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SHORT COMMUNICATION

Haematospirillum and insect *Wolbachia* DNA in avian blood

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Abstract In this study, blood samples of 259 *Acrocephalus* sp. warblers were molecularly analysed for Anaplasmataceae and Rhodospirillaceae based on PCR amplification of 16S rRNA gene fragments. One bird blood sample (from Reed Warbler, *Acrocephalus scirpaceus*) yielded a sequence with 99.8% identity to *Haematospirillum jordaniae*. This is the first molecular evidence for the occurrence of this species in the blood of any vertebrate other than human. Another bird blood sample (from Marsh Warbler: *Acrocephalus palustris*) yielded a *Wolbachia* sequence, closely related to a moth endosymbiont with 99.8% identity. A nematode origin of *Wolbachia* DNA detected here in avian blood can be excluded, because results of phylogenetic analysis showed its closest alignment with insect wolbachiae. This is the first finding of insect *Wolbachia* DNA in the circulatory system of birds, which can be explained either by the inoculation of wolbachiae by blood-sucking vectors, or passing of *Wolbachia* DNA from the gut into the blood of this insectivorous bird species.

Keywords Anaplasmataceae · Aves · Rhodospirillaceae · 16S rRNA gene

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Introduction

Birds (Aves) are the second largest class of vertebrates, with a unique mechanism of flying, which allows them to bridge even large geographical distances. This trait also contributes to their increasingly recognized epidemiological significance, for instance, in the context of spreading avian influenza viruses (Xu et al. 2016). Migratory birds were also shown to play an important role in the dispersal of ticks and tick-borne pathogens (Hornok et al. 2012).

Representatives of the family Anaplasmataceae (Proteobacteria: Alphaproteobacteria: Rickettsiales) are Gram-negative, intracellular bacteria, which are frequently associated with arthropods, most notably as pathogens transmitted by blood-sucking vectors into their vertebrate hosts (hence their veterinary significance: Dumler et al. 2001). Based on molecular

evidence, *Anaplasma* and *Ehrlichia* spp. pathogenic to mammals may occur in avian blood, as demonstrated in Hungary (Hornok et al. 2014) and in Brazil (Machado et al. 2012). However, not only pathogenic microorganisms infect birds. For instance, a broad range of bacteria (predominated by Proteobacteria) can be found even in the blood of healthy chickens (Mandal et al. 2016).

With the aim of screening blood DNA extracts for members of Anaplasmataceae and related Proteobacteria, three *Acrocephalus* spp. were chosen as sample sources for this study, because they are insectivorous and long distance migrants, thus with increased chances of contact with vector-borne bacteria from a broad geographical range. The 16S rRNA gene was used as the target of molecular analyses, on account of its suitability for effective genus/species level classification of relevant bacteria (Chakravorty et al. 2007; Wang and Qian 2009).

Materials and methods

In this study, blood samples of 259 *Acrocephalus* sp. warblers (77 Reed Warblers: *Acrocephalus scirpaceus*, 99 Sedge Warblers: *Acrocephalus schoenobaenus*, 83 Marsh Warblers: *Acrocephalus palustris*) were molecularly analysed. These birds were captured with standard Ecotone mist-nets (12 m in length, 2.5 m in height and with 16 mm mesh) (Gdynia, Poland) at Ócsa bird ringing station (47.2967°N, 19.2101°E) from July till October in 2013. Sample sources included 204 young and 55 more than 1 year old birds.

Approx. 80 µl blood sample was taken from the surface-sterilized brachial vein of each bird with a capillary tube, which (when filled) was immediately put into 96% ethanol. DNA was extracted with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, Massachusetts, USA) according to the manufacturer's instructions. Molecular sexing was performed using the primer pairs P2–P8 (Griffiths et al. 1998).

All samples were preliminarily screened for representatives of the family Anaplasmataceae by a conventional PCR amplifying a 345-bp portion of the 16S rRNA gene and by visualization of the PCR product in a 1.5% agarose gel (Hornok et al. 2008). Based on primer alignments (and personal experience), this method also amplifies some members from a related order, Rhodospirillales (Gupta and Mok 2007), i.e. Acetobacteraceae and Rhodospirillaceae.

According to the results of sequencing, which identified *Haematospirillum* and *Wolbachia* DNA, all samples were re-tested with two conventional PCRs amplifying a longer (approx. 700 bp) fragment of the 16S rRNA gene of the relevant taxa. For the detection of *Haematospirillum* sp. two newly designed primers, HSPIR-F (5′—CGC GTG GGA ACA TGC CCT GA—3′) and HSPIR-R (5′—TGG CAC TCA TCG TTT ACA GC—3′) were used. Five µl of extracted DNA were added to 20 µl of reaction mixture containing 1.0 U HotStar Taq Plus DNA Polymerase (5U/µl) (QIAGEN, Hilden, Germany), 0.5 µl dNTP Mix (10 mM), 0.5 µl of each primer (50 µM), 2.5 µl of 10 × Coral Load PCR buffer (15 mM $MgCl_2$ included), and 15.8 µl DW. An initial denaturation step at 95 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 7 min then kept at 4 °C.

In addition, from the sample yielding the short 16S rRNA gene sequence of *Wolbachia*, the amplification of a longer (approx. 650 bp fragment) was also attempted with the following PCR. The two primers were the newly designed WOLB-F (5′—TAT AGG AAT CTA CCT AGT AG—3′) and the primer EHR16SR (5′—TAG CAC TCA TCG TTT ACA GC—3′) (Brown et al. 2001). The 25 µl PCR mix included 5 µl DNA template, 15.8 µl DW, 2.5 µl CoralLoad PCR buffer (15 mM $MgCl_2$ incl.), 0.5 µl PCR nucleotid mix, 0.5 µl of each forward and reverse primers and 0.2 µl HotStar Taq Plus DNA Polymerase (5U/µl). An initial denaturation step at 95 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 40 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 7 min then kept at 4 °C. Sequence-verified DNA extracts containing *Haematospirillum jordaniae* and *Wolbachia* served as positive controls.

PCR products were sequenced at BIOMI Inc. (Gödöllő, Hungary). Obtained sequences were compared to data in GenBank by nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Sequences were submitted to GenBank (Accession Numbers MF374623-4). Phylogenetic analysis was conducted with the Maximum Likelihood method (Jukes-Cantor model) by using MEGA version 6.0.

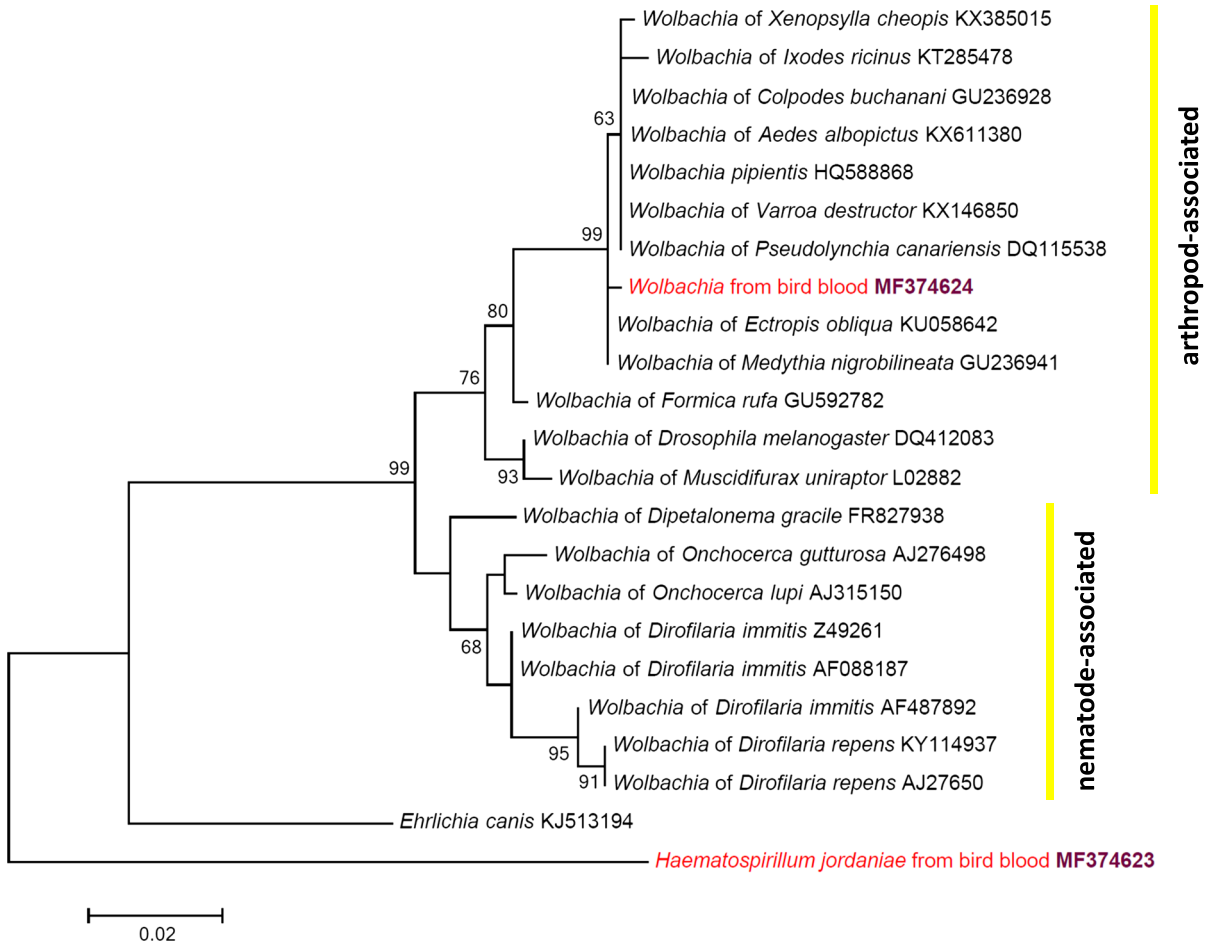

Fig. 1 Phylogenetic tree of *Wolbachia* according to isolation sources, showing the position of *Wolbachia* from bird blood in this study (red colour) within the cluster of arthropod-associated wolbachiae. All sequences written in black colour were retrieved from GenBank, and their sources, i.e. arthropod and nematode species, are shown according to the GenBank database (irrespectively to whether they were published in peer-reviewed journal or not). Two sequences were used as outgroups: one from *Ehrlichia canis* (within family Anaplasmataceae) and that of *Haematospirillum jordaniae* (red colour) obtained in this study (outside family Anaplasmataceae). Branch lengths represent the number of substitutions per site inferred according to the scale shown. (Color figure online)

Results and discussion

Samples analysed in this study included DNA extracts from the blood of 125 male and 134 female birds. Sequencing was successful from two samples, which were positive in the PCR amplifying the shorter (345 bp) fragment of the 16S rRNA gene of Anaplasmataceae and related families. Their positivity was confirmed by sequencing a longer portion of their 16S rRNA gene.

In particular, one bird blood sample (from *A. scirpaceus*, male, older than 1 year) yielded a sequence of *Haematospirillum jordaniae* (family Rhodospirillaceae), with 99.8% (i.e. 636/637 bp) identity to that of strain H5569 (GenBank Accession Number KM083603) recently discovered and described from human patients (Humrighouse et al. 2016). This is the first molecular evidence of the occurrence of this species in the blood of any vertebrate other than human. For instance, Rhodospirillaceae were not found in the blood of chickens, although a broad range of Proteobacteria were identified (Mandal et al. 2016). *Haematospirillum* was hitherto only detected in men of advanced age (Humrighouse et al. 2016). Its pathogenic role is uncertain, although human patients infected with this

species had clinical signs of febrile illness, relevant to septicaemia (Humrighouse et al. 2016).

In the present context it may also be relevant to note that *Novispirillum*, close relatives of *Haematospirillum* (Humrighouse et al. 2016) abound in the environment of the *Acrocephalus* spp. studied here, i.e. in the water of ponds (Yoon et al. 2007). This justifies further investigations of the possible environmental origin of these bacteria (or at least of their DNA) in avian blood.

The second PCR-positive bird blood sample (from *A. palustris*, young male) yielded a *Wolbachia* sequence, closely related to an endosymbiont of the moth *Ectropis obliqua* (Lepidoptera: Geometridae), with 99.8% identity (i.e. 647/648 bp in comparison with KU058642). *Wolbachia* (family Anaplasmataceae) are intracellular symbiotic bacteria of mainly arthropods and nematodes. Arthropod- and nematode-associated wolbachiae belong to different genetic lineages and phylogenetically cluster separately (traditionally divided into clades A + B and D + C, respectively: Fenn et al. 2006).

Wolbachia DNA from filaroid nematodes (which are in contact with the haemolymphatic system) can be detected in the blood circulation of mammals (Rossi et al. 2010). Birds may also harbour filaroid nematodes in their blood (Hamer et al. 2013), but (at least) some of their ornithophilic species in Onchocercinae do not have *Wolbachia* infection (McNulty et al. 2012). However, a nematode origin of *Wolbachia* DNA in avian blood can be excluded here in another way. The results of sequence comparison showed the highest similarity to an insect *Wolbachia*, and this was confirmed by the phylogenetic analysis, because the *Wolbachia* sequence amplified here from bird blood belonged to the group of arthropod- (closest to insect-) associated wolbachiae (Fig. 1).

Another member of Anaplasmataceae, the tick endosymbiont *Midichloria mitochondrii* is known to occur in the blood of animals and humans, possibly after inoculation by the tick (Bazocchi et al. 2013; Serra et al. 2016). However, to the best of our knowledge, inoculation of *Wolbachia* by blood sucking arthropods which harbour it (e.g. the mosquitoe *Culex pipiens*) was not reported. On the other hand, it is also possible, that the *Wolbachia* DNA detected here in avian blood originates from insect food items, as *Acrocephalus* spp. are insectivorous and the DNA from food might pass the gut barrier at least in mammals (Spisák et al. 2013).

Although only a single positive sample was found for each taxon identified, the actual population level prevalence maybe higher for these bacteria, taking into account the very small volume of blood used for molecular analyses.

Data availability

The sequences generated during the current study are available in the GenBank repository, under Accession Numbers MF374623-4. All other relevant data are contained in the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Research was permitted by Middle-Danube-Valley Inspectorate for Environmental Protection, Nature Conservation and Water Management (under Registration Number KTF: 27251-1/2014). Blood sampling was performed by Nóra Ágh (Certificate Registration Number 6/2015, issued by the Institutional Animal Welfare Committee).

Informed consent Informed consent was obtained from all individual participants included in the study.

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