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Detection and molecular status of Isospora sp. from the domestic pigeon (Columba livia domestica).

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Detection and molecular status of *Isospora* sp. from the domestic pigeon (*Columba livia domestica*)

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Abstract

The domestic pigeon, *Columba livia domestica*, is reared for meat production, as a pet, or for racing. Few reports have characterized the parasitic protists from the genus *Isospora* isolated from Columbiformes. We detected *Isospora*-like oocysts from *C. livia* reared for racing. The oocyst contained two sporocysts, and each sporocyst included four sporozoites. The sporulated oocysts (n=4) were spherical; their mean diameters were 25.6 (24.0–27.2) × 24.7 (23.4–26.0) µm. Micropyles, polar granules, and oocyst residuum were absent. The mean length and width of the sporocysts (n=8) were 19.5 (18.5–20.5) and 11.2 (10.2–12.1) µm, respectively. Stieda and sub-Stieda bodies were observed. Single-oocyst PCR revealed two different 18S rRNA gene sequences and one 28S rRNA gene sequence in a single oocyst of *Isospora* sp. Based on a phylogenetic analysis of the 18S rRNA gene, the two sequences made a group which fell within a cluster of known avian *Isospora* species. A tree based on the 28S rRNA gene sequence indicated that sequences from the pigeon *Isospora* sp. fell within a cluster of avian *Isospora* species. Both trees failed to clarify the phylogenetic relationships among the avian

Isospora species due to limited resolution. Because the morphological description of *Isospora* sp. is based on only four oocysts, *Isospora* sp. is not proposed as a novel species here. This is the first description of *Isospora* sp. isolated from the domestic pigeon *C. livia*.

Keywords:

Isospora sp., *Columba livia domestica*, domestic pigeon, coccidia, *Atoxoplasma columbae*

The genus *Isospora* (Coccidia: Eimeriidae) comprises parasitic protists belonging to the phylum Apicomplexa and is distributed worldwide. Coccidian parasites are highly host specific and taxonomic studies have relied mostly on host specificity [1]. The domestic pigeon, *Columba livia domestica*, is reared for meat production, as pets, and for racing. Coccidian parasites are important pathogens in pigeon rearing because the parasites cause intestinal disease, such as diarrhea, and are sometimes lethal [2]. Two *Eimeria* species have been characterized as causative agents of coccidiosis in pigeons, while no *Isospora* species have been isolated from *C. livia* [3]. Recently, we found *Isospora*-like oocysts detected in the feces of *C. livia*. In this report, we describe morphological characteristics and perform a phylogenetic analysis of the *Isospora* sp. from *C. livia* based on the single-oocyst method.

Unsporulated coccidian oocysts were obtained from droppings gathered from 16 domestic pigeons reared in Tokyo, Japan, and kept at 4°C within a humid tube. To identify these oocysts, we performed sporulation by referring to a standard technique for avian coccidia [4] equipping some modifications. In brief, the feces

were placed in 2.5% (w/v) potassium dichromate solution ($K_2Cr_2O_7$) and incubated at 25°C for 5 days under aerobic conditions. The material was then passed through a fine mesh and the oocysts were purified by sucrose gradient centrifugation [5] and identified. In a result, *Eimeria* oocysts were dominant and oocysts of *Isospora* sp. were hardly detectable. We identified the *Eimeria* oocysts as *E. labbeana* [6] and *E. columbarum* [7] based on morphological features. Despite *Isospora* sp. was less frequent in the population, we could identify these based on the size, and the number of sporocysts per oocyst. The purified oocysts were stored at 4°C in phosphate-buffered saline until examination.

Sporulated oocysts were suspended in Milli-Q purified water (Millipore, Billerica, MA, USA). Diluted drops containing oocysts were placed on plastic dishes. Each *Isospora* oocyst was isolated from the drops under an inverted microscope (TMD300; Nikon, Tokyo, Japan) using a micropipette or glass capillary. To eliminate additional oocysts as contamination picked up oocysts were passed through several water drops, and we checked contamination at each step by microscopic observation. The isolated oocysts were individually placed onto a cover

glass and verified by thorough observation, and were then photographed using a differential interference contrast inverted microscope (TMD300) with a ×40 objective lens. In total, four oocysts were observed for the morphological description.

A representative oocyst is shown in Fig. 1. Table 1 summarizes the morphological features of four isolated oocysts and eight isolated sporocysts. The oocysts were spherical, consisted of two sporocysts, and each sporocyst included four sporozoites. The mean oocyst diameters were 25.6 (24.0–27.2) × 24.7 (23.4–26.0) μm, and the length/width (L/W) ratio was 0.99–1.1. The monolayer wall was ~1.5 μm thick. The oocysts were light pink and with a rough surface. The average diameters of the sporocysts were 19.5 (18.5–20.5) × 11.2 (10.2–12.1) μm, respectively, the L/W ratio was 1.6–2.0, and the wall was ~0.5 μm thick. The texture was that of a single, smooth layer. The Stieda body was nipple-like and 1.8 (1.4–2.2) μm in width, the sub-Stieda body was 5.1 (4.6–5.6) × 2.9 (2.6–3.2) μm in width, and the sporocyst residuum consisted of loosely clustered homogeneous granules of various sizes. The sporozoites (n=16) contained two refractile bodies: the smaller at

94 the apical end and the larger at the posterior end. None of the pigeons presented with
95 clinical symptoms in the poultry house; therefore, the *Isospora* sp. would be
96 nonpathogenic or rarely pathogenic. However, to address the pathogenicity, an
97 experimental infection to pathogen-free pigeons will be required.
98 We then reviewed the literature on *Isospora* spp. infecting birds of the genus
99 *Columba* or related genera. *Isospora gallicolumbae* was previously isolated from
100 Beccari's ground dove, *Gallicolumba beccarii*, in Papua New Guinea [8]. We
101 compared the taxonomic information of *I. gallicolumbae* with that of the *Isospora* sp.
102 from domestic pigeons. The reported sizes of oocysts and sporocysts of *I.*
103 *gallicolumbae* are 16.0 × 20.0 µm and 8.0 × 12.0 µm, respectively. These sizes are
104 smaller than those of the *Isospora* sp. detected in this study. Furthermore, sporocysts
105 of *I. gallicolumbae* possess no sub-Stieda bodies, while such structures were clearly
106 observed in the pigeon *Isospora* sp. (Fig. 1). *Gallicolumba beccarii* and *C. livia*
107 belong to different genera in the family Columbidae, suggesting different host ranges
108 of *I. gallicolumbae* and the pigeon *Isospora* sp. due to the strict host specificity of
109 isosporoid species. In addition, we observed that the morphological features of the

pigeon *Isospora* sp. differed in many ways from *I. gallicolumbae*. Thus, the pigeon *Isospora* sp. is morphologically and biologically distinguishable from *I. gallicolumbae*.

After recording the morphological features, each oocyst was subjected to single-oocyst polymerase chain reaction (PCR) according to previously described methods with modifications [9]. Prior to freeze-fracture we carefully observed drops again and verified no additional oocyst existed. Each isolated oocyst was transferred to a PCR tube; the tubes were then subjected to five freeze-thaw cycles to rupture the oocyst wall. To improve the efficiency of PCR amplification, we used a total genomic DNA amplification technique with a GenomiPhi V2 Whole-Genome Amplification Kit (GE Healthcare, Tokyo, Japan). The enriched DNA product was diluted 10–100-fold with Milli-Q water and used as the PCR template. The 18S rRNA gene was amplified with the primers E18SF and E18SR [10], and the 28S rRNA gene was amplified with the forward and reverse primers E28S-4Fw (5'-GACCTGAAATCAGTCGAGGTTAC-3') and E28S-974Rv (5'-CTTGGTCCGTGTTTCAAGACGC-3'), respectively. The primers targeting the

28S rRNA gene (corresponding to positions 4–974 bp of *Eimeria tenella*) were designed manually based on an alignment of the 5' region of the 28S rRNA gene sequences of related species *Eimeria tenella* (AF026388), *E. alabamensis* (AF076861), *Atoxoplasma* sp. (AY283869), *Toxoplasma gondii* (AF076901, L25635), *Plasmodium falciparum* (U48228), and *Cryptosporidium parvum* (AF040725).

We subjected all four oocysts to single-oocyst PCR; however, for both genes PCR amplification was successful for only one same oocyst. The low efficiency of PCR amplification was probably due to the small amount of genomic DNA in a single oocyst. Because direct sequencing was not able to clearly determine the sequence of the 18S rRNA gene, which exhibited heterogeneity, we used the standard TA cloning technique prior to sequencing and obtained two sequences. In contrast, the nucleotide sequence of the 28S rRNA gene could be determined by direct sequencing. The sequences have been deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers AB757860 and AB757862 for the 18S rRNA, and AB757865 for 28S rRNA genes, respectively. Two different 18S rRNA gene

142 sequences were obtained from one oocyst suggesting two alternative possibilities.
143 Coccidian oocyst is formed from diploid zygote. During sporogony, eight haploid
144 sporozoites are produced as the result of meiosis and mitosis. The two individual
145 sequences might be derived from each haploid genome of sporozoites within one
146 oocyst. The other possibility is the heterogeneity of the 18S rRNA genes existing in
147 the haploid genome of *Isospora* sp. It has been suggested that the rapid
148 birth-and-death evolution of the apicomplexan genome generated multiple
149 heterogeneous copies of the 18S rRNA gene in the haploid genome [11]. Ikarashi *et*
150 *al.* (2013) discovered that *Cryptosporidium andersoni* carries two different
151 genotypes heterogeneously in its haploid genome [12].
152 There were other eimerian oocysts in the same pigeon-stool specimen. We
153 also isolated more than 15 eimerian oocysts, performed the single-oocyst PCR and
154 sequencing (Matsubara et al., Unpublished). The 18S rRNA gene sequences obtained
155 from *E. labbeana* showed high homology (99%; 1218–1227 nucleotides were
156 identical among 1229 bp) to the available sequence of *E. labbeana*-like oocyst
157 (KT305927) [13]. These sequences of the coexisting pigeon *Eimeria* were not

identical to the two sequences from *Isospora* sp. This evidently indicated that the *Isospora* was not insufficiently or abnormally sporulated oocyst of *Eimeria* coexisting in the pigeon-stool specimen.

Multiple alignment analyses of the 18S rRNA gene were performed using both Clustal-Omega [14] and MUSCLE [15] in the SeaView 4.6 application [16], and the MUSCLE alignment generated superior results. Gaps and ambiguous positions were edited and eliminated manually. After editing, 1,162 nucleotides of the 18S rRNA gene remained and were subjected to a phylogenetic analysis. A maximum likelihood (ML) tree was constructed using RAxML v8.1.17 [17] with the GTRGAMMAI substitution model (a thorough bootstrap analysis was conducted). In total, 1,000 tree replicates were generated to evaluate bootstrap support. The constructed tree is depicted in Fig. 2A. Pigeon *Isospora* sequences 1 and 3, located beside the *Isospora/Atoxoplasma* clade, formed a monophyletic clade but were not identical to other known species, indicating that the sequences of pigeon *Isospora* are distinct from the others. However, the *Isospora/Atoxoplasma* clade was not strongly supported by bootstrap values. Also, we could not determine the relationships of

operational taxonomic units (OTUs) within the *Isospora/Atoxoplasma* clade due to the low bootstrap support throughout. Nevertheless, *E. labbeana* and pigeon *Isospora* belonging to the different clades again indicates that pigeon *Isospora* was not the insufficiently or abnormally sporulated oocyst of *E. labbeana.*

For the 28S rRNA gene, the sequence from the isolated *Isospora* oocysts (using the same oocyst that provided the 18S rRNA information) did not show heterogeneity. The ML tree based on the 28S rRNA gene sequences was constructed using the same method as the 18S rRNA gene tree, and the MUSCLE alignment provided superior results. The constructed tree is depicted in Fig. 2B. The overall branch lengths are short, suggesting that this locus is not suitable for analyses of the phylogenetic relationships of *Isospora*.

Based on our results, we cannot clearly discuss the phylogenetic characteristics of pigeon *Isospora*. Other loci, including mitochondrial COIa and COIb [18, 19], or other protein-coding genes may help increase the phylogenetic resolution. In this study, we attempted to amplify these sequences but the trials were unsuccessful, probably due to limitations of the template genome. Further

190 improvement of whole-genome amplification techniques may increase the efficacy
191 of PCR amplification and enable us to improve our understanding of *Isospora*.
192 Mandour *et al*. (1986) described *Atoxoplasma columbae* as a parasite from
193 Columbiformes in Assiut, Egypt [20]. The genus *Atoxoplasma* was previously
194 recognized as a blood parasite and was recently divided into two groups: one
195 transmitted by mites considered to be a synonym of *Lankesterella* and the other with
196 fecal-oral transmission as a synonym of *Isospora* [21, 22]. In the original description
197 of *A. columbae*, only a rough line drawing of intra-erythrocyte merozoites from the
198 thin blood smear of a pigeon was available; none of the morphological features of
199 other stages were recorded, and no molecular information is available. In particular,
200 the lack of oocyst information raises the question of whether *A. columbae* can be
201 transmitted via an insect vector or fecal oocyst; in the latter case, it would be
202 recognized as *Isospora*. In the present study, we did not make blood smears or
203 perform a dissection to clarify the infection site of the pigeon *Isospora* sp. Hence we
204 only mention the possibility that the detected pigeon *Isospora* sp. is *A. columbae*.

In conclusion, we detected an isosporoid oocyst from the domestic pigeon *C. livia*. It had distinct morphology compared with other species isolated from the same host order. This is the first case in which an *Isospora* sp. has been reported from *C. livia* worldwide, and that a coccidium has been reported from Columbiformes in Japan.

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Ethical standards

All experiments herein comply with the current laws of Japan.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1. Morphology of the pigeon *Isospora* sp. isolated in this study

(**A)** Differential interference contrast image of a sporulated oocyst of the pigeon *Isospora* sp. (**B)** Line drawing of sporulated oocysts of the pigeon *Isospora* sp. Note the obvious sub-Stieda bodies. Bar: 10 μm.

Fig. **2**. **Phylogenetic tree of *Isospora* sp**. **and related taxa**

(**A**) A maximum likelihood (ML) phylogenetic tree of the pigeon *Isospora* sp. and related taxa was constructed using 1,162 bp of the 18S rRNA gene. The confidence in the tree topology is shown with ML nonparametric bootstrap values (values >50 are shown). The pigeon *Isospora* sp. is underlined. (**B**) ML tree of *Isospora* sp. and related taxa is shown. A total of 598 bp of the 28S rRNA gene were analyzed. ML nonparametric bootstrap values (>50) are shown. The pigeon *Isospora* sp. is underlined.