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Molecular characterization of *Haemoproteus sacharovi* (Haemosporida, Haemoproteidae), a common parasite of columbiform birds, with remarks on classification of haemoproteids of doves and pigeons

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Abstract

Haemoproteus (Haemosporida, Haemoproteidae) is the largest genus of avian haemosporidian parasites, some species of which cause lethal diseases in birds. Subgenera *Parahaemoproteus* and *Haemoproteus* are usually accepted in this genus; these parasites are transmitted by biting midges (Ceratopogonidae) and hippoboscid flies (Hippoboscidae), respectively. As of yet, species of *Parahaemoproteus* have not been reported to infect doves and pigeons (Columbiformes), parasites of these birds have not been reported to be transmitted by biting midges (Ceratopogonidae). Applying microscopy and PCR based methods, we identified mitochondrial cytochrome *b* (cyt *b*) sequences of *Haemoproteus sacharovi*, a widespread parasite of doves and pigeons. Phylogenetic relationships of dove haemoproteids, which traditionally have been classified in the subgenus *Haemoproteus*, showed that *H. sacharovi* and *H. turtur*, common parasites of doves, branch in the clade with *Parahaemoproteus* species, indicating that these haemoproteids may belong to this subgenus and are likely transmitted by biting midges. This study provides barcodes for *H. sacharovi*, clarifies the taxonomic positions of *H. sacharovi* and *H. turtur*, and indicates directions for development of classification of avian haemoproteid species. Our analysis shows that the current subgeneric classification of avian haemoproteids is generally effective, but the position of some species may need to be revised.

Key words: *Haemoproteus*, *Parahaemoproteus*, haemosporidians, Columbidae, Hippoboscidae, Ceratopogonidae, barcoding

Introduction

Avian haemosporidians (Protista, Haemosporida) are a phylogenetically distinct and evolutionarily successful group of heteroxenous protists exclusively transmitted by blood-sucking dipterans (Levine, 1988). These protists parasitize many birds throughout the world (Greiner *et al.* 1975; McClure *et al.* 1978; Bishop & Bennett, 1992; Valkiūnas 2005) and have been the subject of extensive research for over 120 years. In some bird populations, between 50–100% of the individuals are infected with these blood parasites (Valkiūnas *et al.* 2003; Fernandez *et al.* 2010; Belo *et al.* 2011). Lethal cases of haemosporidian infections occur both in domestic and wild birds (Garnham 1966; Ferrell *et al.* 2007; Atkinson & LaPointe 2009) emphasizing the importance of the extended investigations of these parasites, particularly in light of emerging highly virulent haemoproteosis recently documented among captive parrots in Europe (Olias *et al.* 2011).

According to current taxonomy (Valkiūnas 2005), *Haemoproteus* is the largest genus of avian haemosporidian parasites; over 140 morphologically distinct parasites have been described and are categorized into subgenera *Haemoproteus* and *Parahaemoproteus*. Species of these subgenera are transmitted by hippoboscid flies

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(Hippoboscidae) and biting midges (Ceratopogonidae), respectively. Most of avian haemoproteids belong to the subgenus *Parahaemoproteus* and parasitize birds of many different orders; they are particularly diverse and prevalent in terrestrial birds, but have not been reported in doves and pigeons (Columbiformes). According to current knowledge, the subgenus *Haemoproteus* contains eight species, which parasitize numerous bird species of Columbiformes (Valkiūnas *et al.* 2010a), one species of Pelecaniformes (Levin *et al.* 2011) and one species of Charadriiformes (Levin *et al.* 2012). Barcoding of the majority of these parasites has not been developed.

Recent molecular studies, particularly of the mitochondrial genes, have revealed a huge genetic diversity of avian haemosporidians (Perkins & Shall 2002; Waldenström *et al.* 2002; Bensch *et al.* 2004; Ricklefs *et al.* 2004; Hellgren *et al.* 2007a; Martinsen *et al.* 2008). Furthermore, phylogenies based on mitochondrial lineages have demonstrated that many species belonging to the subgenera *Parahaemoproteus* and *Haemoproteus* can be clearly distinguishable in the phylogenetic trees, which can be used for determining the subgeneric position of avian haemoproteids (Hellgren *et al.* 2007b; Valkiūnas *et al.* 2007; Martinsen *et al.* 2008; Levin *et al.* 2011, 2012). Determining subgenera of these parasites is difficult using microscopy because blood stages of *Parahaemoproteus* and *Haemoproteus* parasites are similar, so the time and labor consuming detection of vector stages have been used for determining the subgeneric identity of these pathogens. Thus, the development of barcodes for identification of these parasites is an important task.

According to the MalAvi database (Bensch *et al.* 2009) the majority of the reported haemoproteid lineages represent species within the subgenus *Parahaemoproteus*; only a few lineages of *Haemoproteus columbae* represent the subgenus *Haemoproteus* in most of the former phylogenetic analyses (Waldenström *et al.* 2002; Križanauskienė *et al.* 2006; Martinsen *et al.* 2008). Recently, Santiago-Alarcon *et al.* (2010) showed that the genetic diversity of the subgenus *Haemoproteus* is also vast. The morphospecies *Haemoproteus multipigmentatus*, which exhibits high intraspecific genetic divergence, was described in the endemic Galapagos dove *Zenaida galapagoensis* (Valkiūnas *et al.* 2010a). Unexpectedly, it was recently shown that *Haemoproteus iwa*, a parasite of frigate birds (Fregatidae, Pelecaniformes) and *Haemoproteus jenniae*, a parasite of gulls (Lariidae, Charadriiformes) also belongs to the hippoboscid-transmitted parasites of subgenus *Haemoproteus* (Levin *et al.* 2011, 2012). However, molecular data about other species placed in the subgenus *Haemoproteus* are absent and their position in the phylogenetic tree remains unknown. Using sequence data from 3 genomes (mitochondrial, nuclear and plastid genomes) Martinsen *et al.* (2008) showed that *Haemoproteus* (*Haemoproteus*) *turtur*, a common parasite of doves, clusters with *Parahaemoproteus* lineages in the phylogenetic tree. This unexpected taxonomic issue needs clarification because *H. turtur,* the widespread parasite of doves, was reported to complete development in the hippoboscid fly, *Pseudolynchia canariensis* (Rashdan 1998). So, it should belong to the subgenus *Haemoproteus*. This point has been discussed in several recent studies (Valkiūnas *et al.* 2008b; Santiago-Alarcon *et al.* 2010; Levin *et al.* 2011), but remains unexplained.

The main aim of this study was to acquire sequence data of *Haemoproteus sacharovi* (Novy & MacNeal 1904) and to clarify the classification of avian haemoproteids on the levels of genera and species groups. For the first time we obtained mitochondrial cytochrome *b* gene (cyt *b*) sequences of *H. sacharovi*, identified phylogenetic relationships of this parasite with other haemosporidians, and conclude that this haemoproteid likely belongs to *Parahaemoproteus.*

Materials and methods

Study sites, collection of blood samples and examination of blood smears. The blood samples from 38 mourning doves (*Zenaida macroura*) and 11 Socorro ground doves (*Columbina passserina socorroensis*) were collected in Northern California and Socorro Island in 2009, respectively. Birds were caught with mist nets. Approximately 50 µl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were fixed in lysis buffer (Sehgal *et al.* 2001); they were held at ambient temperature in the field and later at –20 C in the laboratory. Blood films were air-dried, fixed in absolute methanol in the field and stained with Giemsa in the laboratory, as described by Valkiūnas *et al.* (2008a). All birds were released after collecting blood samples.

An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides and to prepare illustrations. Blood films were examined for 10–15 min at low magnification (×400),

and then at least 100 fields were studied at high magnification (×1,000), as described by Valkiūnas *et al.* (2008a). Because lineage of *H. sacharovi* was determined for the first time during this study, we examined entire blood films with this parasite to ensure that this parasite is present in single infections in our samples. Haemosporidian parasites were identified according to Valkiūnas (2005). Images of *H. columbae*, *H. iwa*, *H. multipigmentatus*, *H. sacharovi*, and *H. turtur* (Fig. 1) were prepared from type and voucher preparations deposited in the Institute of Ecology, Nature Research Centre Vilnius, Lithuania (accession nos. 2905.87, 47741NS, 47727NS, 47739NS, 1315.87 respectively).

Molecular analysis. Total DNA from stored blood was extracted following a DNeasy kit protocol (Qiagen, Valencia, California). For genetic analysis, a nested-PCR protocol (Waldenström *et al.* 2004) was used to amplify a segment of the parasite cyt *b* gene using the two pairs of initial primers HaemNF and HaemNR2. These primers amplify longer fragments of cyt *b* gene of *Haemoproteus* and *Plasmodium*. For the second PCR, we used primers specific to *Haemoproteus* and *Plasmodium* spp., HaemF and HaemR2 (Bensch *et al.* 2000). One negative control (nuclease-free water) was used per every 8 samples to control for false amplification. No cases of false positive samples were found.

For sequencing we used procedures as described by Bensch *et al.* (2000). Fragments were sequenced from the 5´ end with the primer HaemF, and new lineages were sequenced from 3´ end with the primer HaemR2. We used dye terminator cycling sequencing (big dye) and the samples were loaded on an ABI PRISM ™ 3100 capillary sequencing robot (Applied Biosystems, Florida, USA). The new sequence from *H. sacharovi* was deposited in GenBank under accession number JX073258.

Phylogenetic analysis. To compose a data set for phylogenetic analysis, we used new sequences and those of morphologically characterized haemosporidian parasites, which were deposited in MalAvi database (Bensch *et al.* 2009). To increase the number of sequences of hippoboscid transmitted haemoproteids in our analysis, we also used partial mitochondrial cyt *b* gene sequences of two recently identified hippoboscid transmitted avian haemoproteids, *H. multipigmentatus* (Santiago-Alarcon *et al.* 2010) and *H. iwa* (Levin *et al.* 2011) and also several unidentified dove *Haemoproteus* spp., which likely belong to this subgenus. This provided an opportunity to extensively compare the sequences of parasites belonging to the subgenus *Haemoproteus* with those of *Parahaemoproteus* parasites and to clarify several taxonomic issues of avian haemoproteids. Sequences were carefully selected to avoid species misidentifications. Examples of studies linking parasite lineages with their morphospecies, see Križanauskienė *et al.* 2006, 2010; Palinauskas *et al.* 2007; Krone *et al.* 2008; Martinsen *et al.* 2008; Valkiūnas *et al.* 2008b, 2008c, 2010a, 2010b; Zehtindjiev *et al.* 2008, 2012; Bensch *et al.* 2009; Santiago-Alarcon *et al.* 2010. GenBank accession numbers and, when available, the lineage reference codes of MalAvi database (Bensch *et al.* 2009) are provided in Figure 2.

We used sequences from 23 unique partial mitochondrial cyt *b* lineages belonging to the *Haemoproteus* genus, 14 lineages belonging to *Plasmodium* genus and 7 lineages belonging to the *Leucocytozoon* genus. A *Plasmodium falciparum* lineage has been used as the outgroup. The sequences were edited and aligned using BioEdit 7.0.9.0 (Hall 1999). In total, sequences from 45 species were used in the alignment for Bayesian and maximum likelihood analysis.

The Bayesian phylogeny was constructed using mrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). We used the General Time Reversible model including invariable sites and variation among sites (GTR+I+G), as the best-fit model of DNA evolution, suggested by the jMODELTEST (Version 0.1.1) (Posada 2008). Two simultaneous runs were conducted with a sample frequency of every 100th generation over 3 million generations. Convergence in phylogeny estimation for each analysis was assessed using the program Tracer (Software available from http://tree.bio.ed.ac.uk/software/tracer/ accessed 1 September 2012) and used to indicate the appropriate ‘‘burn-in” period. The 25% of the trees were discarded as “burn-in” period. The remaining trees were used to construct a Majority rule consensus tree.

We used PAUP* (PAUP*: phylogenetic analysis using parsimony (* and other methods)) Version 4.0 (Swofford 2001), and the same General Time Reversible model including invariable sites and variation among sites (GTR+I+G), to estimate phylogenetic relationships among genera using maximum likelihood. Bootstrap support for branches was estimated based on 300 replicates. Both phylogenies were visualized using Tree View 1.6.6. (Software available from http://evolution.genetics.washington.edu/phylip/software.html accessed 1 September 2012). Genetic distances between the different lineages were calculated in MEGA 3.1 (Kumar *et al.* 2004).

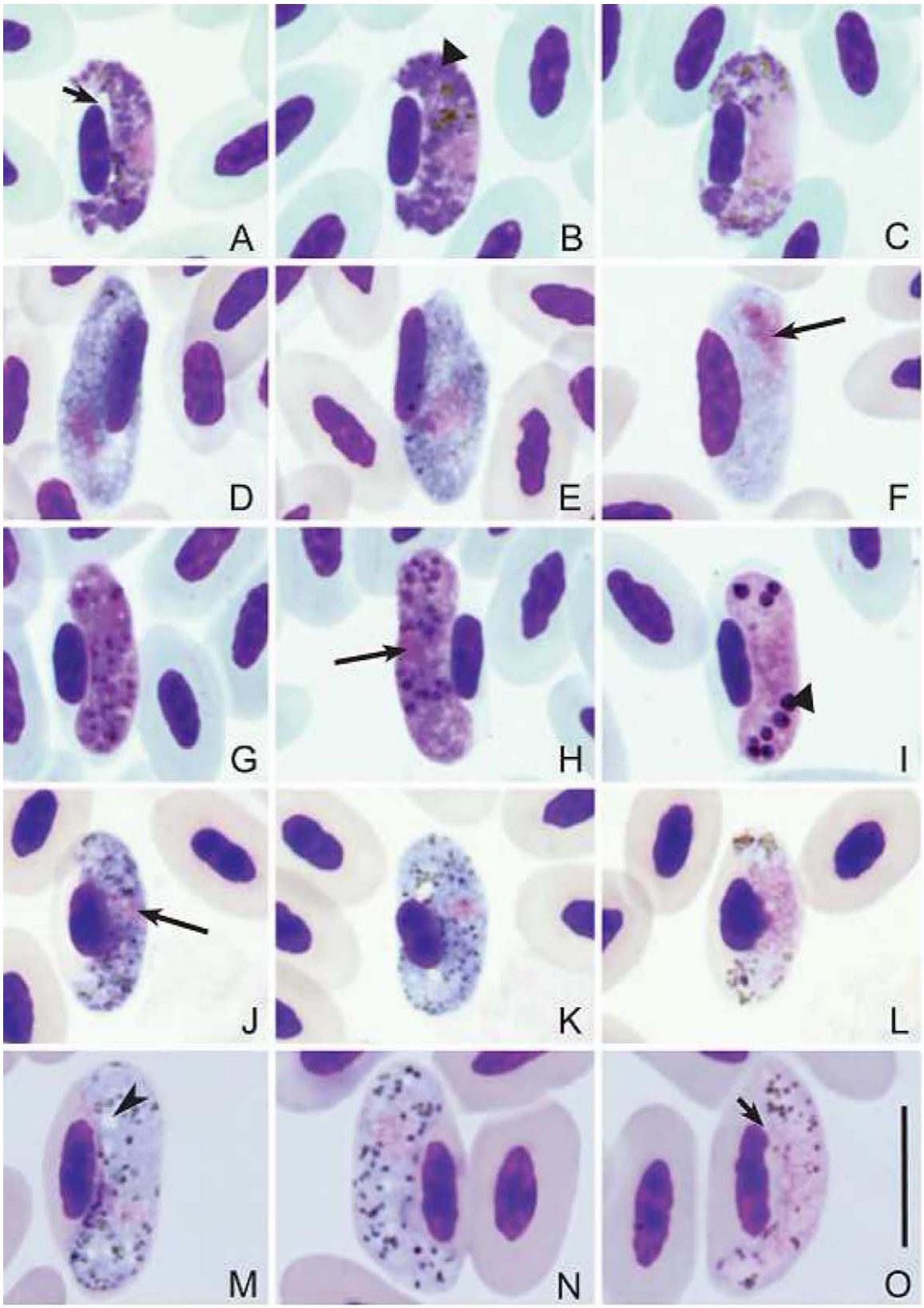

FIGURE 1. Mature gametocytes of haemoproteids of columbiform birds: *Haemoproteus* (*Parahaemoproteus*) *turtur* (A–C), *Haemoproteus* (*Parahaemoproteus*) *sacharovi* (D–F), *Haemoproteus* (*Haemoproteus*) *columbae* (G–I), *Haemoproteus* (*Haemoproteus*) *multipigmentatus* (J–L), and *Haemoproteus* (*Haemoproteus*) *iwa* (M–O). A, B, D, E, G, H, J, K, M, N—macrogametocytes; C, F, I, L, O—microgametocytes. Long arrows—nuclei of parasites; short arrows—unfilled spaces between gametocyte and nucleus of infected erythrocyte; triangle arrow heads—volutin granules; simple arrow head—vacuole. Giemsa-stained thin blood films. Bar = 10 µm.

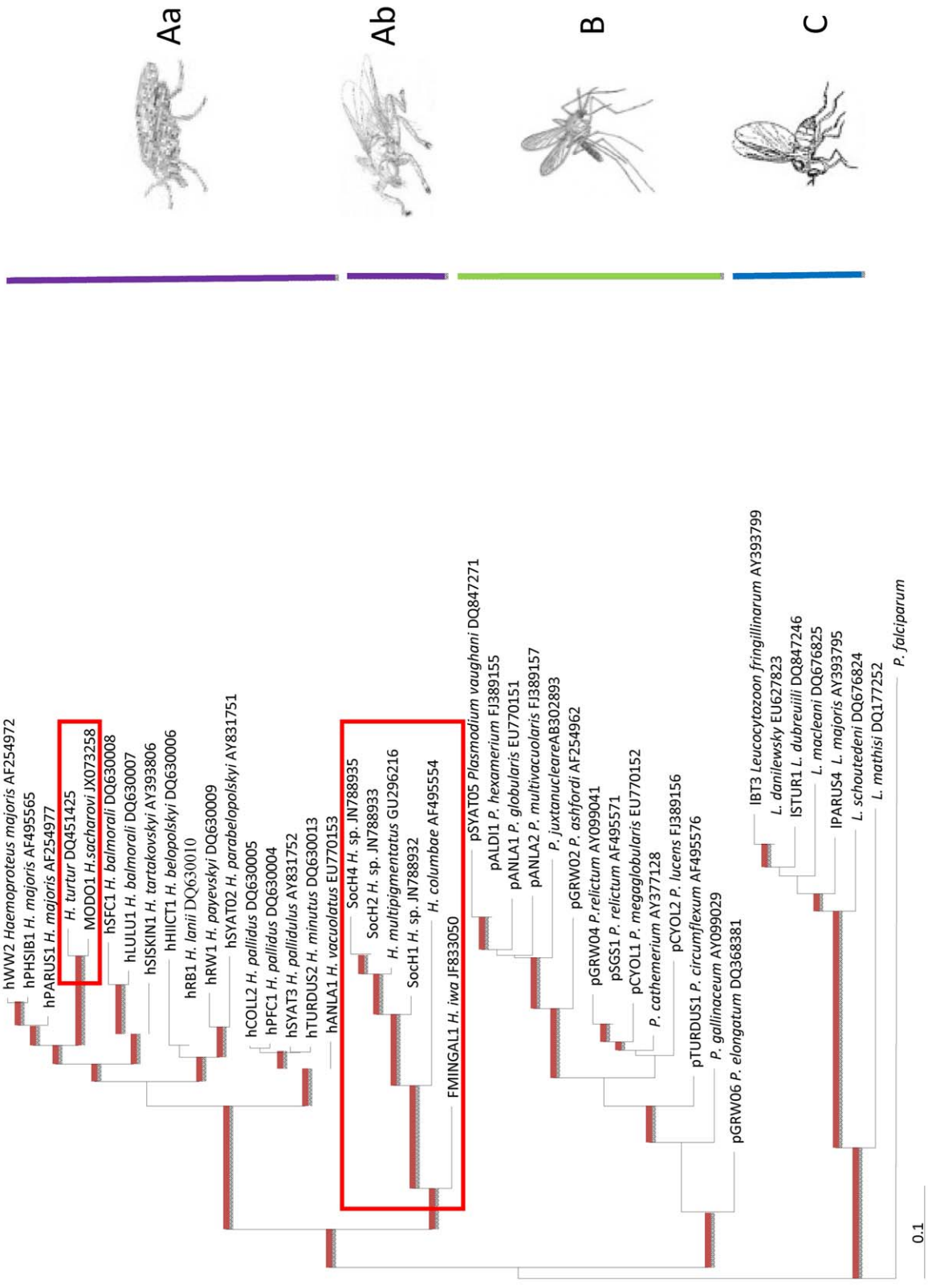

FIGURE 2. Evolutionary relationships among haemosporidian parasite (Haemosporida) mitochondrial cytochrome *b* gene lineages estimated using Bayesian and maximum likelihood analyses. Nodes with bootstrap support > 90% are highlighted in red. Names of the lineages (when available) are given before the species names of parasites; GenBank accession numbers of the lineages are provided after the parasite species names. Clade Aa representing species of *Parahaemoproteus* subgenus transmitted by biting midges (Ceratopogonidae), Ab—species of *Haemoproteus* subgenus transmitted by hippoboscid flies, B—species of *Plasmodium* genus transmitted by mosquitoes (Culicidae) , C—species of *Leucocytozoon* genus transmitted by black flies (Simulidae).

Results

Both Bayesian and Maximum likelihood phylogenies revealed three well supported major clades representing species of the genera *Plasmodium* (Fig. 2, B), *Leucocytozoon* (Fig. 2, C) and *Haemoproteus*, with two monophyletic subclades representing *Parahaemoproteus* and *Haemoproteus* (Fig. 2, Aa and Ab, respectively). The presence of highly supported monophyletic subclades Aa and Ab are in accord with the subgeneric classification of avian *Haemoproteus* spp., indicating that species of the subgenera *Haemoproteus* and *Parahaemoproteus* are closely related. Fourteen distinct lineages of identified morphospecies representing *Plasmodium* species, and 7 lineages representing *Leucocytozoon* species are clearly distinguishable in the phylogenetic tree and represent well-supported clades B and C (Fig. 2).

Blood stages of haemoproteids, which have been frequently recorded in columbiform birds and traditionally attributed to the subgenus *Haemoproteus* are shown in Fig. 1, A–O. Presence of a single infection *H. sacharovi* in mourning dove samples (Fig. 1, D–F) was proved by microscopic examination of entire blood films. DNA sequences of this parasite were reported for the first time during this study.

Surprisingly, not only the sequence of *H. turtur* (Fig. 1, A–C), but also *H. sacharovi* (lineage hMODO1) (Fig. 1, D–F), both common parasites of doves, appeared in the subclade Aa, which represents only biting midge transmitted haemoproteids (Fig. 2). Additionally, lineages of *H. turtur* and *H. sacharovi* clustered together with a genetic distance of 2.6 % between them and with mean distance of 5% from other lineages within this subclade.

The subgenus *Haemoproteus* (subclade Ab) includes 6 lineages representing at least three morphological species. The hippoboscid transmitted *H.* (*H*) *columbae* (Fig. 1, G–I), which is the type species of subgenus *Haemoproteus* and has been often used in phylogenetic studies of avian haemosporidians, clusters within this group of species. *Haemoproteus* (*H.*) *multipigmentatus* (Fig. 1, J–L) shows an intraspecific divergence with 4 distinct lineages (lineages SocH4, SocH2, hJH003W and SocH1) with a mean genetic distance of 1.8 % between them.

The gametocytes of the 5 species of haemoproteids used in this study have clear morphological differences and can be easily distinguishable from each other (Fig. 1). Fully-grown gametocytes of *H. turtur* usually do not touch the nucleus of erythrocytes (Fig. 1, A–C), they grow around the nucleus and markedly displace it laterally. Also they possess small or of medium size slightly elongated pigment granules. *Haemoproteus sacharovi* has a unique morphology among bird haemoproteids. Gametocytes of this parasite are similar to gametocytes of *Leucocytozoon* spp. and even can be confused with leucocytozoids, but they possess a few small pigment granules (Fig. 1, D–F). The average width of fully grown gametocytes of *H. sacharovi* is significantly greater than gametocytes of other species of haemoproteids. Mature gametocytes of *H. columbae* markedly displace the nuclei of infected erythrocytes and slightly enclose the nuclei with their ends; volutin and pigment granules are aggregated into large roundish masses (1 µm in diameter in microgametocytes), which mask pigment granules (Fig. 1, G–I). Fully grown gametocytes of *H. multipigmentatus* extend around nuclei of erythrocytes, enclose them with their ends, but do not encircle nuclei completely; pigment granules are small and numerous (Fig. 1, J–L). Gametocytes of *H. iwa* extend along nuclei of the erythrocytes and displace the nuclei laterally from early stages of their development; cytoplasm often possesses prominent vacuoles of variable size, volutin granules not seen (Fig. 1, M–O). It is worth noting, that based upon the gametocytes and other morphological features, it is straightforward to identify species of these haemoproteids. However, it is impossible to attribute them to *Haemoproteus* or *Parahaemoproteus* subgenera; molecular identification of these subgenera is essential.

Discussion

Species identification of haemosporidian parasites is based mainly on morphology of gametocytes and their host cells (Bennett & Peirce 1988; Atkinson 1991; Valkiūnas 2005). Numerous readily distinguished morphospecies of avian haemoproteids have been described, including haemoproteids of columbiform birds (Fig. 1). However, there are no gametocyte morphological characters, which allow subgeneric identification of avian haemoproteids. It is essential to develop barcodes for the subgeneric identification of haemosporidian parasites. Available data show that the use of mitochondrial sequences provides a convenient tool to distinguish species of *Parahaemoproteus* and *Haemoproteus* (Santiago-Alarcon *et al.* 2010; Levin *et al.* 2011, 2012). Mainly, species belonging to these two subgenera usually group in clearly different clades in phylogenetic trees (Fig. 2).

The subgeneric classification of several avian haemoproteid species has been recently revised due to application of molecular markers. Levin *et al.* (2011) showed that species of *Haemoproteus* subgenus are not only parasites of columbiform birds as traditionally believed; they provided evidence that *H. iwa* the haemoproteid of frigatebirds (Pelecaniformes) belongs to the *Haemoproteus* subgenus and is transmitted by the hippoboscid fly *Olfersia spinifera* (Hippoboscidae). This was an unexpected finding showing that host range of *Haemoproteus* species is not limited to species of Columbiformes. Additionally, Levin *et al.* (2012) recently described *H. jenniae*, adding additional species parasitizing non-columbiform birds and belonging to the subgenus *Haemoproteus*.

We confirmed results by Martinsen *et al.* (2008), according to which *H. turtur* appeared in the clade of species belonging to *Parahaemoproteus* subgenus. Furthermore, we found that *H. sacharovi* also appears in the same clade; moreover, it clusters with *H. turtur* and also many other species of *Parahaemoproteus*, which were proved experimentally to be transmitted by biting midges, i. e. *H. balmorali, H. lanii, H. tartakovskyi, H. parabelopolskyi* (Valkiūnas 2005). The presence of 2 species of haemoproteids of columbiform birds readily distinguishable by morphology of gametocytes (Fig. 1, A–C, D–F) in the clade of *Parahaemoproteus* species (Fig. 2) indicate that these 2 species are likely transmitted by biting midges. However, that contradicts available literature information (Huff 1932; Rashdan 1998). Here we provide a summary of previous literature data.

Transmission of *H. sacharovi* was studied by Huff (1932) who provided a morphological re-description of blood stages and did experimental work using *Pseudolynchia maura* (Hippoboscidae) as a possible vector of this haemoproteid. Huff (1932) concluded that *P. maura* is a likely vector, but neither detected nor described the development of *H. sacharovi* in this fly. It is important to note that, biting midges were unknown as vectors of haemoproteids at that time, so experimental birds could not protected from natural bites of these insects during Huff's (1932) experiments. Only later, Fallis and Wood (1957) proved evidence that biting midges can transmit avian haemoproteids. If the doves were kept unprotected from biting midges in Huff's (1932) study, then naturally infected biting midges could reach recipient birds and contaminate his experiments. Hence, the results of Huff's (1932) study should be interpreted with caution. This is particularly true because Huff (1932) mentioned that hippoboscid flies have not been recorded on the mourning doves (*Z. macroura*), the type host of *H. sacharovi* at the study site. Thus, the mentioned Huff's (1932) information and our data about position of *H. turtur* and *H. sacharovi* in the clade of *Parahaemoproteu*s spp. indicate that both these parasites are likely transmitted by biting midges. To clarify vectors of this parasite, additional experimental studies are needed.

Experimental studies performed by Rashdan (1998) showed, that the hippoboscid fly *Pseudolynchia canariensis* can be a vector of *H. turtur*; a weak infection was induced experimentally in the raised turtle dove *Streptopelia turtur.* In Rashdan's (1998) study, three groups of flies, previously infected with *H. turtur*, were allowed to feed on recipient birds *Columba livia*, *Streptopelia senegallus*, and *S. turtur.* Only turtle doves developed light parasitemia. It is important to note that all these experimental birds were kept in hippoboscid fly-proof pens, but were not protected from natural exposure to biting midges. Hence, the experimental vector study by Rashdan (1998) is questionable because it is difficult to rule out that *H. turtur* might have be transmitted by biting midges; that is in accord to phylogenetic data (Fig. 2) and needs additional experimental investigation.

Despite some uncertainties about phylogenetic relationships and the validity of haemosporidian subgenera and genera, the classification of haemosporidian parasites has been quite well developed (Garnham 1966; Valkiūnas 2005); it was supported in many points by recent molecular studies (Martinsen *et al.* 2008; Santiago-Alarcon *et al.* 2010; Levin *et al.* 2011, 2012). Levine and Campbell (1971) suggested the current subgeneric classification of avian haemoproteids by following a widely accepted scheme of subgeneric classification of malaria parasites of mammals and birds (Corradetti *et al.* 1963; Garnham 1966). Levine and Campbell (1971) attributed subgeneric status to two formerly described genera *Parahaemoproteus* and *Haemoproteus.* Parasites of both subgenera undergo markedly different sporogony (Baker 1966; Garnham 1966; Atkinson 1991; Valkiūnas 2005), but morphology of gametocytes of parasites belonging to these subgenera is similar, so cannot be used for subgeneric identification.

In spite of clear differences between species of *Haemoproteus* and *Parahaemoproteus*, the establishment of the subgeneric classification of avian haemoproteids (Levine & Cambell 1971) was accepted by many authors (Bennett & Peirce 1988; Atkinson 1991; Desser & Bennett 1993; Valkiūnas 2005). Such classification simplified the generic identification of species whose life cycles were incompletely studied, especially in the vectors, so it provided an opportunity to apply binomial nomenclature in species taxonomy and description of many new species without investigating parasite vectors. Importantly, the subgeneric classification of haemoproteids was in accord

with widely used subgeneric classification of genus *Plasmodium*, including human malaria parasites (Corradetti *et al.* 1963; Garnham 1966) and thus contributed to the uniformity of classification in different families of haemosporidians.

Our phylogenic analysis clearly separates major clades associated with particular vector groups (Fig. 2), and it supports a conclusion made by Martinsen *et al.* (2008) that the evolution of avian haemoproteids is related to vectors. However, it does not support their conclusion regarding the paraphyly of the subgenus *Haemoproteus* in relation to *Parahaemoproteus*. Our study shows that the subgenera *Haemoproteus* and *Parahaemoproteus* are likely monophyletic (Fig. 2) and the subgeneric classification of avian haemoproteids (Levine & Cambell 1971) is worth using, but the position of some species in certain subgenera needs clarification.

In conclusion, this study provides barcodes for the detection of *H. sacharovi* and shows the importance of combining microscopic and molecular methods, not only in taxonomy, ecology and evolutionary biology studies, but also in vector research of haemosporidian parasites. In addition, our work indicates new directions for experimental vector and vertebrate-host association research.

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