3, 5'-CMAACCAAGATGTCACGTA-3' (Schrenzel et al., 2005); and the mitochondrial cytochrome C oxidase subunit 1 (cox1) sequence, primers: Sdce_COX1_260F, 5'-GACCTTTATGGTATRTGCC-3' and Sdce_COX1_1147R, 5'-CAT-TACCCATAACYGACCC-3' (Ogedengbe et al., 2016).

PCR was performed in 20 μL volume containing 2 μL of 10 μL × Ex Taq® buffer, 1.6 μL of dNTPs (2.5 mM each), 0.2 μL of each primer (50 μM), 0.2 μL of Ex Taq® polymerase (Takara Bio, Japan), 1.0 μL of DNA extract, and 14.8 μL of PCR grade water. PCR thermal cycling included 95 °C (18S and 28S) or 96 °C (cox1) for 5 min, followed by various cycles (18S: 30 cycles; 28S: 35 cycles; cox1: 40 cycles) of denaturation at 94 °C for 30 s, annealing at different temperatures (18S: 60 °C; 28S: 45 °C; cox1: 50 °C) for 30 s, and extension at 72 °C for 1 min. The final extension was done at 72 °C for 10 min (18S) or 5 min (28S and cox1), followed by a holding step at 4 °C. Negative and positive control templates were included in all reactions. PCR products were electrophoresed on 1.5% agarose gels in 1× Tris-acetate-EDTA buffer at 100 V for 25 min, visualized using an LED transilluminator and stained with Green loading buffer 6 × (Bio Craft, Japan), following which product size was estimated by comparing with 100-bp plus DNA ladder (Mastrogen, Taiwan). PCR products were sent to a sequencing service (Macrogen Corp., Japan) and analyzed with an ABI 3730XL DNA Analyzer (Thermo Fisher Scientific, USA) using the abovementioned PCR primers. Sequence similarity was determined separately using the BLASTN program via a nucleotide database provided by the National Center for Biotechnology Information within the Geneious Prime 2020.2.2 (https://www.geneious.com) bioinformatics software.

2.4. Data treatment and multiple sequence assembly

Six genome datasets were analyzed in this study: single datasets included 18S, 28S, and cox1; concatenated datasets contained 18S with 28S (18S + 28S), 18S with cox1 (18S + cox1), and 28S with cox1 (28S + cox1), respectively. Publicly available related sequences were retrieved from nucleotide databases and consensus sequences were generated for each related species for the purpose of reducing the number of sequences to be analyzed. For a single coccidia species with ≥10 available sequences as described by Ogedengbe et al. (2018) and the accession numbers of sequences used are shown in Supplementary Table S1. In order to reveal the phylogenetic relationship between *Eumonospora* spp., available associated sequences (KJ634019, KT037081, MN629229–30) were used independently, with the exception of *E. henryae*. Accession number KJ634020 (E. dacelo) was excluded from 28S analyses due to a 948-bp long sequence being 100% identical to that of KF766053 (Isospora antsohaeae), possibly due to a mis-submitted sequence.

MAFFT (Katoh and Toh, 2010) was used for multiple alignment of 18S and 28S sequences, while Geneious Alignment algorithm (Cost matrix = 65% similarity; Gap open penalty = 12; Gap extension penalty = 3) was used for cox1 sequences. Both MAFFT and the Geneious Alignment algorithm were executed within Geneious Prime. Obvious errors were collected with visual inspection of each alignment. Concatenation sequences were created without modification of each alignment.

2.5. Phylogenetic analyses

Phylogenetic analyses were performed via Bayesian inference (BI) using MrBayes 3.2.6 (Husonbeck and Rontqui, 2001) and maximum likelihood (ML) using PHYML 3.3.2 (Guindon et al., 2010), both of which were executed within Geneious Prime. The best-fit model and parameters for the analyses of 18S, 28S, and cox1 alignments were evaluated in JModelTest 2.1.10 based on Akaike Information Criterion (Darriba et al., 2012). The best fit model for all datasets was the General Time Reversible (GTR) model with a Gamma distribution for rate variation among sites, with the proportion of invariable sites (I) being 0.16, rate variation among sites (R) being 0.094, and a log-likelihood of -4041.54673 for the 18S dataset, -1903.50369 for the 28S dataset, and -1457.89682 for the cox1 dataset.
Time Reversible model (GTR), which accounts for the proportion of invariant sites (+I) with gamma distributed rate variation among sites (+G). The BI analysis consisted of $1.1 \times 10^6$ generations of Markov Chain Monte Carlo searches containing four chains, a heated chain temperature of 0.2, and a burn-in of 100,000 generations. ML bootstrap analysis from 1000 replicates was performed to estimate node support. Gaps were treated as missing in all datasets.

3. Results

Microscopic examination of sporulated oocysts revealed a large quantity of monosporocystic and octasporozoic coccidia. Based on morphological and molecular characters of sporulated oocysts, these were identified as *Eumonospora henryae* (Table 1).

3.1. Description of sporulated oocysts

Oocyst shape: ellipsoidal or sub-spherical; 40.4 ± 2.2 × 34.3 ± 2.0 (37.0–44.27 × 30.5–37.9); L/W 1.18 ± 0.06 (1.08–1.34). Oocyst wall: bilayered, outer and inner layer smooth, ~2 μm thick; MC, OR, PG: all absent.

3.2. Description of sporocyst and sporozoites

Sporocyst shape: spherical to sub-spherical; 23.0 ± 1.8 × 22.6 ± 1.8 (20.3–28.4 × 20.0–27.9); length of SP/width of SP 1.02 ± 0.02 (1.08–0.96). Sporocyst wall: bilayered, outer and inner layer smooth, ~1 μm thick; SB, SSB, PSB: all absent. SR: present, diffuse or compact (Fig. 1B, C, 1D); SZ number: eight; shape: stout and ellipsoidal; 13.6 ± 1.9 × 4.9 ± 0.7 (10.7–17.1 × 3.7–6.2); length of SZ/width of SZ 2.85 ± 0.4 (2.08–3.83); arranged parallel or randomly within SP (Fig. 1A).

3.3. Description summary

**Type host:** Bubo bubo (Linnaeus, 1758).
**Detection host:** Falco columbarius Linnaeus, 1758
**Other hosts:** *Athene noctua* (Scopoli, 1769), *Bubo scandiacus* (Linnaeus, 1758), *Ptilosis leucotis* (Temminck, 1820), *Sistrurus miliarius* (Linnaeus, 1769), *Strix nebula* Foster, 1772

**Prepatent and patent time:** Unknown
**Site of infection:** Unknown. Oocysts recovered from feces.

**Sporulation:** Endogenous. Most oocysts were passed in the feces unsporulated and sporulated by day 4 in 2.5% K₂Cr₂O₇ solution at room temperature.

3.4. Genetic analysis

A 1672-bp fragment of 18S rRNA gene, a 1427-bp fragment of 28S rRNA gene, and a 885-bp fragment of cox1 from the coccidia collected from the merlin were amplified, sequenced and deposited in the DNA Data Bank of Japan (DDBJ) (accession nos., 18S: LC595644, 28S: LC595645, cox1: LC595646). Moreover, re-analysis of *E. henryae* from previous study was performed (Chou et al., 2020) with newly obtained 18S (1584 bp), 28S (1431 bp), and cox1 (885 bp) sequences. These sequences were also deposited in DDBJ (accession nos., 18S: LC595641, 28S: LC595642, cox1: LC595643). The coccidian 18S and 28S sequences obtained from the merlin were 100% identical to *E. henryae*. Comparison of sequences in available nucleotide databases indicated that, 18S sequence was 99.0% identical to that of *Besnoitia besnoiti* (Franco and Borges, 1916): AY833646, 28S sequence was 92.2% identical to that of *B. besnoiti*: AF076900, and cox1 sequence was 100%, and 99.86% identical to that of *E. henryae* from *Athene noctua* (Scopoli, 1769): LC521950, and from other Strigiformes, *Bubo scandiacus* (Linnaeus, 1758); LC521952 and *Ptilopsis leucotis* (Temminck, 1820): LC521951, respectively. A single synonymous substitution occurred in between the coccidian sequences obtained from *Pt. leucotis*, *B. scandiacus* (G) and from *A. noctua*, *F. columbarius* (T) with cox1 region.

The 18S (25 sequences, 1821-bp long), 28S (16 sequences, 3259-bp long), and cox1 (14 sequences, 1039-bp long) datasets were used for phylogenetic analyses with BI and ML methods. The consensus sequence of *Sarcocystis rileyi* (Stiles, 1893) was used as the out group in all datasets and illustrated similar topologies with both methods, by which 4 major clades were consistently supported as follows: (1) a clade of *Cystoisospora* spp.; (2) a clade of *Eumonospora* spp.; (3) a clade comprising genera *Hammondia*, *Heydornia*, *Neospora*, and *Toxoplasma*; and (4) a clade of *Besnoitia* spp. While *Hyloklis kaylebuerkuehni* (Labbé, 1896) branched earliest in the 28S dataset and *Nephriopora* sp. *epsteii* Wünschmann, 2010 clustered in the *Cystoisospora* spp. clade with the cox1 dataset, the 18S dataset failed to obtain highly-supported phylogenetic positions for *H. lieberkuehni* and *N. epsteii* (Supplementary Fig. 1).

3.5. Genetic analysis–concatenated sequences

The original concatenated alignment lengths for 18S + 28S, 18S + cox1, and 28S + cox1 were 5080 bp, 2860 bp, and 4298 bp, respectively. The phylogenetic trees illustrated with BI and ML methods were similar for all datasets with the four well supported major clades as mentioned above, having *H. lieberkuehni* branching earliest among all taxa in 18S + 28S and 28S + cox1 datasets (Fig. 2). On the other hand, *N. epsteii* was found within the *Cystoisospora* clade in 18S + cox1 and 28S + cox1, but within the genera *Hammondia*, *Heydornia*, *Neospora*, and *Toxoplasma* clade in the 18S + 28S dataset. The phylogenetic position of *Eumonospora* clade was identical in all concatenated datasets that branched early among the genera *Hammondia*, *Heydornia*, *Neospora*, and *Toxoplasma* clade and the genus *Besnoitia* clade. Phylogenetic trees of the genus *Eumonospora* (Fig. 3) largely corresponded with avian host phylogeny (McClure et al., 2019; Prum et al., 2015).

4. Discussion

Monosporocystic and octasporozoic coccidia without Stieda bodies, belonging to the genus *Eumonospora*, are found in various avian orders, including Accipitriformes, Charadriiformes, Coraciiformes, Falconiformes, Otidiformes and Strigiformes. The host specificity of *Eumonospora* spp. is ambiguous, although it was considered to be mesostenoxenous (more than one hosts, but restricted to one genus) based on a series of unsuccessful, attempted transmissions between avian hosts of various families or orders (Allen, 1933; Bégé, 1982; Cawthorn and Stockdale, 1982; Upton et al., 1990). On the contrary, although questioned by Upton et al. (1990), Wetzel and Enigk (1939) reported a successful transmission of *E. falconis* collected from Falconiformes to Strigiformes. Moreover, Pavlik et al. (1998) identified *E. neofalcons* in Accipitriformes suggesting a mild infection or merely intestinal passage, while *E. megafalconis* was recovered from wild as well as captive bustards (Otidiformes: Aves), a favorite prey of falcons in the United Arab Emirates (Schuster et al., 2016), and *E. henryae* was detected in five owl genera of the family Strigidae (Chou et al., 2020) indicating some *Eumonospora* spp. may be metastenoxenous (more than one host, but restricted to one family) or euryxenous (more than one family of hosts). Among members of the genus *Eumonospora*, *E. henryae* is one of the most taxonomically confusing species as it has been described in falcons, kites, and owls by Yamamoto and Matsushisky (1936). However, species with oblong oocysts found in falcons was interpreted by Bo; er (1982) as being *E. kunzerti*, while species with spherical oocysts as *E. falconis*, leaving *Bubo bubo* (Strigiformes: Aves) as the typical host of *E. henryae* (Upton et al., 1990). In this study, *E. henryae* was identified by the characteristic morphology of sporulated oocysts (monosporocystic and octasporozoic) and molecular analyses from a merlin, *Falco columbarius* (Falconiformes: Aves), resulting in a new host being recorded and evidence of a broad host spectrum across order boundaries.
Table 1
Morphological characters, host, and localities of genus *Eumonospora* infecting avian hosts.

| Species          | Oocyst Size (μm) | L/W | sporocyst Size (μm) | L/W | sporozoite Size (μm) | L/W | Host                | Locality     | Ref                      |
|------------------|------------------|-----|---------------------|-----|----------------------|-----|---------------------|--------------|-------------------------|
| *E. aquilae*     | 43.0 × 37.5 (40.0-49.0 × 34.0-39.0) | 1.15 (1.03-1.26) | 23.8 × 23.3 (23.0-25.0 × 22.0-25.0) | 1.02 (1.00-1.45) | 13.5 × 4.5 (13.0-14.0 × 4.0-5.0) | NA | Aquila chrysaetos | Czech Republic | Volf et al. (2000) |
| *E. arcayae*     | 32.9 × 29.4 (30.3-37.6 × 29.0-31.8) | NA | 21.9 × 21.8 | NA | NA | NA | Buteo plaptypterus | Venezuela Volcán and Medrano (1986) |
| *E. dacelo*      | 32.1 × 28.3 (29.0-36.0 × 26.4-30.4) | 1.13 (1.06-1.22) | 20.0 | 1 | 12.0 × 5.1 (10.4-13.6 × 4.8-5.6) | NA | Buteo magnirostris | USA | Upton et al., 1990 |
| *E. argentini*   | 20.3 × 16.9 (18.5-23.8 × 15.1-20.5) | NA | NA | NA | NA | NA | Larus argentatus | Germany Schwalbach (1959), Upton et al. (1986) |
| *E. biarmicaxis* | 40.2 × 34.7 (37.5-42.4 × 32.9-35.7) | 1.16 (1.08-1.31) | 20.1(18.6-21.3) | 1 | NA | NA | Falco biarmicaxis | Saudi Arabia Alyonif et al. (2011) |
| *E. boeri*       | 36.6-33.4 (33.2-39.6 × 31.1-36.6) | 1.09 (1.0-1.14) | 27.8 × 19.6 (25.8-30.9 × 17.9-21.2) | 1.41 (1.24-1.51) | 16.6 × 4.7 (15.0-17.0 × 4.0-5.0) | NA | Falco tinnunculus | Europe Klíh (1994) |
| *E. bubonii*     | 43.9 × 40.2 (38.0-52.0 × 30.0-47.0) | 1.1 (1.0-1.3) | 26.8 × 25.6 (20.0-33.0 × 20.0-32.0) | 1.1 (1.0-1.2) | 15.5 × 2.5 (13-20.8 × 2.3-3) | 6.2 (4.6-8.7) | Bubo virginianus | Canada Stockdale (1981) |
| *E. cherrug*     | 32.1 × 29.3 (31.0-35.0 × 28.0-30.0) | 1.1 (1.0-1.3) | 24.0 × 20.0 (23.0-26.0 × 19.0-21.0) | 1.1 (1.0-1.1) | 15.0 × 4.0 (14.0-17.0 × 3.0-6.0) | NA | Falco cherrug | Saudi Arabia Alafeih et al. (2013) |
| *E. circi*       | 23.7 × 28.0 (29.0-35.0 × 23.0-32.0) | NA | 22.6 × 18.9 (20.0-25.0 × 15.0-22.0) | 1.04 (1.00-1.07) | 10.4 × 4.3 (9.0-11.0 × 4.0-5.0) | NA | Falco cherrug | UAE Mateuda and Samour (2017) |
| *E. dacelo*      | 31.4 × 29.3 (30.0-32.0 × 28.0-31.0) | 1.05 (1.01-1.1) | 21.2 × 20.6 (20.0-24.0 × 20.0-21.0) | 1.03 (1.0-1.14) | 17.0 × 4.8 (16.0-18.0 × 4.0-6.0) | 3.54 | Dacelo novaeguineae | Australia Yang et al. (2014) |
| *E. falconis*    | 29.5 × 36.5 NA | NA | 21.0-23.0 | NA | 13.0-14.0 (10.0-17.0 × 2.0-5.0) | NA | Falco peregrinus | Europe Wetzel and Enigk (1957), Upton et al. (1990) |
|                  |                  |     |                  |     | Falco tinnunculus | Falco subbuteo | Falco peregrinus | UAE Mateuda and Samour (2017) |
| *E. hambripnicki*| 48.1 × 42.1 (42.0-54.0 × 37.0-50.0) | 1.2 (1.0-1.4) | 24.8 (23.8) | 1 | 18.6 × 5.6 (16.0-20.0) | NA | Falco tinnunculus | Falco peregrinus | USA McAlistier et al. (2013a) |
| *E. henryae*     | 41.0 × 34.5 (36.6-43.5 × 31.0-37.1) | 1.16 (1.08-1.28) | 22.0 × 22.6 (20.3-28.4 × 20.0-27.9) | 1.02 (0.96-1.08) | 13.5 × 4.9 (10.7-17.1 × 3.7-6.2) | 2.84 (2.07-3.83) | Falco insignis | Japan This study |
| *E. japonica*    | 41.0 × 37.0 (39.6-43.2 × 36.0-39.6) | 1.11 | 21.6-25.2 × 19.8-21.6 | NA | 10.8-14.4 × 2.7-5.4 | NA | Falco peregrinus | Russia Yakimoff and Matschulsky (1932) |
| *E. leucocephalus*| 41.2 × 35.2 (37.0-44.0 × 33.0-38.0) | 1.17 (1.03-1.26) | 23.8 × 23.3 (21.0-26.0 × 20.0-25.0) | 1.02 (1.00-1.09) | NA | NA | Buteo superciliosus | Japan Chou et al. (2020) |
| *E. montana*     | 43.2 × 37.8 (41.0-46.0 × 34.0-41.0) | 1.14 (1.05-1.22) | 23.8 × 23.3 (22.0-26.0 × 21.0-25.0) | 1.02 (1.00-1.10) | NA | NA | Buteo superciliosus | Japan Chou et al. (2020) |
| *E. norvegica*   | 42.6 × 35.7 (40.4-46.0 × 34.0-38.0) | 1.19 (1.13-1.27) | 24.2 × 24.0 (23.0-25.0 × 23.0-25.0) | 1.01 (1.00-1.04) | NA | NA | Pilosis leucopogon | Japan Chou et al. (2020) |
| *E. planci*      | 42.8 × 37.6 (40.0-46.0 × 33.0-38.0) | 1.16 (1.05-1.27) | 24.2 × 23.8 (21.0-26.0 × 21.0-25.0) | 1.02 (1.00-1.10) | NA | NA | Pulsatrix perspicillata | Japan Chou et al. (2020) |
| *E. sericea*     | 42.2 × 36.2 (38.0-45.0 × 33.0-39.0) | 1.16 (1.04-1.25) | 24.0 × 23.5 (22.0-26.0 × 22.0-26.0) | 1.02 (1.00-1.10) | NA | NA | Serix nebulosa | Japan Chou et al. (2020) |

(continued on next page)
| Species          | Oocyst Size (μm) | Oocyst L/W | Sporocyst Size (μm) | Sporocyst L/W | Sporozoite Size (μm) | Sporozoite L/W | Host | Locality | Ref                  |
|-----------------|-----------------|----------|---------------------|--------------|----------------------|--------------|------|----------|----------------------|
| E. kansasensis  | 37.2 × 32.6     | 1.14     | 22.5                | 1            | 14.4 ± 5.4           | NA           | USA  | Upton et al. (1990) | Boer (1982)          |
| E. smithi       | 38.7 × 34.1     | NA       | 24.6 ± 2.0          | NA           | 4.6 ± 2.0            | NA           | NA   | USA       | Upton et al. (1990)  |
| E. megeつけ | 37.58 × 32.54   | 1.15     | 24.17 ± 21.96       | 1.1          | 15.5 ± 4.8           | NA           | NA   | USA       | Mateuta and Samour (2017) |
| E. lindsayi     | 33.7 × 31.6     | 1.07     | 20.5 ± 19.2         | 1            | 16.5 ± 4.2           | NA           | NA   | USA       | Upton et al. (1990)  |
| E. megfalconis | 43.6 × 35.8     | 1.12     | 22.4 ± 24.2         | NA           | 18.6 ± 4.6           | NA           | NA   | Europe    | Klüh (1994)          |
| E. neofalconis | 42.25 × 37.77   | 1.19–1.23 | 22.7–23.93          | NA           | 13.0–15.0 × 5.0–6.0  | NA           | NA   | USA       | Schuster et al. (2016) |
| E. mochagalloni| 42.2 × 35.9     | NA       | 23.6 ± 22.7         | NA           | 16.5 ± 4.2           | NA           | NA   | USA       | Mateuta and Samour (2017) |
| E. peregrinus   | 38.9 × 32.9     | 1.18     | 21.1 ± 20.1         | 1.02         | 16.0 ± 4.7           | NA           | NA   | Europe    | Boer (1982)          |
| E. cherrug      | 27.0 × 23.8     | NA       | 18.8 ± 14.8         | NA           | 9.9 ± 2.4            | NA           | NA   | USA       | Mateuta and Samour (2017) |
| E. peregrineiroi| 47.1 ± 37.6    | 1.25     | 25.1 ± 24.3         | 1.03         | 17.0 ± 4.2           | NA           | NA   | Portugal  | Cardozo et al. (2017) |
| E. petersoni    | 43.1 ± 39.8     | 1.08     | 23.4 ± 23.3         | 1.05         | 15.6 ± 4.2           | NA           | NA   | USA       | McAllister et al. (2013b) |
| E. stripsi      | 13.8 × 10.9     | NA       | NA                  | NA           | 7.5 ± 3.4            | NA           | Tyto alba | Europe   | Gotschalk (1972); Upton et al. (1990) |
| E. tremula      | 33.0 × 35.0     | NA       | 23.5–25.0           | NA           | 20.0 ± 6.0           | NA           | USA   | Allen (1933) | C. auroreus | USA         |
| E. rusticolus   | 33.4 × 30.0     | 1.2      | 20.4 ± 20.1         | 1.01         | 16.3 ± 5.2           | NA           | NA   | USA       | Lindsay et al. (1994) |
| E. undata       | 30.6 ± 29.3     | NA       | 22.0 ± 21.0         | NA           | 15.0 ± 2.4           | NA           | NA   | Germany   | Schwabach (1959)     |
| E. australis    | 32.5 × 29.2     | NA       | NA                  | NA           | 9.9 ± 2.4            | NA           | NA   | Netherlands | Poolma and Stirk (1966) |
| E. pacificus    | 30.5 × 27.6     | 1.1      | 20.3                | NA           | 15.0 ± 2.4           | NA           | L. cinnhata | USA | Upton et al. (1992) |
| E. utahensis    | NA              | NA       | NA                  | NA           | 15.0 ± 2.4           | NA           | NA   | USA       | (continued on next page) |
Based on phylogenetic analyses, *Eumonospora* spp. formed a well-supported monophyletic group, including a taxon of *E. neofalconis* (type A: KT037081 and type B: MN629229) found in Falconiformes, except type C: MN629230, and a clade consisting of *Eumonospora* spp. found in other avian species. Unfortunately, detailed information on type C is unavailable. Nevertheless, results of molecular analyses performed on 18S sequences showed that it was 98.59% and 98.87% identical to types A and B, respectively. Moreover, it showed high identical rates of over 99% to *E. dacelo* and *E. henryae*, suggesting that such taxonomic identification was highly questionable. This branching pattern of the parasite phylogenetic tree is highly congruous with that of the phylogram of core land birds (Fig. 3). Telluravus, a recently defined controversial clade of birds, including Astrapalae (Falconfornes, Psittaciformes and Passeriformes) and Afroaves comprising Accipitriformes, Cathariformes, Strigiformes and Coraciformes (McClure et al., 2019; Prum et al., 2015). The molecular analyses of two separate *Eumonospora* taxa, from which type C was excluded due to the reason mentioned above, showed great similarity to the Australalves clade (*E. neofalconis*) and the Afroaves clade (*E. dacelo* and *E. henryae*). Whereas the Australalves clade is comprised of only Falconiformes parasites, the Afroaves clade includes those of Falconiformes, Strigiformes, and Coraciformes. Moreover, molecular identification of *E. henryae*, found in various avian hosts which cross order boundaries, indicated host switching, raising the possibility of the low host specificity of this coccidia, which is similar to those conditions observed in avian *Plasmodium* and some *Cryptosporidium parvum* strains (Morgan et al., 1999; Bensch et al., 2000). Since *E. henryae* exhibits a broad host spectrum, identifying this species by morphological characteristics and host information alone is not sufficiently persuasive. Molecular characterization of this confusing genus should be mandatory for purposes of taxonomic identification. In addition, characterization of 18S, 28S, and cox1 sequences of *Eumonospora* species would help resolving the taxonomic status of this genus. On the other hand, successive detection of *E. henryae* from imported avian species in Japan indicates the possibility of this pathogen emerging in Asia and therefore its biological effects on domestic animal populations requires careful investigation.

Although the genus *Eumonospora* was reinstated recently, it was synonymous with the genus Caryospora Léger, 1904 (Apicomplexa: Eimeriidae), which was considered for decades as the third largest genus in the family Eimeriidae, according to monosporocytic and octasporocytic oocyst morphology (Chou et al., 2020). Indeed, molecular analyses of *Caryospora* sp. in the magpie-lark, *Grallina cyanoleuca* (Latham, 1801) (Aves: Passeriformes), possessing Stieda bodies in their sporocysts exhibits a broad host specificity. This branching pattern of the parasite phylogenetic tree is highly congruous with that of the phylogram of core land birds (Fig. 3). Telluravus, a recently defined controversial clade of birds, including Astrapalae (Falconfornes, Psittaciformes and Passeriformes) and Afroaves comprising Accipitriformes, Cathariformes, Strigiformes and Coraciformes (McClure et al., 2019; Prum et al., 2015). The molecular analyses of two separate *Eumonospora* taxa, from which type C was excluded due to the reason mentioned above, showed great similarity to the Australalves clade (*E. neofalconis*) and the Afroaves clade (*E. dacelo* and *E. henryae*). Whereas the Australalves clade is comprised of only Falconiformes parasites, the Afroaves clade includes those of Falconiformes, Strigiformes, and Coraciformes. Moreover, molecular identification of *E. henryae*, found in various avian hosts which cross order boundaries, indicated host switching, raising the possibility of the low host specificity of this coccidia, which is similar to those conditions observed in avian *Plasmodium* and some *Cryptosporidium parvum* strains (Morgan et al., 1999; Bensch et al., 2000). Since *E. henryae* exhibits a broad host spectrum, identifying this species by morphological characteristics and host information alone is not sufficiently persuasive. Molecular characterization of this confusing genus should be mandatory for purposes of taxonomic identification. In addition, characterization of 18S, 28S, and cox1 sequences of *Eumonospora* species would help resolving the taxonomic status of this genus. On the other hand, successive detection of *E. henryae* from imported avian species in Japan indicates the possibility of this pathogen emerging in Asia and therefore its biological effects on domestic animal populations requires careful investigation.

Table 1 (continued)

| Species       | Oocyst Size (μm) | Oocyst L/W | Sporocyst Size (μm) | Sporocyst L/W | Sporozoite Size (μm) | Sporozoite L/W | Host | Locality | Ref                        |
|---------------|-----------------|------------|---------------------|---------------|----------------------|---------------|------|----------|---------------------------|
| Eumonospora sp. | 44.5–49.5 × 39.6 | NA         | NA                  | NA            | NA                   | NA            | Buteo jamaicensis          | Greece | Papazahariadou (2001)     |

Fig. 1. Optical (A, B) and differential interference contrast photomicrographs (C, D) of oocysts and sporocysts of *Eumonospora* sp. detected from *Falco columbarius*. Fig. 1A. Sporulated oocyst with stout sporozoites (SZ) inside a sporocyst (SP). Fig. 1B. A collapsed oocyst with a compact sporocyst residuum (SR) within an SP. Fig. 1C. Randomly diffused SR within an SP. Fig. 1D. Eight SZs with diffused SR. Scale bars = 10 μm.
Genus *Cystoisospora* Frenkel, 1977 (Apicomplexa: Sarcocystidae) shared a similar history of taxonomic confusion with the genus *Eumonomospora*. Traditionally, the family Sarcocystidae Poche, 1913 is divided into two subfamilies, Sarcocystinae Poche, 1913 and Toxoplasmatinae Biocca, 1956, that are differentiated from other coccidia based on their heteroxenous life cycle, oocyst morphology (disporocystic and tetrasporozoic), and the ability to form tissue cysts in intermediate hosts (Frenkel, 1977). Meanwhile, the genus *Cystoisospora* and subfamily *Cystoisosporinae* Frenkel et al., 1979 were proposed within the family Sarcocystidae for mammalian *Isospora* spp., which form monozoic cysts in lymphoid and other tissues of intermediate or paratenic hosts (Frenkel, 1977; Frenkel et al., 1979). Nevertheless, the genus *Cystoisospora* was not widely accepted and was frequently synonymized with the genus *Isospora* Schneider, 1881 (Mugridge et al., 2000), until Barta et al. (2005) concluded, with considerable molecular evidence, that transferring those disporocystic and tetrasporozoic oocysts without Stieda bodies infecting mammals to genus *Cystoisospora* following which, this genus and subfamily were finally accepted. Since the characteristic of monozoic cysts was considered as a sufficient proof for creating subfamily *Cystoisosporinae* (Frenkel et al., 1979), the unique monosporocystic and octasporozoic oocyst morphology of the genus *Eumonomospora* is suggested as satisfactory for the creation of Eumonomosporinae n. subfam., which can be obviously differentiated from subfamilies Sarcocystinae, Toxoplasmatinae, and Cystoisosporinae in the family Sarcocystidae. Although cyst-forming ability, cyst location with morphological description and life cycle pattern of this genus are needed for further confirmation, highly-supported monophyletic topology by various molecular analyses is of taxonomic significance. Moreover,
Fig. 3. Phylograms of the genus Eumonaspora on the left and core land birds modified from McClure et al. (2019) on the right. The boxes under Eumonaspora spp. represent detected host species and the shaded boxes encompass the Afroaves. The lines connect parasites and hosts encountered, with the dotted line indicating host switching across order boundaries.

defining the family Sarcocystidae based on criteria such as having oocysts with two sporocysts, each with four sporozoites, should be modified.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property. We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript. We understand that the Corresponding Author is the sole contact email address which is accessible by the Corresponding Author and that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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Appendix A. Supplementary data

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References

Alfaleh, F.A., Alyousif, M.S., Al-Shawa, Y.R., Al-Quraishy, S., 2013. Caryosporida sternungi sp. n. (Apicomplexa: Eimeriidae) infecting Falco cherrug in Saudi Arabia. Parasitol. Res. 112, 971–974. https://doi.org/10.1007/s00436-012-3217-y.
Allen, E.A., 1933. Caryosporida sternungi gen. et sp. nov., a coccidium from the intestine of the Turkey buzzard, Cathartes aura septentrionalis Wied. Trans. Am. Microsc. Soc. 52, 192–194. https://doi.org/10.2307/3222553.
Alyousif, M.S., Alfaleh, F.A., Al-Shawa, Y.R., 2011. Caryosporida sternungi sp. n. (Apicomplexa: Eimeriidae) infecting falcon from the genus Falco in Saudi Arabia. J. Egypt. Soc. Parasitol.
Barta, J.R., Schrenzel, M.D., Carreno, R., Rideout, B.A., 2014. Studies on coccidian oocysts (Apicomplexa: Eimeriidae) and Eimeria bubonis in old world. Rev. Bras. Parasitol. Vet. 25, 206–202. https://doi.org/10.1590/s1984-296120160300.
Bartsch, S., Stérmann, M., Hasselquist, D., Ostman, O., Hansson, B., Westerdahl, H., Pinheiro, R.T., 2000. Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. Proc. Biol. Sci. 267, 1583–1589. https://doi.org/10.1098/rspb.2000.1181.
Berto, B.P., McIntosh, D., Lopes, C.W.G., 2014. Studies on coccidian oocysts (Apicomplexa: Eucoccidiidae). Rev. Bras. Parasitol. Vet. 23, 1–15. https://doi.org/10.1590/1984-296120140011.
Boer, R., 1982. Untersuchungen über das Vorkommen von Kokzidien bei Greifvögeln und über die Entwicklung von zwei Caryospora-Arten der Falken (Caryospora neofalcons n. sp. and Caryospora kuzerti n. sp.). Dissertation, Tierärztliche Hochschule Hannover.
Cardozo, S.V., Berto, B.P., Caetano, I., Maniero, V.C., Fonseca, I.P., Lopes, C.W.G., 2016. Caryospora tenera sp. n. (Apicomplexa: Eimeriidae) in the common kestrel, Falco tinnunculus (Falconiformes: Falconidae), in mainland Portugal. Rev. Bras. Parasitol. Vet. 25, 202–206. https://doi.org/10.1590/s1984-296120160300.
Cardozo, S.V., Berto, B.P., Caetano, I., Maniero, V.C., Santos, M., Da Fonseca, I.P., Lopes, C.W.G., 2017. Aviparas cherrugsp. n. sp. (Apicomplexa: Caryosporidae) in the little owl, Athene noctua (Strigiformes: Strigidae), in mainland Portugal. Rev. Bras. Parasitol. Vet. 26, 348–351. https://doi.org/10.1590/s1984-296120170533.
Cardozo, S.V., Berto, B.P., Caetano, I., Thomas, A., Santos, M., Da Fonseca, I.P., Lopes, C.W.G., 2019. Caryosporids parasites from birds at rehabilitation centers in Portugal, with notes on Aviparas bubonis in old world. Rev. Bras. Parasitol. Vet. 28, 187–193. https://doi.org/10.1590/s1984-296120190232.
Cawthorn, R.J., Stockdale, P.H.G., 1981. Description of Eimeria bubonis sp.n. (Protozoa: Eimeriidae) and Caryosporida bubonis sp.n. (Protozoa: Eimeriidae) in the great horned owl, Bubo virginianus (Gmelin), of Saskatchewan. Can. J. Zool. 59, 170–173. https://doi.org/10.1139/z81-030.
Cawthorn, R.J., Stockdale, P.H.G., 1982. The developmental cycle of Caryospora bubonis Cawthorn and Stockdale 1981 (Protozoa: Eimeriidae) in the great horned owl, Bubo virginianus (Gmelin). Can. J. Zool. 60, 152–157. https://doi.org/10.1139/z82-019.
Chou, S., Tokiwa, T., Hadano, S., Izawa, M., Kojima, A., Ike, K., 2020. Resurrection of the genus Eumonaspora (Apicomplexa: Sarcocystidae) for Icke sp. (Protozoa: Sarcocystidae) in the Turkey buzzard, Buteo buteo, in Saudi Arabia. Parasitol. Int. 77 https://doi.org/10.1016/j.parint.2019.09.006.
Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and high-performance computing. Nat. Methods 9, 772. https://doi.org/10.1038/nmeth.2108.
Forbes, N.A., Simpson, G.N., 1997. Caryospora neofalcons: an emerging threat to captive-bred raptors in the United Kingdom. J. Avian Med. Surg. 11, 110–114. https://doi.org/10.2307/30134522.
Lindsay, D.S., Blagburn, B.L., 1986. Caryospora – Like isolate (Apicomplexa: Eimeriidae) from the magpie-lark (Grallina cyanoleuca) – an avian coccidian. Can. J. Zool. 68, 1256–1260.

McAllister, C.T., Duszynski, D.W., McKown, R.D., 2013a. A new species of Caryospora from the laughing kookaburra (Dacelo novaeguineae). Exp. Parasitol. 135, 334–339.

McAllister, C.T., Duszynski, D.W., McKown, R.D., 2013b. A new species of Caryospora (Apicomplexa: Eimeriidae) from the bald eagle, Haliaeetus leucocephalus (Accipitriformes). J. Parasitol. 99, 490–492. https://doi.org/10.1645/GE-3236.1.

McClure, C.J.W., Schulwitz, S.E., Anderson, D.L., Robinson, B.W., Mojica, E.K., 2000. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321. https://doi.org/10.1080/00948346.2006.10498365.

McKelvey, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic relationships among the avialae. Mol. Biol. Evol. 18, 1842–1853. https://doi.org/10.1093/oxfordjournals.molbev.a025685.

Mogg, S.J., Current, W.L., Barnard, S.M., 1986. A review of the genus Caryospora (Apicomplexa). J. Parasitol. 72, 762–765. https://doi.org/10.2307/3281470.

Mogab, H., 1994. Untersuchungen zur Therapie und Prophylaxe der Infektionen der Falken (Falconiformes: Falconidae) mit Tolttrazuril sowie die Bewertung von zwei neuen Caryospora-Arten (Caryospora neofalconis und Caryospora arcayae). Dissertation, Tierarztliche Hochschule Hannover.

Molnar, P., Morgan, P.T., Monis, R., Moreira, C., 1999. Phylogenetic relationships among isolates of Cryptosporidium: evidence for several new species. J. Parasitol. 85, 1126–1133. https://doi.org/10.2307/3285679.

Mugridge, N.B., Morrison, D.A., Jakes, I., Heckeroth, A.R., Tenter, A.M., Johnson, A.M., 2000. Effects of sequence alignment and structural domains of ribosomal DNA on phylogeny reconstruction for the protozoan family Sarcocystidae. Mol. Biol. Evol. 17, 1842–1853. https://doi.org/10.1093/oxfordjournals.molbev.a025685.

Mogab, H., 2000. Untersuchungen zur Therapie und Prophylaxe der Infektionen der Falken (Falconiformes: Falconidae) mit Tolttrazuril sowie die Bewertung von zwei neuen Caryospora-Arten (Caryospora neofalconis und Caryospora arcayae). Dissertation, Tierarztliche Hochschule Hannover.

Morgan, U.M., Monis, P.T., Fayer, R., Deplazes, P., Thompson, R.C.A., 1999. Phylogenetic relationships among isolates of Cryptosporidium: evidence for several new species. J. Parasitol. 85, 1126–1133. https://doi.org/10.2307/3285679.