Isospora hypoleucae sp. n. (Apicomplexa: Eimeriidae), a new coccidian parasite found in the Pied Flycatcher (*Ficedula hypoleuca*)

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SUMMARY

A new Coccidia species is reported from the natural population of Pied Flycatcher (*Ficedula hypoleuca*) in northern Germany. Sporulated oocysts were found in faeces from 6 of 8 sampled adults. The spherical oocysts of the new *Isospora* species have a brownish, smooth, bi-layered wall. Average size of sporulated oocysts was 19·4 × 19·3 μm (17·5–22·8 μm × 17·5–22·8 μm) with a shape index (length/width) of 1·0. The sporulated oocysts have no micropyle or residuum, but enclose several small polar granules that often cluster into 2–3 dumbbell-shaped formations. Sporocysts are slightly elongated, rounded at the end opposite the Stieda body, 15·3 μm × 9·2 μm in size (13·8–16·1 μm × 8·5–10·3 μm), and have a shape index of 1·7 (1·6–1·8). The Stieda body has a prominent knob-like cap, whereas the substieda body is absent. Sporocysts contain a small compact sporocyst residuum and 4 sporozoites. COI haplotypes identical to those isolated from faecal oocysts were PCR amplified from the blood of 13-day-old nestlings, suggesting that the newly described species has extra-intestinal stages in blood. This represents the first description of a new avian *Isospora* species supported by molecular sequence data from the same oocysts that are described morphologically.

Key words: *Isospora hypoleucae*, *Ficedula hypoleuca*, mitochondrial COI gene.

INTRODUCTION

Passerine bird species are frequently found to be infected with Coccidian parasites. In fact all passerines are likely to be potential hosts for at least 1 coccidian species (Svobodová, 1994; Dolnik, 2002*a*; Schrenzel *et al*. 2005). Infection is transmitted via sporulated oocysts that are shed with faeces and then swallowed by the next host in contaminated food or water (Long, 1982). Host-specific foraging behaviour thus plays an important role in the transmission ecology of these parasites (Dolnik, 2002*a*). Consequently, isosporan parasites of aerial feeding birds face exceptional transmission difficulties, usually resulting in low prevalence and low infection intensities compared to parasites of bird species with other foraging modes (Dolnik, 2002*a*). However, this does not necessarily mean that parasite diversity is low in these host species (Pellerdy, 1974).

The Pied Flycatcher (*Ficedula hypoleuca*) is an aerial feeder or foliage gleaner, preying on various kinds of arthropods. This small migratory songbird breeds in tree holes and nest boxes across the Palaearctic Region, and winters in West- and Central-Africa south of the Saharan desert (Lundberg and Alatalo, 1992). At least 3 species of coccidia have been described from Pied Flycatchers, and several additional unnamed species have been reported (Schwalbach, 1959; Cringoli and Quesada, 1990).

Descriptions of new isosporan species are traditionally based solely on oocyst morphology (e.g. Pellerdy, 1974; Upton *et al*. 2001) and only recently has photomicrograph documentation become common (Duszynski, 1999). Modern molecular methods enable a new level of documentation; morphological descriptions and photomicrographs are supported by molecular haplotypes determined from the same individual oocysts that were photographed, measured and described (Dolnik *et al*. 2009). In this article, we describe a new species of *Isospora* Schneider, 1875 parasitizing Pied Flycatchers, based on oocyst morphology and confirmed by mitochondrial sequence data acquired from the same individual oocysts.

MATERIALS AND METHODS

Samples were collected between April and June 2006 in a breeding population of Pied Flycatchers near Itzehoe in northern Germany (54°01′ N; 9°33′ E). Adult birds where captured during incubation or

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while feeding their young, as part of a larger project investigating the population ecology of this species. Upon capture, each bird that had not been marked previously was provided with a uniquely numbered aluminium ring, measured, sampled and released. Nestlings were ringed, measured and sampled when 13 days old (hatching day = day 0). A small blood sample (10–100 μl) was collected from each bird by venepuncture of the brachial vein. Blood samples were kept at 4 °C in the field and subsequently stored in 96% ethanol.

Faecal droplets, 1 pellet per bird, were collected immediately after shedding from 8 adult Pied Flycatchers that were trapped in the afternoon hours. Samples were kept in 2·5% aqueous potassium dichromate ($K_2Cr_2O_7$) until processed. After flotation centrifugation in saturated NaCl solution for 5 min at 375 **g**, the surface layer was placed on slides and immediately examined under × 100 magnification to determine the presence and the number of oocysts. The whole slide was checked to avoid errors caused by oocyst clustering. Infection intensity was measured in o.p.d. (oocysts per defecation) according to the method described by Dolnik (2006). The detailed morphological structure of oocysts was studied under × 1000 magnification with oil immersion.

To isolate individual oocysts for photographing and genetic analysis, we used the following method (Dolnik *et al*. 2009). From a positive sample, a drop with oocysts was transferred to a new object glass using a sterile micropipette. Next it was serially rinsed through several 1 μl drops of ddH_2O by transferring part of each drop to the next with microscopic control, until we achieved a single oocyst in a drop (Dolnik *et al*. 2009). Each oocyst separated in this way was individually photographed and measured to the nearest 0·1 μm. Measurements are in micrometers, with range and number (*n*) of stages measured in parentheses.

Abbreviations used in the species descriptions are as suggested by Duszynski and Wilber (1997). Oocyst characters include length (L), width (W), and their ranges and ratio (L/W); micropyle (M); residuum (OR); and polar granule (PG). Sporocyst characters include length (L), width (W), and their ranges and ratio (L/W); Stieda body (SB); sub-Stieda body (SSB); para-Stieda body (PSB); residuum (SR); sporozoites (SP); refractile bodies (RB); and nucleus (N) in SP.

After photographing, 22 sporulated oocysts (1–6 oocysts per individual bird) were individually collected in separate vials and frozen at −80 °C. DNA of each individual oocyst was extracted by adjusted Chelex extraction (Dolnik *et al*. 2009). Molecular analysis of oocysts was performed individually using a nested PCR method to amplify a 250 bp long fragment of the alpha subunit of the mitochondrial cytochrome oxidase gene (COI) of the parasite using external primers COX tenella F4/R and internal primers COX tenella F2/R2 (Dolnik *et al*. 2009). Positive controls with genomic DNA from *Isospora* sp. oocysts from Blackcap *Sylvia atricapilla*, and negative no-template controls were included in each PCR reaction.

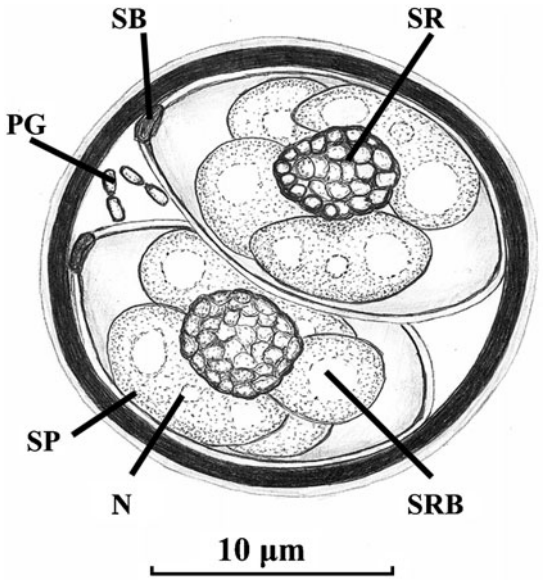

Fig. 1. Composite line drawing of a sporulated oocyst of *Isospora hypoleucae* sp. n. PG – polar granule; SB – Stieda body; SR – sporocyst residuum; SP – sporozoites; RB – sporozoite refractile bodies; N – nucleus.

Total DNA from blood was extracted using a standard phenol-chloroform method (Sambrook *et al*. 2002), and 2 μl of diluted genomic DNA (25 ng/μl) was used as template in the same PCR protocol as for the oocysts. The success of PCR amplifications was checked on 2% agarose gels and positive samples were sequenced with BigDye (®Applied Biosystems, Foster City, CA, USA) terminator cycle sequencing kit. Sequences were edited and aligned using the software BioEdit (Hall, 1999). We used 'Basic Local Alignment Search Tool' (Blast) to compare obtained sequence fragments with known parasite sequences (http://www.ncbi.nlm.nih.gov/blast).

RESULTS

Isosporan oocysts were found in faeces from 6/8 (75%) adult Pied Flycatchers. All infections detected were monospecific and intensity of infection varied between individuals from 1 to 500 oocysts per defecation. A comparison revealed several morphological differences between the oocysts from this species and those of previously described Isosporan species found in *Ficedula*- and *Muscicapa*-Flycatchers, as described below. From 22 single oocysts, we obtained 14 (64%) high quality COI sequences.

None of the blood samples of the adult birds showed positive PCR reactions. However, blood

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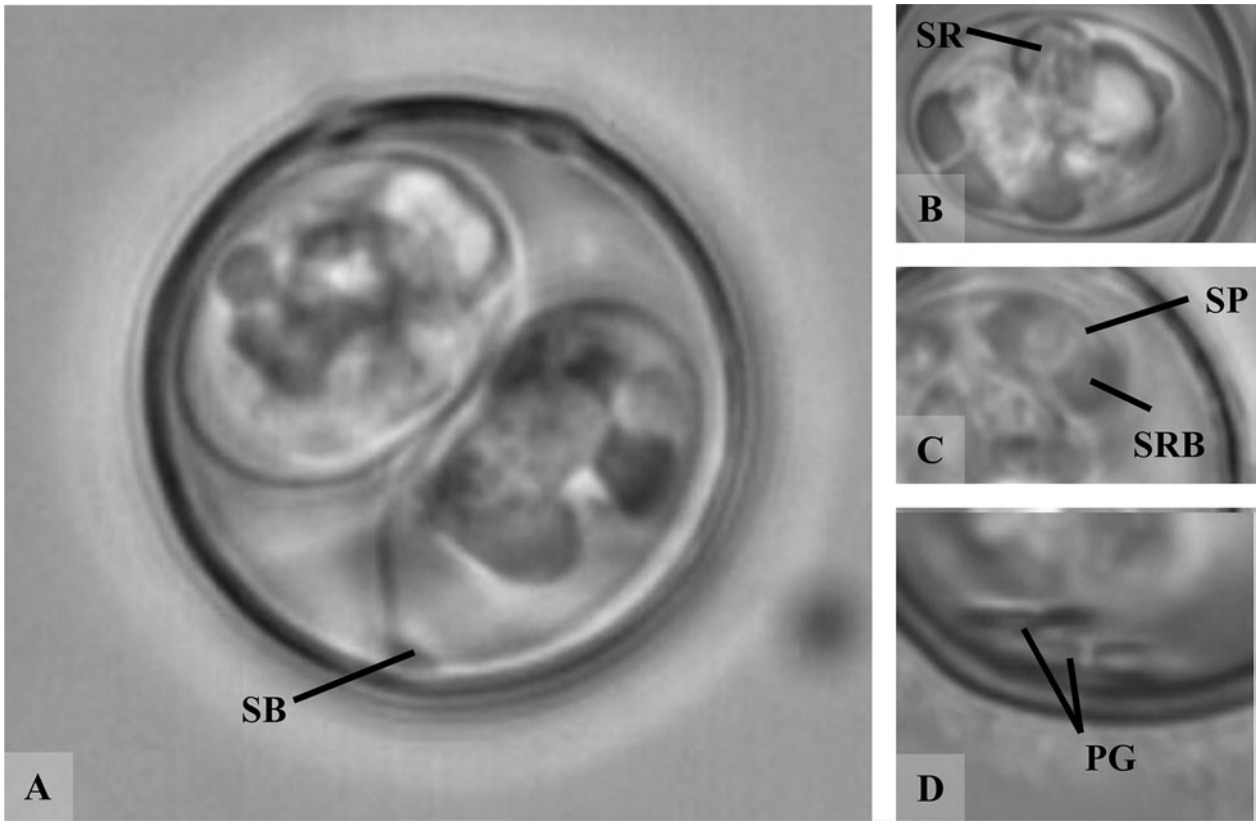

Fig. 2. Photomicrograph of sporulated oocysts of *Isospora hypoleucae* sp. n. A – round oocyst, note the absence of SSB; B – sporocyst, note compact SR; C – sporozoite; D – two dumbbell-shaped PGs. Abbreviations as in Fig. 1.

samples of nestlings from nests with infected parents showed positive PCR reactions, which resulted in high quality sequence. We found infections in 3 out of 12 (prevalence 25%) nestlings belonging to 1 out of 3 nests. We obtained the same *Isospora* haplotype (iFICEHYP1, GenBank No. FJ269363) from both oocysts of adult Pied Flycatchers and blood samples from the nestlings. When comparing iFICEHYP1 to lineages iSAT1-iSAT6 from Blackcaps (Dolnik *et al.* 2009) we found sequence divergences between 2·5 and 4·5%. We observed only minor variation in oocyst size and morphology among isolates from the flycatchers. For morphological comparison and species description we deliberately took measurements only of the 14 oocysts that were individually sequenced and proved to belong to the same haplotype iFICEHYP1.

DESCRIPTION

Isospora hypoleucae sp. n. (Figs 1 and 2)

Description of sporulated oocyst: The spherical oocysts have a brownish smooth bi-layered 1·3 thick wall, M absent. L × W (n = 14), 19·4 × 19·3 (17·5–22·8 × 17·5–22·8) L/W ratio, 1·01 (1·00–1·05), OR absent. Several small PGs ~0·9 × 1·3 often cluster into 2–3 dumbbell-shaped formations.

Description of sporocyst and sporozoites: Sporocysts are slightly elongated, rounded at the end opposite to SB, L × W (n = 14), 15·3 × 9·2 (13·8–16·1 × 8·5–10·3), L/W ratio, 1·66 (1·56–1·79). SB present, with prominent knob-like cap; SSB and PSB absent. Sporocysts contain small compact SR and 4 SP that are usually rolled up into balls. Each SP has 2 RB and N.

Taxonomic summary

Type host: *Ficedula hypoleuca* (Pallas 1764), Pied Flycatcher

Type locality: (54°01′ N; 9°33′ E), North-West Germany.

Prevalence: In faeces: 6/8 (75%) adults (4/5 females and 2/3 males). In blood: 0/8 adults, 3/12 juveniles of infected females.

Sporulation time: Unknown, oocysts were completely sporulated.

Pre-patent and patent periods: Unknown, parasite appears in blood at least on day 13.

Site of infection: Unknown. Oocysts recovered from faeces, haplotypes detected in blood.

Material deposited: Photosyntype (see Duszynski, 1999) of sporulated oocyst deposited in the US National Parasite Collection No. 101305. Haplotype iFICEHYP1 is deposited in GenBank (Accession number FJ269363).

Etymology: The name is derived from the specific epitaph of the scientific name of the type host, *Ficedula hypoleuca*.

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Table 1. Morphological characteristics of isosporan oocysts observed in various Flycatcher species

Isospora species	Type host	Oocyst		Polar granule *n* and form	Sporocyst		
		Average size (μm)	Micropyle		Stieda body form	Substieda body form	Residuum
I. ficedulae Schwalbach 1959	*Ficedula hypoleuca*	20·2 × 20·2	absent	1–2 round	Not projecting	six-edged	compact
I. landauae Cringoli and Quesada 1990	*Ficedula hypoleuca*	18·5 × 20·9	absent	2 round	Projecting	triangle	diffuse
I. parvae Chatterjee and Choudhury 1976	*Muscicapa parva*	19 × 19	present	1–2 round	Projecting	absent	compact
Isospora sp. non *I. phoenicuri* Schwalbach 1959	*Ficedula hypoleuca*	30·6 × 20	absent	2 star-formed	Not projecting	triangle	compact
Isospora sp. non *I. wurmbachi* Schwalbach 1959	*Ficedula hypoleuca*	21·6 × 21·6	absent	4–8 stick-formed	Not projecting	absent	diffuse
I. hypoleucae	*Ficedula hypoleuca*	19·4 × 19·3	absent	2–3 dumbbell-shaped	Not projecting	absent	compact

DISCUSSION

Narrow host specificity of avian *Isospora* was demonstrated by cross-transmission experiments, particularly for *Isospora* species of passerine birds (Černá, 1973; Box, 1981; Dolnik, 2002*b*). Levine (1982*a*) proposed that ''a coccidian species may be transmissible from one species to another in the same genus, but not from one genus to another in the same family until otherwise demonstrated.'' Molecular data later showed that these parasites are predominantly host specific at the species level and only occasionally undergo lateral transfer (Schrenzel *et al*. 2005).

We compared the morphological characteristics of *Isospora hypoleucae* sporulated oocysts with other *Isospora* spp. previously described from 2 closely related Flycatcher genera, *Ficedula* and *Muscicapa*.

Three *Isospora* species have been described previously from *Ficedula* and *Muscicapa* spp. (Table 1): *Isospora ficedulae* Schwalbach 1959 and *I. landauae* Cringoli and Quesada 1990 are described from the Pied Flycatcher, and *I. parvae* Chatterjee and Choudhury 1976 from the Redbreasted Flycatcher *Muscicapa parva*. Further, Schwalbach (1959) reported the presence of *I. phoenicuri* Schwalbach 1959 and *I. wurmbachi* Schwalbach 1959 in Pied Flycatchers. Host specificity for *Isospora* spp. is now thought to be narrower than in the time of Schwalbach's investigations, thus the 2 species he saw were probably not *I. phoenicuri* and *I. wurmbachi*, but other species that resemble them.

The absence of SSB distinguishes the new species from *I. ficedulae* and *I. landauae*, and from *Isospora* sp. non *I. phoenicuri* described by Schwalbach (1959). From all *Isospora* species recorded from Flycatchers, 2 species lack SSB. These are *I. parvae*, found in the Red-breasted Flycatcher in West Bengal, and *Isospora* sp. non *I. wurmbachi*. *I. parvae* resembles the species described here in the size of its oocysts and form of SB (Table 1). However, the oocysts of *I. hypoleucae* can easily be distinguished from those of *I. parvae* by the absence of M, which is a distinct characteristic of *I. parvae*. *I. hypoleucae* can be distinguished from *Isospora* sp. non *I. wurmbachi* the by the smaller size of the oocysts, form and number of PGs, prominent SB and compact SR (Table 1).

Based on these characteristics, we consider the species described here to be a new coccidian. Presence of the COI haplotype in the blood of nestlings suggests that the new species can form blood stages, and in this case follows the *Isospora serini*-like life cycle (Box, 1975, 1977). Some authors place such blood-inhabiting coccidia into genus *Atoxoplasma* (e.g. Levine 1982*b*), yet recent studies consider them rather as a facultative variation of the life cycle within the *Isospora* genus (e.g. Upton *et al*. 2001; Schrenzel *et al*. 2005). It is interesting that despite the fact that adult birds were shedding oocysts, no blood stages of *I. hypoleucae* were detected in these birds, but only in 13-day-old nestlings. The explanation of this age-dependent difference remains unclear; future investigation, preferably employing molecular tools, is required to clarify the situation.

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REFERENCES

Box, E. D. (1975). Exogenous stages of *Isospora serini* (Aragão) and *Isospora canaria* sp. n. in the canary (*Serinus canarius* Linnaeus). *Journal of Protozoology* **22**, 165–169.

Box, E. D. (1977). Life cycles of two *Isospora* species in the Canary, *Serinus canarius* Linnaeus. *Journal of Protozoology* **24**, 56–67.

Box, E. (1981). *Isospora* as an extraintestinal parasite of passerine birds. *Journal of Protozoology* **28**, 244–246.

Černá, Ž. (1973). Interesting coccidians from wild birds. *Journal of Protozoology* **20**, 535–536.

Cringoli, G. and Quesada, A. (1990). *Isospora couderti* sp.n., *Isospora aycardii* sp.n. chez *Cisticola jundicis* (Cisticole des Jones) (Passeriformes: Sylviidae) et *Isospora landauae* sp.n. chez *Muscicapa hypoleuca* (Gobe-mouche) (Passenformes: Muscicapidae). *Annales de Parasitologie Humaine et Comparée* **65**, 167–169.

Dolnik, O. (2002*a*). Some aspects of the biology and host-parasite interactions of *Isospora* spp. (Protozoa: Coccidiida) of passerine birds. Ph.D. dissertation, Carl von Ossietzky University, Oldenburg, Germany. Online: available at: http://docserver.bis.uni-oldenburg.de/publikationen/dissertation/2003/dolsom02/dolsom02.html

Dolnik, O. V. (2002*b*). *Isospora sylvianthina* (Protozoa: Coccidiida), parasite of Blackcap, does not infect Reed Warbler. *Zoosystematica Rossica* **10**, 240.

Dolnik, O. (2006). The relative stability of chronic *Isospora sylvianthina* (Protozoa: Apicomplexa) infection in blackcaps (*Sylvia atricapilla*): evaluation of a simplified method of estimating isosporan infection intensity in passerine birds. *Parasitology Research* **100**, 55–60.

Dolnik, O., Palinauskas, V. and Bensch, S. (2009). Individual oocysts of *Isospora* (Apicomplexa: Coccidia) parasites from avian faeces: from photo to sequence. *The Journal of Parasitology* **95**, 169–174. DOI: 10.1645/GE-1873.1

Duszynski, D. W. and Wilber, P. G. (1997). A guideline for the preparation of species descriptions in the eimeriidae. *Journal of Parasitology* **83**, 333–336.

Duszynski, D. W. (1999). Critical comment: revisiting the code: clarifying name-bearing types for photomicrographs of protozoa. *The Journal of Parasitology* **85**, 769–770.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Levine, N. D. (1982*a*). *Isospora passeris* n. sp. from the house sparrow *Passer domesticus*, *I. lacazei*, and related apicomplexan Protozoa. *Transactions of the American Microscopical Society* **101**, 66–74.

Levine, N. D. (1982*b*). The genus *Atoxoplasma* (Protozoa: Apicomplexa). *The Journal of Parasitology* **68**, 719–723.

Long, P. L. (ed.) (1982). *The Biology of the Coccidia*. Edward Arnold, London, UK.

Lundberg, A. and Alatalo, R. V. (1992). *The Pied Flycatcher*. T & A Poyser, London, UK.

Pellerdy, L. P. (1974). *Coccidia and Coccidiosis*. Akademiai Kiado, Budapest, Hungary.

Sambrook, J., Fritsch, F. J. and Maniatis, T. (2002). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold, Spring Harbor, New York, USA.

Schrenzel, M. D., Maalouf, G. A., Gaffney, P. M., Tokarz, D., Keener, L. L., McClure, D., Griffey, S., McAloose, D. and Rideout, B. A. (2005). Molecular characterization of isosporoid Coccidia (*Isospora* and *Atoxoplasma* spp.) in passerine birds. *The Journal of Parasitology* **91**, 635–647.

Schwalbach, G. (1959). Untersuchungen und Beobachtungen an Coccidien der Gattungen *Eimeria*, *Isospora* und *Caryospora* bei Vögeln mit einer Beschreibung von sechzehn neuen Arten. *Archiv für Protistenkunde* **104**, 431–491.

Svobodová, M. (1994). *Isospora*, *Caryospora* and *Eimeria* (Apicomplexa: Eimeriidae) in Passeriform Birds from Czech Republic. *Acta Protozoologica* **33**, 101–108.

Upton, S. J., Wilson, S. C., Norton, T. M. and Greiner, E. C. (2001). A new species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) from the Bali (Rothschild's) mynah *Leucopsar rothschildi* (Passeriformes: Sturnidae), and comments concerning the genera *Atoxoplasma* Garnham, 1950 and *Isospora*. *Systematic Parasitology* **48**, 47–53.