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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* (Bö;er, 1982) and *E. kutzeri* (Bö;er, 1982) infections, which produce clinical signs such as regurgitation, hemorrhagic diarrhea, anorexia, and acute death without symptoms, have been reported in Falconiformes (Bö;er, 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; Bö;er, 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., 1990; Wetzel and Enigk, 1939). Nevertheless, detection of *E. megafalconis* (Klü;h, 1994) in bustards (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, were 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 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 ($K_2Cr_2O_7$) at room temperature for sporulation.

2.2. Morphological analysis

Most oocysts in the 2.5% $K_2Cr_2O_7$ solution sporulated by day 4, and observed under a BX41microscope (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% $K_2Cr_2O_7$ 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. DNA was stored at 4 °C for immediate use or −20 °C for storage. The following primer pairs were used for PCR amplification of: the nuclear small subunit ribosomal DNA (18S) sequence, primers: CRYPTOF, 5′-AACCTGGTTGATCCTGCCAGT-3′ and CRYPTOR, 5′-GCTTGATCCTTCTGCAGGTTCACCTAC-3' (Herwaldt et al., 2003); the nuclear large subunit ribosomal DNA (28S) sequence, primers: KL1, 5′-TACCCGCTGAACTTAAGC -3′ and KL3, 5′- CMACCAAGATCTGCACTAG -3' (Schrenzel et al., 2005); and the mitochondrial cytochrome C oxidase subunit 1 (*cox1*) sequence, primers: Sdae_COX1_260F, 5′-GATCTTTATGTTYTTRATGCC-3′ and Sdae_COX1_1147R, 5′-CAT-TACCCATAACYACACC-3' (Ogedengbe et al., 2016).

PCR was performed in 20 μL volume containing 2 μL of 10 × 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, 28S: 35, *cox1*: 40) 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 under an LED transilluminator and stained with GRGreen loading buffer 6 × (Bio Craft, Japan), following which product size was estimated by comparing with 100-bp plus DNA ladder (Maestrogen, 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, a 90% majority rule consensus sequence was generated. While a 50% strict consensus sequence was generated 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. daceloe*) was excluded from 28S analyses due to a 948-bp long sequence being 100% identical to that of KF766053 (*Isospora anthochaerae*), 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 (Huelsenbeck and Ronquist, 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 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 × 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 (n = 30)

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 (n = 30)

Sporocyst shape: spherical to sub-spherical; 23.0 ± 1.8 × 22.6 ± 1.8 (20.3–28.4 × 20.0–27.9); lengh 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), *Pulsatrix perspicillata* (Latham, 1790), *Strix nebulosi* Foster, 1772

Prepatent and patent time: Unkown

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% $K_2Cr_2O_4$ solution at room temperature.

3.4. Genetic analysis–18S, 28S and cox1

A 1672-bp fragment of 18S rRNA gene, a 1427-bp fragment of 28S rRNA gene, and a 865-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-analyzation 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. heraye*. 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 *Hyaloklossia lieberkuehni* (Labbé, 1896) branched earliest in the 28S dataset and *Nephroisospora eptesici* 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. eptesici* (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. eptesici* 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; Bö;er, 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, Pavlík et al. (1998) identified *E. neofalconis* 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 Yakimoff and Matschulsky (1936). However, species with oblong oocysts found in falcons was interpreted by Bö; er (1982) as being *E. kutzeri,* 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		Sporocyst		Sporozoite		Host	Locality	Ref
	Size (μm)	L/W	Size (μm)	L/W	Size (μm)	L/W			
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 platypterus*	Venezuela	Volcán and Medrano (1986)
	32.1 × 28.3 (29.0–36.0 × 26.4–30.4)	1.13 (1.06–1.22)	20.0 (18.4–21.0)	1	12.0 × 5.1 (10.4–13.6 × 4.8–5.6)	NA	*Buteo magnirostris*	USA	Upton et al., 1990
E. argentati	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. biarmicusis	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 biarmicus*	Saudi Arabia	Alyousif 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	Klüh (1994)
	38.5 × 31.0 (38.0–39.0 × 30.0–32.0)	NA	24.5 × 20.5 (24.0–25.0 × 20.0–21.0)	NA	19.0 × 3.5 (18.0–20.0 × 3.0–4.0)	NA	*Falco tinnunculus*	UAE	Mateuta and Samour (2017)
E. bubonis	43.9 × 40.2 (38.0–52.0 × 33.0–47.0)	1.1 (1.0–1.3)	26.6 × 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	Cawthorn and Stockdale (1981)
	45.4 × 37.4 (42.0–49.0 × 34.0–40.0)	1.2 (1.1–1.4)	25.1 × 24.2 (24.0–27.0 × 23.0–25.0)	1.04 (1.0–1.1)	NA	NA	*Bubo bubo*	Portugal	Cardozo et al. (2019)
E. cherrughi	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)	NA	15.0 × 4.0 (14.0–17.0 × 3.0–6.0)	NA	*Falco cherrug*	Saudi Arabia	Alfaleh et al. (2013)
	33.7 × 28.0 (29.0–35.0 × 23.0–32.0)	NA	22.6 × 18.9 (20.0–25.0 × 15.0–22.0)	NA	15.0 × 4.0 (12.0–20.0 × 3.0–5.0)	NA	*Falco cherrug*	UAE	Mateuta and Samour (2017)
E. circi	24.5 × 21.8 (23.0–25.0 × 21.0–24.0)	1.12 (1.04–1.19)	16.2 × 15.6 (15.0–17.0 × 15.0–17.0)	1.04 (1.00–1.07)	10.4 × 4.3 (9.0–11.0 × 4.0–5.0)	NA	*Circus aeruginousus*	Czech Republic	Volf et al. (2000)
E. daceloe	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	21.0–23.0	NA	13.0–14.0	NA	*Falco peregrinus* *Falco tinnunculu* *Falco subbuteo*	Europe	Wetzel and Enigk (1937); Upton et al. (1990)
	32.4 × 29.8 (29.0–36.0 × 23.0–35.0)	NA	21.7 × 20.6 (15.0–25.0 × 14.0–25.0)	NA	13.2 × 4.6 (10.0–17.0 × 2.0–4.0)	NA	*Falco peregrinus*	UAE	Mateuta and Samour (2017)
E. hanebrinki	48.1 × 42.1 (42.0–54.0 × 37.0–50.0)	1.2 (1.0–1.4)	24.8 (23–28)	1	18.6 × 5.6 (16.0–20.0 × 4.0–6.0)	NA	*Haliaeetus leucocephalus*	USA	McAllister et al. (2013a)
E. henryae	41.0 × 34.5 (36.6–43.5 × 31.0–37.1)	1.16 (1.08–1.28)	23.0 × 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
	37.0–42.0 × 30.0–35.0	NA	20.0 × 20.0	NA	NA	NA	*Bubo bubo*	Russia	Yakimoff and Matschulsky (1932)
	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	*Bubo bubo*	Russia	Yakimoff and Matschulsky (1936)
	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	*Athene noctua*	Japan	Chou et al. (2020)
	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	*Bubo scandiacus*	Japan	Chou et al. (2020)
	42.6 × 35.7 (40.0–46.0 × 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	*Ptilosis leucotis*	Japan	Chou et al. (2020)
	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)
	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	*Strix nebulosa*	Japan	Chou et al. (2020)

(*continued on next page*)

Table 1 (*continued*)

Species	Oocyst		Sporocyst		Sporozoite		Host	Locality	Ref
	Size (μm)	L/W	Size (μm)	L/W	Size (μm)	L/W			
E. kansasensis	37.2 × 32.6 (32.8–40.0 × 29.6–36.0)	1.14 (1.07–1.24)	22.5 (20.8–24.8)	1	14.4 × 5.4 (13.6–15.2 × 5.0–6.0)	NA	*Buteo sqainsonii*	USA	Upton et al. (1990)
E. kutzeri	38.7 × 34.1 (34.1–44.7 × 29.6–37.9)	NA	24.6 × 21.0 (21.4–27.1 × 19.2–23.5)	NA	NA	NA	*Falco mexicanus* *Falco tinnunculus* *Falco biarmicus* *Falco jugger* *Falco cherrug* *Falco rusticolus* *Falco peregrinus*	Europe	Böer (1982)
	37.58 × 32.54 (32.5–43.13 × 30.0–35.0)	1.15	24.17 × 21.96 (18.75–28.75 × 18.75–28.75	1.1	NA	NA	*Falco tinnunculus*	Germany	Krone (2002)
	39.1 × 33.1 (33.0–46.0 × 28.0–40.0)	NA	22.5 × 21.3 (18.0–26.0 × 16.0–26.0)	NA	4.6 (2.0–7.0 × 2.0–7.0)	NA	*Falco rusticolus* *Falco peregrinus* *Flaco cherrug* *Falco tinnunculus*	UAE	Mateuta and Samour (2017)
E. lindsayi	33.7 × 31.6 (31.2–36.0 × 30.4–32.8)	1.07 (1.02–1.13)	20.5(19.2–22.0)	1	15.5 × 4.8 (14.4–16.8 × 4.4–5.0)	NA	*Buteo jamaicensis*	USA	Upton et al. (1990)
E. megafalconis	43.6 × 35.8 (39.4–48.3 × 32.1–41.0)	1.12 (1.12–1.35)	22.4–24.2	NA	18.6 × 4.6 (17.0–20.0 × 4.0–6.0)	NA	*Falco tinnunculus* *Falco rusticolus* *Falco cherrug*	Europe	Klüh (1994)
	42.25–43.77 × 35.36–37.14	1.19–1.23	22.7–23.93	NA	13.0–15.0 × 5.0–6.0	NA	*Chlamydotis macqueenii* *Chlamydotis undulata* *Falco rusticolus*	UAE	Schuster et al. (2016)
	42.2 × 35.9 (39.0–49.0 × 30.0–40.0)	NA	23.6 × 22.7 (21.0–27.0 × 20.0–26.0)	NA	16.5 × 4.2 (11.0–20.0 × 3.0–6.0)	NA	*Falco rusticolus* *Falco peregrinus* *Falco cherrug*	UAE	Mateuta and Samour (2017)
E. mochogalegoi	38.9 × 32.9 (37.0–43.0 × 31.0–37.0)	1.18 (1.15–1.23)	21.1 × 20.1 (20.0–24.0 × 19.0–23.0)	1.02 (1.00–1.07)	16.6 × 4.7 (15.0–18.0 × 4.0–5.0)	NA	*Athene nocutua*	Portugal	Cardozo et al. (2017)
E. neofalconis	27.0 × 23.8 (23.0–32.4 × 20.4–29.4)	NA	18.8 × 14.8 (16.5–21.8 × 13.7–16.1)	NA	NA	NA	*Falco mexicanus* *Falco subbuteo* *Falco biarmicus* *Falco peregrinus*	Europe	Böer (1982)
	26.3 × 23.9	NA	17.9 × 14.8	NA	NA	NA	*Falco peregrinus*	Mexico	Santana-Sánchez et al. (2015)
	25.6 × 22.5 (20.0–30.0 × 19.0–28.0)	NA	17.2 × 15.2 (13.0–19.0 × 12.0–19.0)	NA	9.9 × 2.4 (8.0–12.0 × 2.0–4.0)	NA	*Falco rusticolus* *Falco p. peregrinator* *Falco cherrug* *Falco sparverius*	UAE	Mateuta and Samour (2017)
E. penerireiroi	47.1 × 37.6 (42.0–49.0 × 34.0–40.0)	1.25 (1.2–1.4)	25.1 × 24.3 (24.0–27.0 × 24.0–25.0)	1.03 (1.0–1.1)	17.0 × 4.2 (15.0–18.0 × 3.0–5.0)		*Falco tinnunculus*	Portugal	Cardozo et al. (2016)
E. petersoni	43.1 × 39.8 (38.0–46.0 × 37.0–42.0)	1.08 (1.0–1.1)	23.4 × 23.3 (21.0–26.0 × 21.0–26.0)	1.05 (1.0–1.1)	15.6 × 4.2 (15.0–16.0 × 4.0–5.0)	NA	*Accipiter striatus*	USA	McAllister et al. (2013b)
E. strigis	13.8 × 10.9 (11.9–15.0 × 10.3–12.5)	NA	NA	NA	7.5 × 3.4	NA	*Tyto alba*	Europe	Gottschalk (1972); Upton et al. (1990)
E. tremula	33.0–35.0 × 28.0–30.0	NA	23.5–25.0	NA	20.0 × 6.0	NA	*Cathartes aura*	USA	Allen (1933)
	33.4 × 28.0 (30.0–38.0 × 25.0–32.0)	1.2 (1.07–1.52)	20.4 × 20.1 (18.6–21.6 × 18.6–21.6)	1.01 (1.00–1.05)	16.3 × 5.2 (15.2–18.4 × 4.8–5.6)	NA	*Cathartes aura*	USA	Lindsay et al. (1994)
E. undata	30.6 × 29.3 (26.6–33.3 × 24.0–32.0)	NA	22.0 × 21.0	NA	NA	NA	*Larus argentatus*	Germany	Schwalbach (1959)
	32.5 × 29.2 (27.0–42.0 × 24.0–35.0)	NA	NA	NA	NA	NA	*Uria aalge aalge*	Netherlands	Poelma and Strik (1966)
	30.5 × 27.6 (29.6–36.0 × 24.0–31.2)	1.1 (1.1–1.3)	20.3 (19.2–23.2)	NA	15.0 × 2.4 (13.6–16.8 × 1.8–3.2)	NA	*Lunda cirrhata*	USA	Upton et al. (1992)
E. uptoni						NA		USA	

(*continued on next page*)

Table 1 (*continued*)

Species	Oocyst		Sporocyst		Sporozoite		Host	Locality	Ref
	Size (μm)	L/W	Size (μm)	L/W	Size (μm)	L/W			
	28.1 × 26.4 (25.5–31.5 × 24.0–28.5)	1.07 (1.0–1.18)	18.2 × 17.9 (18.0–19.5 × 16.5–18.0)	1.01 (1.00–1.09)	12.6 × 4.2 (10.5–15.0 × 3.0–4.5)		*Buteo jamaicensis*		Lindasy and Blagburn (1986)
Eumonospora sp.	44.5–49.5 × 39.6	NA	NA	NA	NA	NA	*Bubo scandiaca*	Greece	Papazahariadou et al. (2001)

NA: Not available.

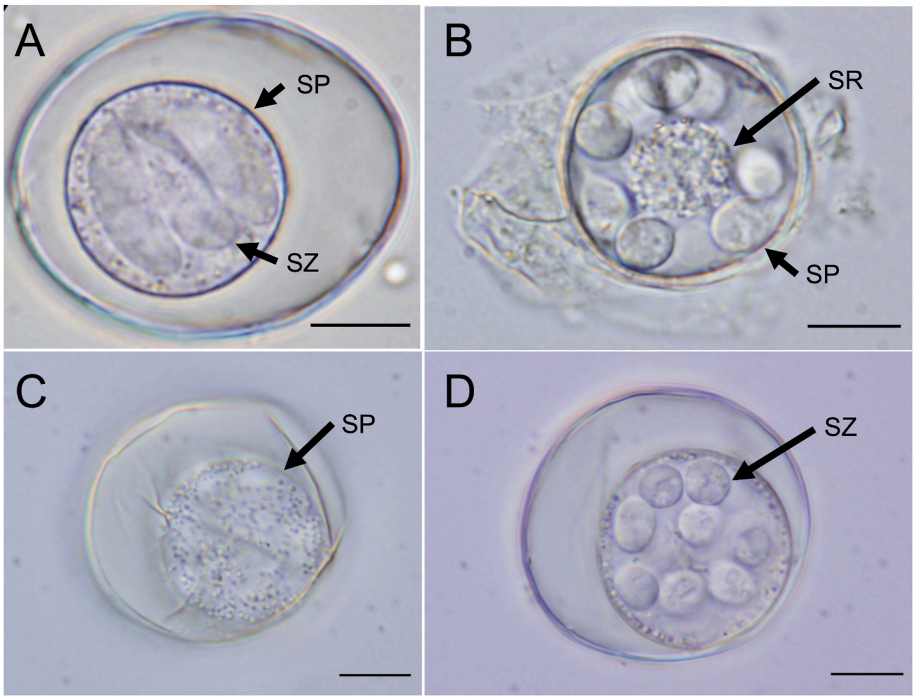

Fig. 1. Optical (A, B) and differential interference contrast photomicrographs (C, D) of oocysts and sporocysts of *Eumonospora* sp. detected from *Falco columbarius*. Fig. 1A. Sporulated oocyst with stout sporozoites (SZ) inside a sporocyst (SP). Fig. 1B. A collapsed oocyst with a compact sporocyst residuum (SR) within an SP. Fig. 1C. Randomly diffused SR within an SP. Fig. 1D. Eight SZs with diffused SR. Scale bars = 10 μm.

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. daceloe* 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), Telluraves, a recently defined controversial clade of birds, including Australaves (Falconiformes, Psittaciformes and Passeriformes) and Afroaves comprising Accipitriformes, Catharitiformes, Strigiformes and Coraciimorphae (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 Australaves clade (*E. neofalconis*) and the Afroaves clade (*E. daceloe* and *E. henryae*). Whereas the Australaves clade is comprised of only Falconiformes parasites, the Afroaves clade includes those of Falconiformes, Strigiformes, and Coraciiformes. 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 monosporocystic and octasporozoic 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 clustered in Eimeriidae more than in Sarcocystidae (Liu et al., 2020). Meanwhile, various *Eumonospora* spp. without Stieda bodies clustered in Sarcocystidae more than in Eimeriidae, revealing through sharing, the close phylogenetic relationships between hosts (Falconiformes and Passeriformes) and similar oocyst morphologies (monosporocystic and octasporozoic), in addition to indicating that *Caryospora* and *Eumonospora* are two completely taxonomically separated genera.

Fig. 2. Phylogenetic trees based on three concatenated datasets (A: 18S + *cox1*, B: 18S + 28S, and C: 28S + *cox1*). Phylogenetic analyses are performed via Bayesian inference (BI) and maximum likelihood (ML) methods. Nodes are labelled with probability for BI method node support (left) and bootstrap value support for the ML method (right). Similar phylograms are illustrated with both methods in all datasets. Monophyletic clade of *Eumonospora* spp. branches off earlier than the clade of *Besnoitia* spp. and the clade comprising genera *Hammondia*, *Heydornia*, *Neospora*, and *Toxoplasma*. 18S: nuclear small subunit ribosomal DNA; 28S: nuclear large subunit ribosomal DNA; *cox1*: mitochondrial Cytochrome C oxidase subunit 1; NA: not available.

Genus *Cystoisospora* Frenkel, 1977 (Apicomplexa: Sarcocystidae) shared a similar history of taxonomic confusion with the genus *Eumonospora*. 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 *Eumonospora* is suggested assatisfactory for the creation of Eumonosporinae 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,

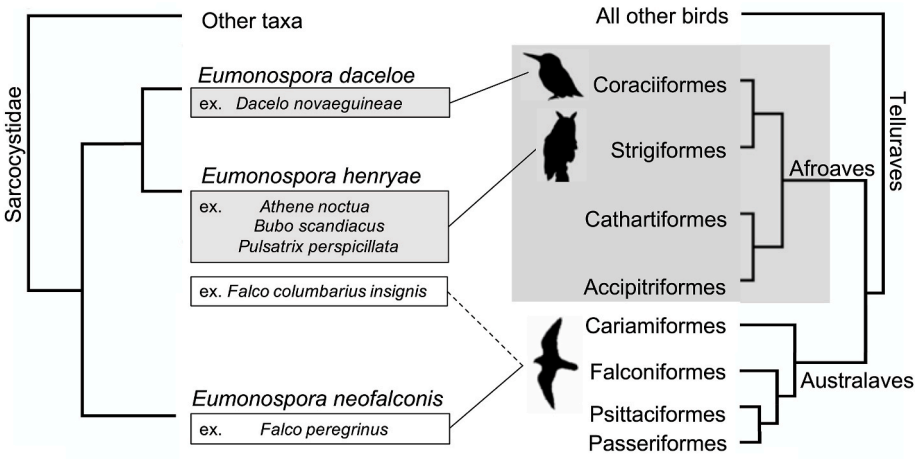

Fig. 3. Phylograms of the genus *Eumonospora* on the left and core land birds modified from McClure et al. (2019) on the right. The boxes under *Eumonospora* spp. represent detected host species and the shaded boxes encompass the Afroaves. The lines connect parasites and hosts encountered, with the dotted 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 for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from (tokiwa@nvlu.ac.jp) Signed by all authors as follows: Shyun Chou, Nobumoto Izawa, Kazunori Ike, and Toshihiro Tokiwa (Oct 14, 2020).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.01.002.

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