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Morphological and molecular characterization of adult worms of *Leucochloridium paradoxum* Carus, 1835 and *L. perturbatum* Pojmańska, 1969 (Digenea: Leucochloridiidae) from the great tit, *Parus major* L., 1758 and similarity with the sporocyst stages

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Abstract

Unlike the sporocyst stages, adult leucochloridiid digeneans are difficult to differentiate. Sporocyst broodsacs can be identified on the basis of their colour and banding pattern, but in the absence of broodsacs and when experimental infection cannot be performed, tentative morphological identification needs to be verified, and molecular techniques offer a tool to do this. In this study, adult leucochloridiid digeneans were collected from the great tit (*Parus major*) found dead at three localities at or near the Baltic Sea coast (Hel, Bukowo-Kopań and Szczecin) in northern Poland. On the basis of differences in their morphological characters, Hel specimens were tentatively assigned to *Leucochloridium perturbatum*, Bukowo-Kopań and Szczecin specimens being identified tentatively as *L. paradoxum*. Subsequent ribosomal DNA sequence analysis confirmed the identification of these leucochloridiid flukes. Nucleotide sequences discriminating between the two species were identical to those used by earlier authors as characteristic of two distinctly different sporocyst broodsacs representing *L. perturbatum* and *L. paradoxum*.

Introduction

Flukes of the genus *Leucochloridium* are fairly common avian parasites, two species – *L. paradoxum* Carus, 1835 and *L. perturbatum* Pojmańska, 1969 – being particularly frequent in Europe (Casey *et al.*, 2003). Birds become infected by ingesting snails carrying leucochloridiid sporocyst broodsacs which, once in the avian body, give rise to adult worms. Morphological identification of adult leucochloridiids relies on several characteristics, some of those relied upon when identifying *L. paradoxum* and *L. perturbatum* being listed in table 1. However, as pointed out by Casey *et al.* (2003), species identification of adult leucochloridiids, including the two species mentioned above, is very difficult due to a number of reasons: the

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Table 1. Morphometrics of adult *Leucochloridium paradoxum* and *L. perturbatum* (measurements are given in μm).

	L. paradoxum Carus, 1835		*L. perturbatum*, Pojmańska, 1969	
Morphometrics	Pojmańska (1967, 1969b, c)[b]	This study ($n = 45$ or fewer)	Pojmańska (1969b, c)	This study ($n = 4$ or fewer)
Relative size of suckers[a]	Oral sucker larger than ventral; 440–540 × 510–630; oral:ventral sucker ratio 1.13:1	Oral sucker 331 ± 39 × 357 ± 17 (257–379 × 331–399), larger than ventral 296 ± 39 × 294 ± 32 (236–348 × 246–344); oral:ventral sucker ratio 1.11:1 to 1.24:1 (1.2:1)	Suckers equal in size or oral sucker (280–670 × 240–720) somewhat larger than ventral (240–700 × 270–710)	Suckers equal in size; oral sucker 550 ± 26 × 557 ± 35 (519–577 × 522–602); ventral sucker 543 × 502; oral:ventral sucker ratio 1.08:1
Anterior extent of uterus	Uterine branches extending to or beyond the proximal end of vitellaria	Uterus extending to or beyond proximal end of vitellaria	Uterus not extending beyond proximal end of vitellaria	Uterus in its anterior part extending beyond anterior intestine loop, but not extending beyond proximal end of vitellaria
Size and location of cirrus sac	Size variable, but generally smaller than testes and ovary; 110–160 × 80–120, located at end of body, completely or almost completely beyond intestine	Cirrus sac 102 ± 13 × 73 ± 12 (90–131 × 57–100) located at end of body beyond intestine	Cirrus sac 120–320 × 80–230, usually equal in size to testes and ovary or a little larger, located between the ends of caeca, sometimes overlapping posterior part of testis	Cirrus sac 223 ± 37 × 172 ± 27 (193–265 × 142–180), located between lower parts of intestine (at body end, between intestinal branches)
Posterior extent of vitellaria	Usually extending somewhat beyond intestine, branched; individual variation in extent of vitellaria	Vitellaria asymmetrical or equal, extending somewhat beyond intestine	Extending somewhat beyond intestine, individual variation in extent of vitellaria	Vitellaria ending below intestine
Body shape	Barrel-shaped	Barrel-shaped and oval	Oval	Oval
Cirrus sac shape	Fairly bulged, sometimes nearly spherical	Spherical, bulged, partly covered by uterine branches	Usually elongated	Elongated, poorly visible, partly covered by uterine branches
Body dimensions	1510–1900 × 980–1270	1111 ± 195 × 591 ± 71 (909–1709 × 438–775)	250–2650 × 400–1470	1743 ± 139 × 856 ± 42 (1557–1875 × 827–886)
Pharynx	160–200 × 170–270	Not visible, covered by uterine loops overlapping anterior end of oral sucker	90–210 × 70–280	In lower part obscured by uterine branches
Anterior testes	150–200 × 200–270	Ends obscured by uterine branches	80–330 × 90–370	318 × 270; visible in a single specimen only
Ovary	150–200 × 200–270	Ends obscured by uterine branches	70–290 × 90–370	Ends obscured by uterine branches
Posterior testes	140–200 × 190–210	Ends obscured by uterine branches	70–330 × 90–360	Poorly visible; 188 and 318 × 209 and 318 (253 ± 92 × 264 ± 77)
Eggs	24 × 16	25 ± 1 × 17 ± 1 (22–29 × 13–19)	18–30 × 11–21	22–28 × 13–17 (24 ± 1 × 15 ± 1)

[a] According to Bakke (1980), the specific sucker ratio must also be used with caution at the individual level.
[b] Biometric characters given are those found in *L. paradoxum* collected from *Parus major* (Pojmańska, 1967).

parasites possess few autapomorphic characters and lack hard structures, their size ranges overlap, and the internal organs are, in gravid individuals, hidden behind egg-filled uterine loops. In contrast, leucochloridiid broodsacs differ distinctly, the differences being large enough to warrant species descriptions based on colour and banding patterns of sporocyst broodsacs. Specifically, *L. paradoxum* sporocysts occur in green- and those of *L. perturbatum* in brown-banded broodsacs (Pojmańska, 1967, 1969a, b; Bakke, 1978). However, parasitologists frequently face a difficulty of assigning adult specimens they find in birds to sporocysts encountered in organisms preyed upon by those birds (e.g. snails).

There are two ways out of this difficulty. First, the life cycle needs to be completed by experimental infection (Pojmańska, 1969a, 1975, 1978; Rietschel, 1972, 1979; Bakke, 1980). This pathway was taken by Pojmańska (1963, 1969a) who cultured brown-banded sporocysts of *Leucochloridium* and experimentally infected birds to produce adult worms. This approach, however, is seldom feasible, as the field-collected material may consist of adult flukes only and no additional sampling for sporocysts is possible. The second option is to use molecular techniques to, first, establish a genetic match between a sporocyst and an adult, and then to apply the molecular characteristics revealed to identify the adults found.

So far, genetic studies on leucochloridiid flukes have focused on their phylogenetic status within the Digenea (Olson *et al.*, 2003). The first genetic analysis performed on leucochloridiid sporocysts (Casey *et al.*, 2003) allowed comparison of the variability within the sequence of the 5.8S ribosomal RNA gene and internal transcribed spacers (ITS1 and ITS2) of the two species mentioned above. The green-banded *L. paradoxum* and the brown-banded *L. perturbatum* sporocysts differed in 6.8% of their ITS sequences. As there were no intraspecific differences, the ITS region was considered a reliable molecular marker with which to differentiate between the two species represented by sporocysts.

However, no corresponding molecular study has been carried out on adult leucochloridiids. Therefore, this study was aimed at finding DNA sequences of adult *Leucochloridium* specimens matching the sequences described by Casey *et al*. (2003) from the two morphologically distinct sporocyst broodsacs (green- and brown-banded). We hypothesized that a match between adult *Leucochloridium* and sporocysts would be reflected at the molecular level; in other words, the sequences revealed for sporocysts would be found in adults as well. Additionally, we assumed that rDNA sequences would differ between the two adult *Leucochloridium* morphotypes, and would remain identical within each morphotype.

Materials and methods

Collection and examination of great tits

Adult *Leucochloridium* were collected from cloaca of the great tit (*Parus major*) found dead on the Polish Baltic coast. Birds were collected in March 2009 and 2010 at the Hel Peninsula (54°46′N, 18°28′E) (two birds; worm range, 6–9) as well as in October and November 2008 and 2009 near Bukowo-Kopań (54°28′N, 16°25′E) (three birds; worm range, 62–220) and in the city of Szczecin (53°26′N, 14°32′E) (one bird, 132 worms). Digeneans were fixed in 70–75% ethanol, examined under a stereomicroscope and initially assigned to morphotypes using the characteristics given by Pojmańska (1969b), with a due consideration to descriptions provided by Bakke (1980, 1982).

Morphological and molecular analyses

For the morphometric study, 4, 36 and 9 flukes were collected from the great tits found in Hel, Bukowo-Kopań and Szczecin, respectively. Flukes were stained with alum carmine, mounted in Canada balsam, and examined under a light microscope. For morphological identification, constant (sucker relative size, uterus extension, cirrus sac size and location, vitellaria extension) and additional characters (*sensu* Pojmańska, 1969b) (body shape, cirrus sac shape) were used. In addition, the fluke body was measured (length and width), as were suckers, cirrus sac, anterior and posterior testes, and eggs in those specimens in which the organs were visible.

Molecular analyses were performed on flukes representing the infrapopulations supplying specimens for morphological analyses, although the individuals used were not the same. For sequencing, 11, 75 and 25 specimens from Hel, Bukowo-Kopań and Szczecin were used, respectively. DNA of 70% ethanol-preserved flukes was isolated using the peqGOLD Tissue Mini Kit (PeqLab, Erlangen, Germany) and stored at −20°C until analysis. The targeted DNA regions (ITS1, partial sequence; 5.8S ribosomal DNA gene and ITS2, complete sequence; and 28S ribosomal DNA gene, partial sequence) were amplified with L18S1 and L28SR1 primers following Casey *et al.* (2003). Polymerase chain reaction (PCR) products were identified by their electrophoresis on 1% agarose gel; each PCR product of approximately 1200 bp was sequenced bidirectionally by Genomed (Warszawa, Poland). Sequences were analysed with BioEdit, BLAST and MEGA 4.0 software (Altschul *et al.*, 1990; Hall, 1999; Tamura *et al.*, 2007).

Results

Flukes collected in Hel (oval; 1743 ± 139 × 856 ± 42 μm) were morphologically different from those found in Bukowo-Kopań and Szczecin (table 1). Suckers were almost equal in size; vitellaria extended from the oral sucker centre to below the intestine; the uterus reached beyond the anterior intestinal loop, but did not extend beyond the proximal end of vitellaria; the cirrus sac was located at the lower part of the intestine; and eggs measured 24 ± 1 × 15 ± 1 μm. Hel specimens were tentatively assigned to *L. perturbatum* (table 1).

Specimens from Bukowo-Kopań and Szczecin were morphologically similar, and differed from those found in Hel by being barrel-shaped, shorter (1111 ± 195 μm), and narrower (591 ± 71 μm); the oral sucker was larger than the ventral; the uterus was substantially enlarged, reaching to the proximal end of vitellaria or extended beyond them; the cirrus sac was located at the posterior end of the body; vitellaria were asymmetrical or identical, ending just beyond the intestine; and eggs measured

$25 \pm 1 \times 17 \pm 1$ μm. Bukowo-Kopań and Szczecin specimens were tentatively assigned to *L. paradoxum* (table 1).

Molecular assays of flukes produced characteristic nucleotide sequences deposited in the National Center for Biotechnology Information (NCBI) under accession numbers JF346883 (Szczecin), JF274482 (Bukowo-Kopań) and JF331664 (Hel). Comparison made with the BLAST software showed sequences of Szczecin and Bukowo-Kopań digeneans to be identical. On the other hand, those sequences differed by 1.7% from Hel specimen sequences. Subsequent comparison of sequences obtained in this study with those published by Casey *et al.* (2003) showed the Szczecin and Bukowo-Kopań specimen sequences to be identical, except for a single adenine deletion, to those of *L. paradoxum* (AY258145) of Casey *et al.* (2003). Moreover, the Hel specimen sequences were 100% identical to those of *L. perturbatum* (AY258144). Similar results were obtained by comparing the sequences analysed with other sequences in the NCBI database described as *L. paradoxum* (JN639012) and *L. perturbatum* (JN639011). This provides additional confirmation of species identity of the flukes studied. Analysis conducted with the BioEdit software showed the changes (transitions, transversions and deletions) to have been restricted to ITS1 and ITS2 regions. No differences in 5.8S rDNA and in partial 28S rDNA regions were detected. The sequences in question, when analysed with the neighbour joining algorithm of the MEGA 4.0 software, grouped into two clades, one consisting exclusively of specimens representing *L. paradoxum* and the other, *L. perturbatum*.

Thus, molecular diagnostics of Hel infrapopulation flukes showed them to represent *L. perturbatum.* On the other hand, Bukowo-Kopań and Szczecin specimens were diagnosed, with the molecular technique used, as representing *L. paradoxum*.

Discussion

Tentative morphological identification of leucochloridiid flukes found in dead *P. major* relied on a number of morphological characteristics which Pojmańska (1967, 1969a, b, c) described from adults that had been cultured from distinctly identifiable sporocysts. Those characteristics proved useful for our study, but, unfortunately, their usefulness is not universal. Descriptions, figures and dimensions provided by various authors (Pojmańska, 1967; Rutkowska, 1973; Machalska, 1980; Sitko, 1993, 2001; Macchioni, 2003) differ widely. For example, descriptions published by those authors show that adult *L. paradoxum* and *L. perturbatum* can be easily distinguished by their relative sucker size. However, Bakke (1980) reported this character as variable as well. Therefore, we agree with Pojmańska (1969b) that certain characters are manifested sufficiently frequently to be regarded as diagnostic in abundant leucochloridiid collections only. It is conceivable that, in large collections from different localities, multivariate mathematical techniques might be helpful in identifying sets of morphological characters that would eventually assist in morphological diagnosing of adult leucochloridiids.

When a collection is small, uncertainties prevail and can be resolved by using molecular techniques. Molecular analyses to distinguish between morphologically distinct forms of leucochloridiid sporocyst broodsacs representing two unambiguous forms, brown-banded *L. perturbatum* and green-banded *L. paradoxum*, were run successfully by Casey *et al.* (2003). Not only did they confirm genetic identity of the two species represented by sporocysts, but also provided genetic markers (ITS regions of rDNA) that might be applied to adult flukes of uncertain identity, such as those in our study, where no matching sporocyst broodsacs were available. DNA sequences of adult *Leucochloridium* flukes proved identical to those from sporocysts of the respective species collected in Denmark and Norway by Casey *et al.* (2003). Genetic variation of the flukes we studied was at a level identical to that described by Casey *et al.* (2003). Similarity between the genetic make-up of sporocysts and adult flukes confirmed utility of the rDNA region used as marker. Flukes identified as one morphotype (our tentative species) showed an identical level of genetic diversity, whereas the two morphotypes were sufficiently different to be regarded as separate species.

Thus, when there is a need to assess the extent of avian infection with different leucochloridiid species and the actual collection is small, matching sporocyst broodsacs are not available and no experimental infection can be performed, morphological characters can be used as an initial approximation, but the final diagnosis will depend on the results of molecular assays.

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Bakke, T.A. (1978) Intraspecific variation of adult *Leucochloridium* sp. (Digenea) from natural and experimental infections. *Canadian Journal of Zoology* **56**, 94–102.

Bakke, T.A. (1980) A revision of the family Leucochloridiidae Poche (Digenea) and studies on the morphology of *Leucochloridium paradoxum* Carus 1835. *Systematic Parasitology* **1**, 189–202.

Bakke, T.A. (1982) The morphology and taxonomy of *Leucochloridium* (L.) *variae* McIntosh (Digenea, Leucochloridiidae) from the Nearctic as revealed by light and scanning electron microscopy. *Zoologica Scripta* **11**, 87–100.

Casey, S.P., Bakke, T.A., Harris, P.D. & Cable, J. (2003) Use of ITS rDNA for discrimination of European

green- and brown-banded sporocysts within the genus *Leucochloridium* Carus, 1835 (Digenea: Leucochloriidae). *Systematic Parasitology* **56**, 163–168.

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

Macchioni, F. (2003) Observations on *Leucochloridium paradoxum* Carus, 1835. *Annali della Facoltà di Medicina veterinaria* LVI/**20**, 83–88.

Machalska, J. (1980) Helminth fauna of birds of the genus *Turdus* L., examined during their spring and autumn migration. I. *Digenea. Acta Parasitologica Polonica* **27**, 153–172.

Olson, P.D., Cribb, T.H., Tkach, V.V., Bray, R.A. & Littlewood, D.T.J. (2003) Phylogeny and classification of the digenea (Platyhelminthes: Trematoda). *International Journal for Parasitology* **33**, 733–755.

Pojmańska, T. (1963) Experimental development of *Leucochloridium* sp. (Trematoda, Brachylaemidae). *Acta Parasitologica Polonica* **11**, 153–159.

Pojmańska, T. (1967) Variability of *Leucochloridium paradoxum* Carus (=*L. heckerti* Kagan, 1952) (Trematoda: Brachylaemidae) in natural and experimental conditions. *Acta Parasitologica Polonica* **14**, 381–398.

Pojmańska, T. (1969a) *Leucochloridium perturbatum* sp. (Trematoda: Brachylaimidae). Morphology, individual variability and life cycle. *Acta Parasitologica Polonica* **16**, 153–175.

Pojmańska, T. (1969b) Specific criteria in the genus *Leucochloridium* Carus 1835, emend. Kagan 1952. *Acta Parasitologica Polonica* **16**, 185–192.

Pojmańska, T. (1969c) European species of *Leucochloridium* Carus. *Acta Parasitologica Polonica* **16**, 193–205.

Pojmańska, T. (1975) Life cycle of *Neoleucochloridium holostomum* (Rudolphi, 1819) (Trematoda, Leucochloridiidae). *Acta Parasitologica Polonica* **23**, 23–36.

Pojmańska, T. (1978) Life cycle of *Leucochloridium vogtianum* Baudon, 1881 (=*L. phragmitophila* Bykhovskaja-Pavlovskaja et Dubinina, 1951 in parte) (Trematoda, Leucochloridae). *Acta Parasitologica Polonica* **25**, 11–20.

Rietschel, G. (1972) Untersuchungen über Farbmuster-Typen bei *Leucochloridium*-Sporozysten (Trematoda, Brachylaimidae) und deren Zugehörigkeit zu bestimmten Arten. *Zeitschrift für Parasitenkunde* **40**, 61–68.

Rietschel, G. (1979) Vorkommen larvaler Trematoden in Bernsteinschnecken (Succineidae) Hessens. *Zeitschrift für Parasitenkunde* **58**, 265–274.

Rutkowska, M. (1973) A study of the helminth fauna of Corvidae in Poland. *Acta Parasitologica Polonica* **21**, 183–237.

Sitko, J. (1993) Ecological relations of Trematodes infesting Lariform birds in Czech Republic. *Acta Scientarium Naturalium Academiae Scientarium Bohemicae* **27**, 1–98.

Sitko, J. (2001) Trematodes of owls (Aves: Strigiformes) in the Czech Republic. *Acta Societatis Zoologicae Bohemicae* **65**, 45–56.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.