Molecular characterization of putative *Hepatozoon* sp. from the sedge warbler (*Acrocephalus schoenobaenus*)

ALEKSANDRA BIEDRZYCKA, AGNIESZKA KLOCH*, MAGDALENA MIGALSKA *and* WOJCIECH BIELAŃSKI

Institute of Nature Conservation, Polish Academy of Sciences, al. A. Mickiewicza 33, 31-120 Kraków, Poland

(*Received 14 August 2012; revised 8 October and 13 October 2012; accepted 22 October 2012; first published online 30 January 2013*)

SUMMARY

We characterized partial sequences of 18S rDNA from sedge warblers infected with a parasite described previously as *Hepatozoon kabeeni*. Prevalence was 47% in sampled birds. We detected 3 parasite haplotypes in 62 sequenced samples from infected animals. In phylogenetic analyses, 2 of the putative *Hepatozoon* haplotypes closely resembled *Lankesterella minima* and *L. valsainensis*. The third haplotype grouped in a wider clade composed of *Caryospora* and *Eimeria*. None of the haplotypes showed resemblance to sequences of *Hepatozoon* from reptiles and mammals. Molecular detection results were consistent with those from microscopy of stained blood smears, confirming that the primers indeed amplified the parasite sequences. Here we provide evidence that the avian *Hepatozoon*-like parasites are most likely *Lankesterella*, supporting the suggestion that the systematic position of avian *Hepatozoon*-like species needs to be revised.

Key words: haemogregarines, *Hepatozoon*, *Lankesterella*, avian blood parasites, sedge warbler, *Acrocephalus schoenobaenus*.

INTRODUCTION

The genus *Hepatozoon* consists of apicomplexans infecting a wide range of mammals, birds, reptiles and amphibians (Smith, 1996). In birds, *Hepatozoon* has been reported from various families, and altogether 15 species are considered valid (Peirce, 2005). Generally, the sexual reproduction of *Hepatozoon* occurs in a blood-sucking invertebrate. After ingestion of the invertebrate host by a vertebrate, the sporozoites affect its visceral organs, where they give rise to merogonic stages that later develop into gametocytes circulating in the blood. However, the invertebrate host of most *Hepatozoon* species remains unknown, particularly in the case of bird parasites, although Bennett *et al*. (1992*a*) identified 2 possible intermediate hosts of *H. atticorae* infecting swallows. The limited knowledge of the *Hepatozoon* life cycle results from the fact that most infections are light, and the prevalence studies usually lack investigation of the intermediate hosts (Bennett *et al*. 1992*b*).

Because the life cycles of many of these parasites are poorly known and their blood stages are morphologically similar (Merino *et al*. 2006), the systematics of the genus *Hepatozoon* and other haemogregarines are far from clear (Desser, 1993). Smith and Desser (1997) used a detailed phylogenetic analysis based on morphological, morphometric and developmental characteristics to show that *Hepatozoon* is a paraphyletic group, and suggested that the taxonomy of this group should be modified. A recent analysis of available adeleorinid sequences (Barta *et al*. 2012) added more weight to that side of the argument, but with no molecular data from birds.

The only published molecular analysis of an avian *Hepatozoon*-like isolate is from Merino *et al*. (2006), who obtained it from the blue tit *Cyanistes caeruleus*; in their analysis the parasite was closely related to *Lankesterella* and was grouped outside other *Hepatozoon* species. No such doubts have been raised about the taxonomy of isolates from other terrestrial vertebrates (Barta *et al*. 2012). This suggests that the taxonomic position of avian *Hepatozoon* species should be revised and that more sequences from parasites identified morphologically as hepatozoa in birds need to be examined. Here we give a molecular description of a fragment 18S rDNA from putative *H. kabeeni* taken from the sedge warbler *Acrocephalus schoenobaenus*.

MATERIALS AND METHODS

We collected samples in a sedge warbler population from the Nida marshes (southern Poland) during the 2004–2006 breeding seasons. The birds were mist-netted and blood samples were obtained from the brachial veins of 131 adult birds of both sexes. A drop of blood was used to prepare thin smears, and the rest of the blood was preserved in 95% ethanol for molecular analyses. The smears were air-dried, fixed with 95% methanol and stained with Hemacolor (Merck). The slides were examined microscopically to find blood parasites, including putative *Hepatozoon kabeeni* according to the

* Corresponding author. Tel: +48 608 217 909. Fax: +48 12 632 24 32. E-mail: a.kloch@uj.edu.pl

doi:10.1017/S0031182012002004

Table 1. Measurements of the putative *Hepatozoon* species from the sedge warbler compared with those of *H. kabeeni* (Kruszewicz and Dyrcz 2000), *H. sylvae* (Shurulinkov and Chakarov 2006), and an avian lankesterellid (Merino *et al.* 2006)

(Measurements given in μm, standard deviations given in parentheses, *N* denotes number of measured infected cells examined. The Welch *t*-test shows the difference between measurements of putative *Hepatozoon* from current paper to those reported by other authors. After Bonferroni correction for multiple comparisons, the *P*-value corresponding to $\alpha = 0{\cdot}05$ is 0·016.)

		Avian Lankesterellid		*H. kabeeni*		*H. sylvae*	
	Current paper	measurements	Welch *t*-test	measurements	Welch *t*-test	measurements	Welch *t*-test
Length	8·9 (0·98)	9·0 (1·0)	$t = -0{\cdot}52$ $P = 0{\cdot}605$	9·82 (0·58)	$t = -4{\cdot}12$ $P < 0{\cdot}001$	9·2 (1·0)	$t = -1{\cdot}17$ $P = 0{\cdot}248$
Width	2·8 (0·42)	3·4 (0·7)	$t = -5{\cdot}87$ $P < 0{\cdot}001$	2·52 (0·24)	$t = 2{\cdot}98$ $P < 0{\cdot}001$	3·5 (0·5)	$t = -5{\cdot}75$ $P < 0{\cdot}001$
Length:width ratio	3·17	2·64	na	3·89	na	2·62	na
Area	21·7 (2·89)	27·2 (5·7)	$t = -7{\cdot}04$ $P < 0{\cdot}001$	nd	na	26·2 (4·8)	$t = -4{\cdot}16$ $P < 0{\cdot}001$
	$N = 40$	$N = 81$		$N = 13$		$N = 24$	

Fig 1. *Hepatozoon*-like parasite (arrow) from the sedge warbler, magnification 1600×.

description given by Kruszewicz and Drycz (2000). For each slide 100 fields at 1600× (Nikon 50i light microscope) were checked. In each of a random subset of 40 smears 1 parasite was measured (length, width, area) using ImageJ v. 1.42 software (Wayne Rasband, National Institutes of Health, USA).

Genomic DNA was extracted with the Nucleospin Tissue Kit (Macherey and Nagel, Germany). Part of 18S rDNA was amplified by PCR using primers Hep800F/Hep1615R as described by Merino *et al.* (2006). The PCR reaction contained 10 ng template DNA, 1·5 mM $MgCl_2$, 0·2 mM of each dNTP, 1 mM of each primer, and 0·5 U AmpliTaq (Applied Biosystems, Foster City, CA, USA). The reaction started from initial denaturation for 3 min at 94 °C followed by 40 cycles: 95 °C for 40 s, 60 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 10 min. Products were separated on 2% agarose gel to check whether amplification was successful.

All samples indicating infection with the putative *Hepatozoon* were sequenced in both directions using Hep800F/Hep1615R primers in an automated sequencer (ABI 310, Applied Biosystems). DNA sequences were aligned in CLUSTAL W (Larkin *et al.* 2007) and edited using BIOEDIT (Hall, 1999).

The phylogenetic relationship between the putative *Hepatozoon* and other apicomplexans was analysed using a maximum likelihood (ML) phylogenetic tree based on sequences characterized in the current study and those taken from GenBank. A set of trees was reconstructed in TREEFINDER (Jobb *et al.* 2004), and the best-fitting model of nucleotide substitution was selected based on the Akaike information criterion (AIC), using the FindModel web application (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). The Tamura-Nei plus gamma model had the lowest AIC, and thus it was implemented in subsequent analyses in TREEFINDER. For ML analysis we used the likelihood-ratchet method. Branch confidence values were estimated using the estimated likelihood weights approach (Strimmer and Rambaut, 2002).

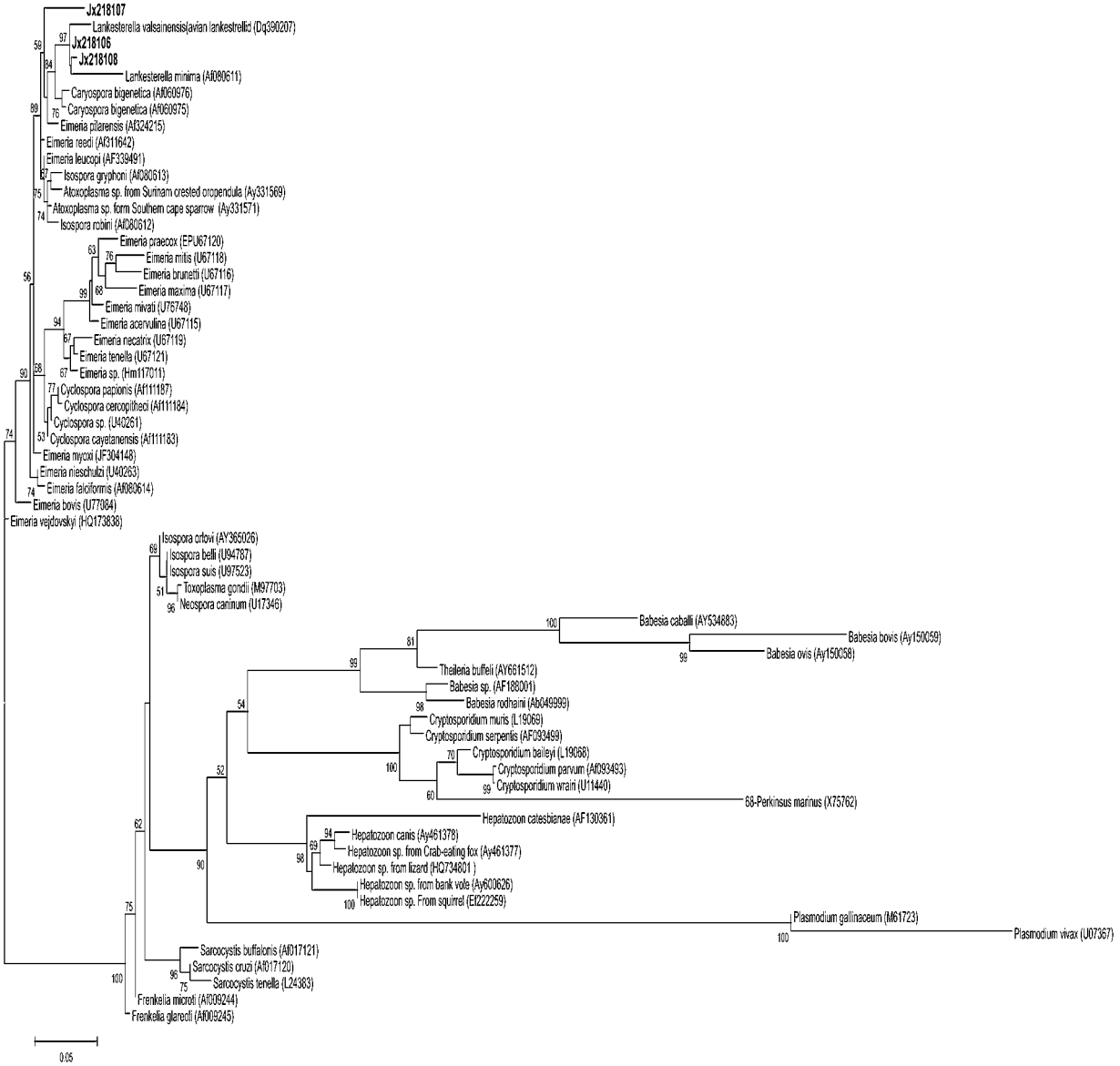

Fig. 2. Phylogenetic relationships of the analysed haplotypes, describing relationships between the putative *Hepatozoon* species from the sedge warbler and other apicomplexans. The sequences reported in the current paper are given in bold.

RESULTS

Overall, 3 parasite species were found: the most prevalent *Haemoproteus* was found in 45·3% of birds, putative *Hepatozoon* in 32·7% (Fig. 1) and *Plasmodium* in 1·6%. Infections with single parasites occurred in 29·7% of animals, with 2 parasites in 23·4%, and 3 parasite species were detected in 3 birds (1·6%). The size of the putative *Hepatozoon* from sedge warbler differed significantly from the measurements of *H. kabeeni* reported previously in sedge warbler, but it also differed in width and area from *H. sylvae* and avian Lankesterellid (Table 1).

Based on PCR, we found putative *Hepatozoon* in 131 sedge warblers (47·3%), and the prevalence was higher than those detected using microscopic examination, which indicates the higher sensitivity of molecular detection. All samples found to be positive by PCR were also identified as infected by microscopic examination.

In 62 sequenced samples we detected 3 haplotypes and submitted the sequences to the GenBank (Accession nos JX218106–JX218108). In the phylogenetic analysis, 2 of the putative *Hepatozoon* haplotypes closely resembled *Lankesterella minima* and *L. valsainensis* (Fig. 2). The third haplotype grouped in a wider clade that also included *Caryospora* and *Eimeria*. The analysed haplotypes did not show resemblance to sequences of hepatozoa from reptiles and mammals, which grouped separately in a distinct clade.

DISCUSSION

In this work we characterized 18S rDNA of a *Hepatozoon*-like parasite previously identified as

H. kabeeni (Kruszewicz and Dyrcz, 2000) from the sedge warbler. Little resemblance of the analysed sequences to those of hepatozoa from other mammalian and reptile hosts was found, but PCR gave positive results for the same samples that were found by microscopy to be infected, confirming that the primers we used amplified the right target. The putative *H. kabeeni* sequences were closely related to *Lankesterella*, and 1 haplotype grouped in a clade composed of various Eimeriidae. Our results are in accordance with the finding of Merino *et al.* (2006) that 18S rRNA sequences from a putative *Hepatozoon* species from the blue tit closely resemble those of *Lankesterella*.

The systematics of haemogregarines is problematic, as it is based mostly on morphological descriptions of the parasites, and knowledge of their life cycle is crucial for proper classification (Levine, 1982). Generally, 2 groups of avian parasites have circulating blood stages: gamonts of members of the suborder Adeleorina, including *Hepatozoon*, and circulating zoites (sporozoites or merozoites) of *Lankesterellidae* and *Eimeriidae* (Merino *et al.* 2006). None of them are easy to distinguish by morphology; because certain lankesterellid sporozoites and haemogregarine gamonts are very similar, some species designated as members of one group have later been assigned to the other (Desser, 1993; Merino *et al.* 2006). The systematic position of *Lankesterella* has been tossed around through the decades (Box, 1975; Desser, 1980; Levine, 1982; Upton, 2000). Addressing the systematics of *Hepatozoon*, Smith (1996) proposed classifying all haemogregarine infections in birds as *Hepatozoon* until we have enough data on the life cycle of these parasites to place them in the correct genus.

Hepatozoon kabeeni was described by Kruszewicz and Dyrcz (2000) in sedge warblers from Poland. Peirce (2005) did not list this species in his revision of the genus, and there are no reports of *H. kabeeni* from any other bird host. In the reed warbler *Acrocephalus scirpaceus*, a host closely related to the sedge warbler, Shurulinkov and Chakarov (2006) reported *H. sylvae*. The parasite we describe here differed in size and shape from both *H. kabeeni* and *H. sylvae*, and it was also smaller and longer than the avian lankesterellid that Merino *et al.* (2006) described. This suggests that it should not be recognized as a *Hepatozoon* species and that most likely it belongs to the genus *Lankesterella*.

The systematic position of this *Hepatozoon*-like parasite from the sedge warbler should be revised, and presumably the same applies to all *Hepatozoon*-like parasites of other avian hosts, as the findings of Merino *et al.* (2006) suggest. To do this, more sequences are needed. Knowledge of the parasite life cycle is considered crucial for proper classification of these species, but our results show that phylogenetic molecular analysis is a good alternative tool, particularly helpful when the parasite life cycle proves elusive.

FINANCIAL SUPPORT

This work was supported by a Polish Science Foundation grant (POMOST 2010-2/1) awarded to Aleksandra Biedrzycka.

REFERENCES

Barta, J. R., Ogedengbe, J. D., Martin, D. S. and Smith, T. G. (2012). Phylogenetic position of the Adeleorinid Coccidia (Myzozoa, Apicomplexa, Coccidia, Eucoccidiorida, Adeleorina) inferred using 18S rDNA sequences. *Journal of Eukaryotic Microbiology* **59**, 171–180.

Bennett, G. F., Earle, R. A. and Penzhorn, B. L. (1992*a*). *Ornithodoros peringueyi* (Argasidae) and *Xenopsylla trispinis* (Siphonaptera), probable intermediate hosts of *Hepatozoon atticorae* of the South African Cliff Swallow, *Hirundo spilodera*. *Canadian Journal of Zoology* **70**, 188–190.

Bennett, G. F., Earle, R. A. and Peirce, M. A. (1992*b*). New species of avian *Hepatozoon* (Apicomplexa: Haemogregarinidae) and a re-description of *Hepatozoon neophrontis* (Todd & Wohlbach, 1912) Wenyon, 1926. *Systematic Parasitology* **23**, 183–193.

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

Desser, S. S. (1980). An ultrastructural study of the asexual development of a presumed *Isospora sp.* in mononuclear, phagocytic cells of the evening grosbeak (*Hesperiphona vespertina*). *Journal of Parasitology* **66**, 601–612.

Desser, S. S. (1993). The Lankesterellidae. In *Parasitic Protozoa*, vol. 4 (ed. Kreier, J. P.), pp. 261–270. Academic Press, New York, USA.

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.

Jobb, G., von Haeseler, A. and Strimmer, K. (2004). TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. *BMC Evolutionary Biology* **4**, 18. doi:10.1186/1471-2148-4-18.

Kruszewicz, A. G. and Dyrcz, A. (2000). *Hepatozoon kabeeni* sp. (Protozoa: Apicomplexa; Hemogregarina) from the sedge warbler, *Acrocephalus schoenobaenus* (Aves: Passeriformes). *Wiadomosci Parazytologiczne* **46**, 507–510.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. (2007). ClustalW and ClustalX version 2. *Bioinformatics* **23**, 2947–2948.

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

Maia, J. P., Harris, D. J. and Perera, A. (2011). Molecular survey of Hepatozoon species in lizards from North Africa. *Journal of Parasitology* **97**, 513–517.

Merino, S., Marinez, J., Martinez-de la Puente, J., Criado-Fronelio, A., Tomas, G., Morales, J., Lobato, E. and Garcia-Fraile, S. (2006). Molecular characterization of the 18S rDNA gene of an avian *Hepatozoon* reveals that it is closely related to *Lankesterella*. *Journal of Parasitology* **92**, 1330–1335.

Peirce, M. A. (2005). A checklist of the valid avian species of *Babesia* (Apicomplexa: Piroplasmida), *Haemoproteus, Laucocytozoon* (Apicomplexa: Haemosporida) and *Hepatozoon* (Apicomplexa: Haemogregarinidae). *Journal of Natural History* **39**, 3621–3632.

Shurulinkov, P. and Chakarov, N. (2006). Prevalence of blood parasites in different local populations of reed warbler (*Acrocephalus scirpaceus*) and great reed warbler (*Acrocephalus arundinaceus*). *Parasitology Research* **99**, 588–592.

Smith, T. G. (1996). The genus *Hepatozoon* (Apicomplexa: Adeleina). *Journal of Parasitology* **82**, 565–585.

Smith, T. G. and Desser, S. S. (1997). Phylogenetic analysis of the genus *Hepatozoon* Moller, 1908 (Apicomplexa: Adeleorina). *Systematic Parasitology* **36**, 213–221.

Strimmer, K. and Rambaut, A. (2002). Inferring confidence sets of possibly misspecified gene trees. *Proceedings of Royal Society of London, B* **269**, 137–142.

Upton, S. J. (2000). Suborder Eimeriorina Lefer, 1911. In *The Illustrated Guide to the Protozoa*, 2nd Edn, Vol. 1. (ed. Lee, J. J., Leedale, G. F. and Bradbury, P.), pp. 318–339. Allen Press, Inc. Lawrence, KS, USA.