Parasitology International xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite

Vaidas Palinauskas [a,*], Tatjana A. Iezhova [a], Asta Križanauskienė [a], Mikhail Yu. Markovets [b], Staffan Bensch [c], Gediminas Valkiūnas [a]

[a] Nature Research Centre, Akademijos 2, Vilnius-21, LT-08412, Lithuania
[b] Biological Station Rybachy, Zoological Institute, Russian Academy of Sciences, Rybachy 238535, Kaliningrad Reg., Russia
[c] Department of Animal Ecology, Ecology Building, Lund University, SE-22362 Lund, Sweden

ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 21 March 2013
Accepted 22 March 2013
Available online xxxx

Keywords:
Haemosporidians
Haemoproteus minutus
hTUPHI1
hTUCHR01
Barcoding
Microscopy
Turdidae
Distribution

ABSTRACT

Recently, the lineage hTURDUS2 of *Haemoproteus minutus* (Haemosporida, Haemoproteidae) was reported to cause mortality in captive parrots. This parasite lineage is widespread and prevalent in the blackbird *Turdus merula* throughout its entire distribution range. Species identity of other closely related lineages recently reported in dead parrots remains unclear, but will be important to determine for a better understanding of the epidemiology of haemoproteosis. Using polymerase chain reaction (PCR)-based and microscopic methods, we analyzed 265 blood samples collected from 52 species of wild birds in Eurasia (23 samples from Kamchatka Peninsula, 73 from Sakhalin Island, 150 from Ekaterinburg and 19 from Irkutsk regions of Russia). Single infections of the lineages hTURDUS2 (hosts are redwing *Turdus iliacus* and fieldfare *Turdus pilaris*), hTUPHI1 (song thrush *Turdus philomelos*) and hTUCHR01 (fieldfare, redwing, song thrush and brown-headed thrush *Turdus chysolaus*) were detected. We identified species of these haemoproteids based on morphology of their blood stages and conclude that these lineages belong to *H. minutus*, a widespread parasite of different species of thrushes (genus *Turdus*), which serve as reservoir hosts of this haemoproteid infection. Phylogenetic analysis shows that the lineages hTURDUS2, hTUCHR01 and hTUPHI1 of *H. minutus* are closely related to *Haemoproteus pallidus* (lineages hPFC1 and hCOLL2), *Haemoproteus pallidulus* (hSYAT03), and *Haemoproteus* sp. (hMEUND3); genetic distance among their mitochondrial cytochrome *b* (cyt *b*) lineages is small (<1% or < 4 nucleotides). All these blood parasites are different in many morphological characters, but are similar due to one feature, which is the pale staining of their macrogametocytes' cytoplasm with Giemsa. Because of the recent publications about mortality caused by the lineages hTUPHI1 and hTURDUS2 of *H. minutus* in captive parrots in Europe, *H. minutus* and the closely related *H. pallidus* and *H. pallidulus* are worth more attention as these are possible agents of haemoproteosis in exotic birds. The present study provides barcodes for molecular detection of different lineages of *H. minutus*, and extends information about the distribution of this blood parasite.

1. Introduction

Species of *Haemoproteus* (Haemosporida, Haemoproteidae) are widespread haemosporidians parasitizing birds on all continents, except Antarctica. Over 140 species of avian *Haemoproteus* have been described [1–6]. The majority of these parasites are transmitted by biting midges (Ceratopogonidae); a few species are transmitted by hippoboscid flies (Hippoboscidae) [7]. Traditionally, these blood parasites are considered to be relatively benign to their avian hosts and vectors [8]. However, some *Haemoproteus* species affect host fitness and cause severe pathology in birds, sometimes even lethal [9–13].

Recent studies have provided further evidence that some lineages of *Haemoproteus* spp. cause lethal disease in captive birds. This has been demonstrated in zoos and private collections in America [13,14] and Europe [15], using both histopathology and PCR-based methods. These studies revealed hepatocellular necrosis and megalomeronts of haemoproteids in dead birds, indicating the parasite-induced pathology. However, the extent of mortality caused by *Haemoproteus* parasites remains insufficiently investigated, especially in wildlife. Such lethal infections [13–15] are difficult to diagnose by method investigating samples from blood because severe haemoproteosis and death of infected birds occur during the tissue stage of parasite development before appearance of blood stages [16] as was the case in *Haemoproteus minutus* (lineage hTURDUS2), which caused mortality of captive parrots in Europe [15]. This parasite is widespread and prevalent in the blackbird *Turdus merula* throughout its entire distribution range, including major parks of European cities [17,18]. However, species identity of the closely related *Haemoproteus* lineages hMEUND3 and hTUPHI1 and the identical *H.* spp. lineages hCYAUR1 and hMEUND1, which were

* Corresponding author. Tel.: +370 5 272 92 69; fax: +370 5 272 93 52.
E-mail address: vaidas@ekoi.lt (V. Palinauskas).

1383-5769/$ – see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.parint.2013.03.006

reported in dead parrots [15], needs clarification; that is important for better understanding of the disease epidemiology.

The aims of this study were (1) to specify species identity, prevalence and natural host range of some *Haemoproteus* spp. lineages, which are responsible for mortality of parrots in Europe, and (2) to identify closely related *Haemoproteus* parasites, which might be causative agents of similar diseases in avian hosts. This study is also one of the first to provide genetic information about the distribution of avian haemoproteids in territories of Russia, including Siberia and the Far East.

2. Materials and methods

2.1. Collection of blood samples

The samples were collected during ornithological expeditions in Ekaterinburg (57° 20′ N, 59° 49′ E, and 56° 50′ N, 58° 36′ E) and Irkutsk districts (51° 34 N, 103° 54′ E) in 2004 (for sampling details see [19]) and in Kamchatka Peninsula and Sakhalin Island (53° 06′ N, 158° 42′ E and 47° 04′ N, 142° 58′ E) in 2007. Information about distribution of avian blood parasites is absent from these regions of Russia. In all, 265 birds belonging to 52 species were sampled (Table 1). The birds were caught with mist nets and banded. Blood samples were collected from the brachial vein and birds were then released; none of them was recaptured.

Approximately 50 μl of whole blood was drawn from each bird for subsequent molecular analysis. The samples were fixed in SET buffer [20]; they were held at ambient temperature in the field and later at −20 °C in the laboratory.

Two or three blood films were prepared from each bird. Blood films were air-dried within 5–10 s after their preparation, fixed in absolute methanol in the field and then stained with Giemsa in the laboratory. Blood samples, which were PCR-positive for lineages reported in parrots [15] or closely related lineages, were examined microscopically.

2.2. Morphological analysis

Blood films were examined for 10–15 min at low magnification (×400) and then at least 100 fields were studied at high magnification (×1000). Detailed protocols of fixation, staining and microscopic examination of blood films were described by [4]. To ensure absence of simultaneous infections with other *Haemoproteus* parasites in our samples, the entire blood films from voucher series were examined microscopically at low magnification.

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine slides, take measurements and prepare illustrations (Fig. 1). Parasite species were identified according to [3] and [5].

2.3. DNA extraction, PCR amplification and sequencing

DNA was extracted from whole blood following a standard phenol–chloroform protocol [21]. For genetic analysis we used a nested-PCR protocol [20]. In the first PCR we used the primers HaemNFI [5′-CATATATTAAGAGAAITATGGAG-3′] and HaemNR3 [5′-ATAGAAAGATAAGAAATACCATTC-3′] [20]. In the second PCR we used the primers HAEMF [5′-ATGGTGCTTTCGATATATGCATG-3′] and HAEMR2 [5′-GCATTATCTGGATGTGATAATGGT-3′] [22]. The PCR conditions with outer primers HaemNFI and HaemNR3 were as described in [20]. Incubation of samples before the cycling reaction was at 94 °C for 3 min and after the cycling reaction at 72 °C for 10 min. The cycling reaction started with 94 °C for 30 s, then 50 °C for 30 s and 72 °C for 45 s. The PCR

Table 1

Birds caught and examined at different study sites in Russia (2004, 2007) with outcomes of the PCR detection of haemosporidian parasites.

Bird species	No. examined/no. infected	District	Lineage (GenBank accession, no. of detected lineages)
Acrocephalus dumetorum	1/1	E	MIX[a] (1)
Carpodacus erythrinus	12/6	E	hPYERY01 (AY172842.1); pPADOM02 (DQ058612.1); hROFI2 (DQ060769.3); MIX (1)
	2/2	K	hPYERY01 (AY172842.2)
Emberiza spodocephala	25/12	S	hEMSPO01 (EU676187.10); hEMSPO02 (EU676188.1); hEMSPO03 (EU676189.1)
Ficedula hypoleuca	28/11	E	hPHSIB1 (AF495565.4); hPFC1(DQ063577.3); pANLAT04 (FJ404718.1); pSGS1 (AF495571.1); MIX (2)
F. parva	13/9	K	hLIN32 (EF380197.5); pBT7(AY393793.1); pFIPAR01 (EU676191.1); MIX (2);
Fringilla coelebs	7/3	E	hCCF6 (DQ368341.2); MIX (1)
F. montifringilla	3/1	E	pBT7 (AY393793.1)
Hippolais caligata	1/1	E	hHIP2 (AF495558.1)
Loxia curvirostra	2/2	E	hSISKIN1 (AY393806.2)
Luscinia calliope	16/7	S	hPHSIB1 (AF495565.7)
Motacilla alba	2/1	E	pSYBOR2 (DQ368392.1)
Muscicapa latirostris	3/2	I	pTURDUS1 (AF495576.1); MIX (1)
Parus ater	12/4	E	pTURDUS1 (AF495576.2); pBT7 (AY393793.2)
P. major	1/1	I	hPHSIB1 (AF495565.1)
P. montanus	17/6	E	pTURDUS1 (AF495576.4); pBT7 (AY393793.1); hWW1 (AF254971.1)
	3/2	S	hPHSIB1 (AF495565.2)
Phylloscopus collybita	12/2	E	pSYBOR2 (DQ368392.1); MIX (1)
Ph. proregulus	3/1	S	hPHSIB1 (AF495565.1)
Prunella modularis	3/1	E	pTURDUS1 (AF495576.1)
Pyrrhula pyrrhula	4/2	E	hPYERY01 (AY172842.1); MIX (1)
Sitta europaea	1/1	E	pTURDUS1 (AF495576.1)
Spinus spinus	5/2	E	hSISKIN1 (AY393806.2)
Sylvia curruca	4/1	E	hLWT2 (DQ368351.1)
Turdus chrysolaus	1/1	S	hTUCHR01 (EU676190.1)
T. iliacus	4/4	E	hTUCHR01 (EU676190.2); hTURDUS2 (DQ060772.1); MIX (1)
T. obscurus	1/1	K	MIX (1)
T. philomelos	6/5	E	hTUPHI1 (GU085191.2); hTUCHR01 (EU676190.2); MIX (1)
T. pilaris	13/9	E	hTUCHR01 (EU676190.4); hTURDUS2 (DQ060772.3); MIX (2)
Total	205/101	4 districts	16 lineages

E — Ekaterinburg district, I — Irkutsk district, K — Kamchatka Peninsula, S — Sakhalin Island.

Uninfected bird species (no of examined, district): *Acrocephalus bistrigiceps* (1, S); *Aegithalos caudatus* (3, E); *Anthus hodgsoni* (1, E); *Certhia familiaris* (1, E); *Cettia diphone* (1, S); *Chloris sinica* (1, K); *Cinclus cinclus* (2, I); *Emberiza rutila* (1, S); *E. rustica* (2, I); *Erithacus rubecula* (4, E); *Gallinago gallinago* (1, K); *Heteroscelus brevipes* (1, S); *Locustella ochotensis* (1, K); *Luscinia calliope* (4, K); *L. cyane* (5, S/I); *L. sibilans* (6, S); *L. sibilatrix* (1, I); *Muscicapa sibirica* (1, S); *Phoenicurus phoenicurus* (2, E); *Phylloscopus fuscatus* (1, I); *Ph. schwarzi* (9, S/I); *Ph. tenellipes* (1, S); *Ph. trochilus* (2, E); *Pyrrhula griseiventris* (3, I); *Sitta europaea* (1, S); *Tarsiger cyanurus* (3, I); *Troglodytes troglodytes* (1, S).

[a] Co-infections were defined by presence of double peaks on the electropherograms of sequences.

Please cite this article as: Palinauskas V, et al, Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite, Parasitology International (2013), http://dx.doi.org/10.1016/j.parint.2013.03.006

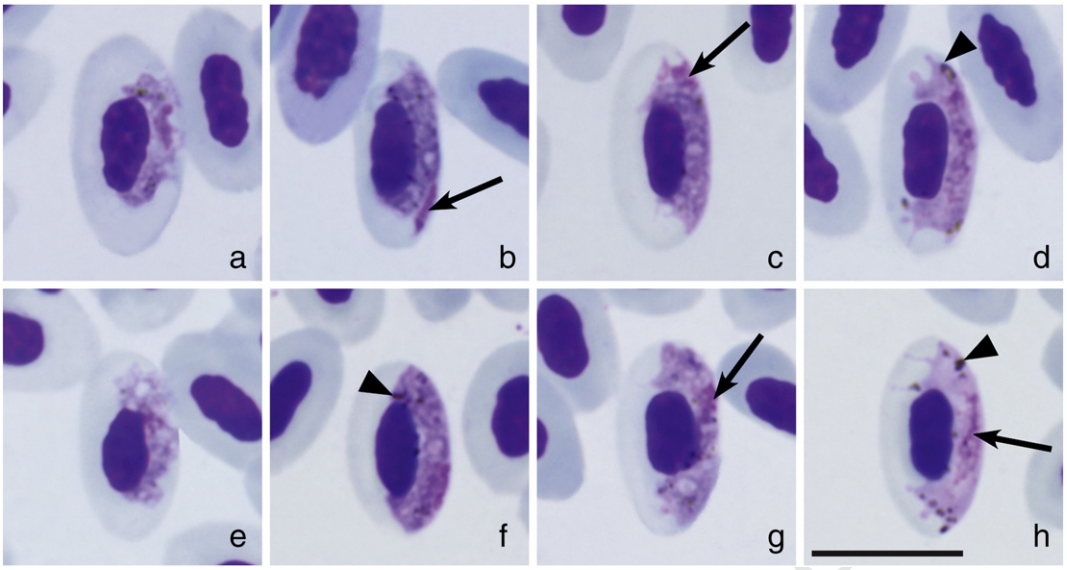

Fig. 1. Gametocytes of *Haemoproteus minutus* (lineage hTUCHR01) from the blood of fieldfare *Turdus pilaris* (a–d), and gametocytes of *H. minutus* (hTUPHI1) from the blood of song thrush *T. philomelos* (e–h); a, b, c, e, f, g — macrogametocytes, d, h — microgametocytes. Arrows — nuclei of parasites. Arrowheads — pigment granules. Giemsa-stained thin blood films. Scale bar = 10 μm.

reaction was performed for 20 cycles. We used 2 μl of the first PCR product as template for the second PCR. The thermal conditions were identical to first PCR just performance was for 35 cycles. For evaluation of amplification we ran 1.5 μl of the final PCR product on a 2% agarose gel. We used dye terminator cycling sequencing (big dye) and loaded on an ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, USA) as described by [22]. Fragments were sequenced from the 5′ end with the primer HAEMF. Sequences were edited and aligned using the program BioEdit [23]. Visualization of "double base calling" in electropherograms of cyt *b* sequences was used to determine the presence of possible haemosporidian co-infections.

Sequences with "double base calling" on the electropherograms were discarded from our analysis. The 'Basic Local Alignment Search Tool' (Blast) was used to determine the lineage of detected parasite sequences in GenBank and the MalAvi database [24].

2.4. Phylogenetic analysis

To determine the position of *H. minutus* and closely related lineages on the phylogenetic tree, we used 21 mitochondrial cyt *b* gene sequences of morphologically described avian *Haemoproteus* (*Parahaemoproteus*) parasites from our survey, GenBank and MalAvi database [24]. Sequences of *Haemoproteus pallidus* and *Haemoproteus pallidulus* were from our former study [5,18]. The GenBank accession numbers and the lineage codes are given in Fig. 2. All individual sequences of *H.* (*Parahaemoproteus*) spp. and two lineages of the subgenus *Haemoproteus* (*Haemoproteus*) were aligned to 479 base pair segments.

The phylogenetic tree was constructed using Bayesian phylogenetics as implemented in mrBayes version 3.1 [25]. We used the General Time Reversible model including invariable sites and variation among sites (GTR + I + G) as suggested by the software Modeltest 3.7 [26]. Two independent runs were conducted with a sample frequency of every 100th generation over 8 million generations. Convergence in phylogeny estimation for each analysis was assessed using the program Tracer (Software available at <http://tree.bio.ed.ac.uk/software/tracer/>) and used to indicate the appropriate "burn-in" period. Before constructing a majority consensus tree, 25% of the initial trees in each run were discarded as "burn-in" periods. The phylogenies were visualized using Tree View 1.6.6. (software available from <http://evolution.genetics.washington.edu/phylip/software.html>). The sequence divergence between the different lineages was calculated with the use of a Jukes–Cantor model of substitution, with all substitution weighted equally, implemented in the program MEGA 3.1 [27].

3. Results

Of the 23 recorded haemosporidian lineages (16 *Haemoproteus* spp. and 7 *Plasmodium* spp.) (Table 1), we focused on the lineages previously described in parrots; these lineages were reported in species of the *Turdidae* (Table 2). The lineage hTURDUS2 of *H. minutus* was identified in the redwing and fieldfare (infection prevalence was 25% and 23%, respectively). The lineage hTUPHI1 was detected in the song thrush (33%). The lineage hTUCHR01 was found in the redwing, song thrush, fieldfare, and brown-headed thrush, with prevalence of 50%, 33%, 30% and 100%, respectively. Co-infections of several *Haemoproteus* spp. or *Haemoproteus* and *Plasmodium* spp. were present in one redwing, one song thrush and 2 fieldfares; these birds were excluded from further analysis.

Main morphological diagnostic characters of gametocytes of the lineages hTUPHI1 and hTUCHR01 (Fig. 1) were the same as in *H. minutus* (hTURDUS2). Mainly, 1) fully-grown gametocytes were small (length of macro- and microgametocytes < 12 μm, Fig. 1, c, d, g, h), 2) medium-grown gametocytes of these parasites were closely associated with erythrocyte nuclei and often did not touch erythrocyte envelope (Fig. 1, a, e), 3) fully-grown gametocytes were closely associated both with nuclei and envelope of erythrocytes but did not fill the erythrocytes up to their poles (Fig. 1, c, d, g, h), 4) pigment granules were few (<10 on average), sometimes aggregated into compact masses, particularly in microgametocytes (Fig. 1, d).

The lineages hTURDUS2, hTUPHI1 and hTUCHR01 clustered together in a well-supported clade (Fig. 2, clade A); that is in accordance with their morphological similarity. Genetic distance among different cyt *b* lineages of *H. minutus* ranged between 0.2% and 0.6%. Because of morphological and genetic similarity, all these lineages were grouped as genetic variation of *H. minutus*. Sequences of these lineages can be used for PCR-based detection of *H. minutus*.

The phylogenetic analysis (Fig. 2) shows that *H. pallidus* and *H. pallidulus* are members of the same clade, in which *H. minutus* is clustered, with genetic distance of <0.6% among lineages of this clade.

Please cite this article as: Palinauskas V, et al, Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite, Parasitology International (2013), http://dx.doi.org/10.1016/j.parint.2013.03.006

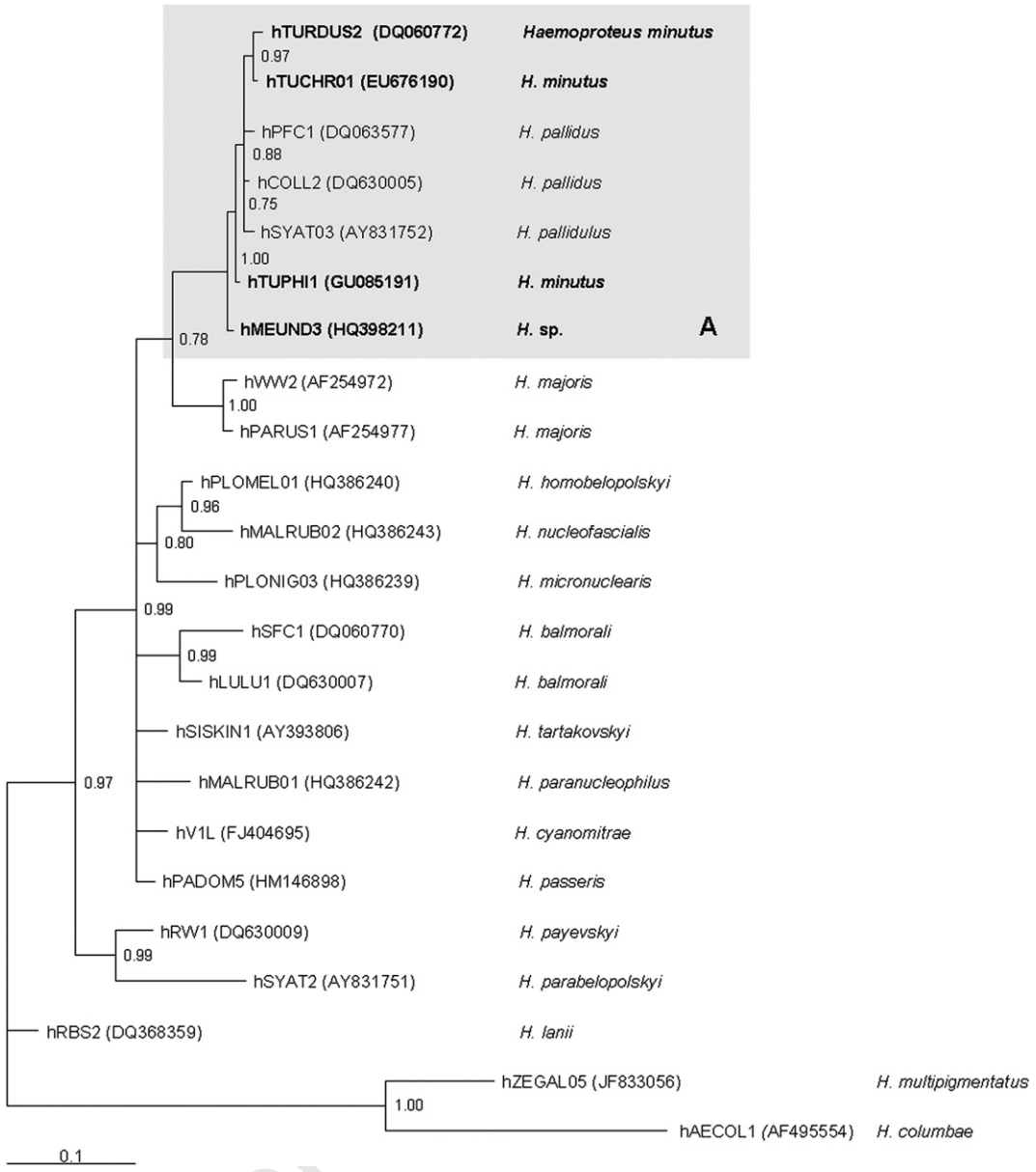

Fig. 2. Bayesian phylogeny of 21 *Haemoproteus* (*Parahaemoproteus*) spp. lineages of cytochrome *b* sequences (479 bp) rooted with two *H.* (*Haemoproteus*) *multipigmentatus* and *H.* (*Haemoproteus*) *columbae* species. Nodal support values indicate Bayesian posterior probabilities. The scale bar indicates the expected substitutions per site. Codes of the lineages were retrieved from the MalAvi database [24]. GenBank accession numbers are provided in the parentheses.

4. Discussion

Of 4 lineages reported in dead parrots in Europe [15], only one *Haemoproteus* lineage (hTURDUS2) has been identified to species level [15]. That is *H. minutus*; the blackbird is the natural host of this parasite lineage [18], which is widespread in Europe [24]. Vector species of *H. minutus* remain unknown, but likely they are biting midges belonging to *Culicoides*, as is the case with other avian *Haemoproteus* (*Parahaemoproteus*) species [3,28]. The blackbird is a common inhabitant of parks and gardens in many European cities and villages. This bird is frequently infected with *H. minutus* (hTURDUS2) and likely serves as the main reservoir host when parrots are exposed to bites of biting midges at sites of active transmission during summer time [16].

Table 2
Haemoproteus minutus lineages recovered from *Turdus* spp. in Ekaterinburg district and Sakhalin Island, Russia.

Bird species	hTUPHI1	hTUCHR01	hTURDUS2
Turdus chysolaus		1	
T. iliacus		2	1
T. philomelos	2	2	
T. pilaris		4	3

The present study shows that the lineages hTUPHI1 and hTUCHR01 also belong to *H. minutus.* This finding contributes to the PCR-based typing of this pathogenic parasite [15]. The lineage hTUPHI1 has been originally detected in the song thrush *Turdus philomelos* in Bulgaria, but its species identity was not determined [29]. We also identified song thrush as the host of the lineage hTUPHI1; this parasite lineage was absent from the other 4 examined *Turdus* species (Table 2). The lineage hTUCHR01 of *H. minutus* was found in 4 species of thrushes (Table 2). This lineage is closely related to the lineage hTURDUS2 (Fig. 2) with genetic divergence between them of 0.2%.

H. minutus and its different lineages have previously been recorded only in thrushes. Other closely related to *H. minutus* lineage, hPFC of

Please cite this article as: Palinauskas V, et al, Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite, Parasitology International (2013), http://dx.doi.org/10.1016/j.parint.2013.03.006

H. pallidus, was detected in Eurasian pied flycatcher *Ficedula hypoleuca* (Table 1). The latter lineage and the lineage of *H. pallidulus* [5], which appeared in the same clade in the phylogenetic tree (Fig. 2 clade A), infect distantly related avian hosts. The hosts of *H. minutus* belong to the family Turdidae, whereas *H. pallidus* and *H. pallidulus* infect Eurasian pied and collared flycatchers (Muscicapidae) and Eurasian blackcaps *Sylvia atricapilla* (Sylviidae), respectively [5,18]. The reason of the restricted distribution of closely related haemoproteid lineages by avian hosts is unclear and needs clarification.

It is worth mentioning that the geographical distribution of the *H. minutus* lineages is extremely broad in Palearctic. Former studies showed that the lineages hTURDUS2 and hTUPHI1 are prevalent in Western and Central Europe [18,29]. The present communication adds the Eurasian territory of Russia, including the Far East of the Palearctic to the range of their distribution (Table 1). All detected lineages are the first reports for our study sites (Table 1). The only unpublished record about identical lineages to hTURDUS2 from other continents is available in GenBank (Acc. nos. JN819383 and JN819388). These two lineages were found in Central America (Costa Rica), in white-throated thrush *Turdus assimilis* and silver-throated tanager *Tangara icterocephala* respectively.

Interestingly, *H. minutus*, *H. pallidus* and *H. pallidulus* are members of the same well-supported clade (Fig. 2, clade A) with small genetic divergence among their cyt *b* lineages. Morphologically, blood stages of these parasites are clearly distinguishable [3,5], but they do possess one common feature. Mainly, when stained with Giemsa, the cytoplasm of macro- and microgametocytes assumes approximately the same pale intensity of staining; in other words, macrogametocytes are pale-blue stained and look like microgametocytes due to this character. This is a characteristic feature of all parasites from the clade A (Fig. 2), but is a rare feature in avian haemoproteids [5]. It is probable that the pale staining of these parasites, in comparison to other avian haemoproteids, is due to less developed cytoplasmic structures in their macrogametocytes (endoplasmic reticulum, ribosomes, and others) [3]. The morphology of parasites of clade A (Fig. 2) clearly indicates three species, but genetic differences in cyt *b* gene among these lineages are negligible. Because of the close phylogenetic relationships of *H. minutus*, *H. pallidus* and *H. pallidulus*, all haemoproteids of clade A (Fig. 2) are worth attention as possible agents of pathology in exotic avian hosts, as is the case with different lineages of *H. minutus* in captive parrots in Europe [15]. This prediction is based on genetic similarity among cyt *b* gene lineages of these parasites and our phylogenetic analysis; further experimental studies are needed to clarify this issue.

Recent findings indicate that some widespread lineages of *Haemoproteus* spp. are probably relatively benign in natural hosts, but might cause lethal disease in exotic birds in zoos and aviaries [11,13–15]. Numerous cases of so-called 'aberrant haemosporidian infections' have been documented in parrots, doves, ducks and other birds worldwide, but etiology of these infections remained unclear because parasites usually were not observed in the blood. Mortality was due to pathology caused by unidentified haemosporidian tissue stages (exoerythrocytic meronts) in skeletal muscles and (or) in the heart [30–37]. Morphologically, these tissue meronts resemble the megalomeronts of *Leucocytozoon* spp.; they were usually described as 'aberrant *Leucocytozoon* infection' [30–34,37]. However, when megalomeronts were documented in *Haemoproteus handai* [9] and *H. mansoni* [10], it became clear that they better suit the description of *Haemoproteus* spp., but there was no convincing proof for that. Recent PCR-based findings [13–15] supplement former histopathology studies [10,38] and show that species of *Haemoproteus* are responsible for some instances of mortality in birds. Therefore, the traditional opinion about insignificant veterinary importance of avian haemoproteids [8] requires partial reconsideration [16].

It remains unclear if haemoproteids cause pathology in birds in the wild. Both microscopic and PCR-based examination of blood samples are not always sufficient to detect such infections because parasite DNA can be absent from blood samples due to abortive parasite development at tissue stages [16]. Experimental studies combined with histopathology research are needed to answer this question.

Further investigations are needed for better understanding the pathology caused by *Haemoproteus* spp. in birds, particularly in wildlife. Linkage of PCR-based information and morphological data is an urgent task because it provides opportunities for molecular typing of agents of avian haemoproteosis. Molecular markers are particularly necessary for detecting and typing haemosporidian tissue stages, which are similar in many parasite species, as these are difficult to identify to species level based on morphology. This study identifies several closely related lineages of *H. minutus*, which naturally parasitize thrushes. These parasites might be potential agents of lethal haemoproteosis in exotic avian hosts [15].

Acknowledgments

The authors are grateful to Vladislav V. Kosarev and Vladislav D. Efremov for their participation in fieldwork. This study was supported by the Global Grant (VPI-3.1.-ŠMM-07-K-01-047).

References

[1] Garnham PCC. Malaria parasites and other haemosporidia. Oxford: Blackwell Scientific Publications; 1966.
[2] Bishop MA, Bennett GF. Host-parasite catalogue of the avian haematozoa: supplement 1; Bibliography of the avian blood-inhabiting haematozoa: supplement 2. Memorial University of Newfoundland Occasional Papers in Biology; 1992.
[3] Valkiūnas G. Avian malaria parasites and other haemosporidia. Boca Raton, Florida: CRC Press; 2005.
[4] Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. Journal of Parasitology 2008;94:1395–401.
[5] Križanauskienė A, Pérez-Tris J, Palinauskas V, Hellgren O, Bensch S, Valkiūnas G. Molecular phylogenetic and morphological analysis of haemosporidian parasites (Haemosporida) in a naturally infected European songbird, the blackcap *Sylvia atricapilla*, with description of *Haemoproteus pallidulus* sp. nov. Parasitology 2010;137:217–27.
[6] Iezhova TA, Dodge M, Sehgal RNM, Smith TB, Valkiūnas G. New avian *Haemoproteus* species (Haemosporida: Haemoproteidae) from African birds, with a critique of the use of host taxonomic information in hemoproteid classification. Journal of Parasitology 2011;97:682–94.
[7] Levin II, Valkiūnas G, Santiago-Alarcon D, Cruz LL, Iezhova TA, O'Brein SL, et al. Hippoboscid-transmitted *Haemoproteus* parasites (Haemosporida) infect Galapagos Pelecaniform birds: evidence from molecular and morphological studies, with a description of *Haemoproteus iwa*. International Journal of Parasitology 2011;41:1019–27.
[8] Bennett GF, Peirce MA, Ashford RW. Avian haematozoa: mortality and pathogenicity. Journal of Natural History 1993;27:993–1001.
[9] Miltgen F, Landau I, Ratanaworabhan N, Yenbutra S. *Parahaemoproteus desseri* n. sp.; Gamétogonie et schizogonie chez l'hôte naturel: *Psittacula roseata* de Thailande, et sporogonie expérimentale chez *Culicoides nubeculosus*. Annales de Parasitologie Humaine et Comparee 1981;56:123–30.
[10] Atkinson CT, Forrester DJ, Greiner EC. Pathogenicity of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. Journal of Parasitology 1988;74:228–39.
[11] Cardona CJ, Ihejirika A, McClellan L. *Haemoproteus lophortyx* infection in bobwhite quail. Avian Diseases 2002;46:249–55.
[12] Marzal A, de Lopes F, Navarro C, Møller AP. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. Oecologia 2005;142:541–5.
[13] Ferrell ST, Snowden K, Marlar AB, Garner M, Lung NP. Fatal hemoprotozoal infections in multiple avian species in a zoological park. Journal of Zoo and Wildlife Medicine 2007;38:309–16.
[14] Donovan TA, Schrenzel MD, Tucker TA, Pessier AP, Stalis IH. Hepatic hemorrhage, hemocoelom, and sudden death due to Haemoproteus infection in passerine birds: eleven cases. Journal of Veterinary Diagnostic Investigation 2008;20:304–13.
[15] Olias P, Wegelin M, Zenker W, Freter S, Gruber AD, Klopfleisch R. Avian malaria deaths in parrots, Europe. Emerging Infectious Diseases 2011;17:950–2.
[16] Valkiūnas G. Haemosporidian vector research: marriage of molecular and microscopical approches is essential. Molecular Ecology 2011;20:3084–6.
[17] Valkiūnas G, Iezhova T. New species of haemoproteids (Haemosporidia: Haemoproteidae) in passerine birds.Zoologicheskii Zhurnal (Moscow) 1992;71:5–15 [in Russian].
[18] Hellgren O, Križanauskienė A, Valkiūnas G, Bensch S. Diversity and phylogeny of mitochondrial cytochrome *b* lineages from six morphospecies of avian *Haemoproteus* (Haemosporida, Haemoproteidae). Journal of Parasitology 2007;93:889–96.

Please cite this article as: Palinauskas V, et al, Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite, Parasitology International (2013), http://dx.doi.org/10.1016/j.parint.2013.03.006

[19] Palinauskas V, Markovets M, Kosarev V, Efremov V, Sokolov L, Valkiūnas G. Occurrence of avian haematozoa in Ekaterinburg and Irkutsk districts of Russia. Ekologija 2005;4:8–12.
[20] Hellgren O, Waldenström J, Bensch S. A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium,* and *Haemoproteus* from avian blood. Journal of Parasitology 2004;90:797–802.
[21] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1989.
[22] Bensch S, Stjernman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, et al. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. Proceedings of the Royal Society of London - Series B: Biological Sciences 2000;267:1583–9.
[23] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 1999;41:95–8.
[24] Bensch S, Hellgren O, Pérez-Tris J. A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Molecular Ecology Resources 2009;9:1353–8.
[25] Ronquist F, Heulsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003;19:1572–4.
[26] Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. Bioinformatics 1998;14:817–8.
[27] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics 2004;5:150–63.
[28] Santiago-Alarcon D, Palinauskas V, Schaefer HM. Diptera vectors of avian Haemosporidian parasites: untangling parasite life cycles and their taxonomy. Biological Reviews of the Cambridge Philosophical Society 2012;87:928–64.
[29] Dimitrov D, Zehtindjiev P, Bensch S. Genetic diversity of avian blood parasites in SE Europe: cytochrome *b* lineages of the genera *Plasmodium* and *Haemoproteus* (Haemoproteida) from Bulgaria. Acta Parasitologica 2010;55:201–9.
[30] Borst GHA, Zwart P. An aberrant form of *Leucocytozoon* infection in two quaker parakeets (*Myiopsitta monachus* Boddaert, 1783). Zeitschrift für Parasitenkunde 1972;40:131–8.
[31] Fowler NG, Forbes GB. Aberrant *Leucocytozoon* infection in parakeets. Veterinary Record 1972;91:345–7.
[32] Markus MB. *Leucocytozoon* infection in parakeets. Veterinary Research 1972;91:323.
[33] Smith GA. Aberrant *Leucocytozoon* infection in parakeets. Veterinary Record 1972;91:106.
[34] Walker D, Garnham PCC. Aberrant *Leucocytozoon* infection in parakeets. Veterinary Record 1972;91:70–2.
[35] Garnham PCC. Epizootics of *Leucocytozoon* infections in parakeets in England. Progress in parasitology: Abstr. of papers read at the 4-th Int. Congr, 149. 1973.
[36] Gardiner CH, Jenkins HJ, Mahoney KS. Myositis and death in bobwhites, *Colinus virginianus* (L.), due to hemorrhagic cysts of a haemosporozoan of undetermined taxonomic status. Journal of Wildlife Diseases 1984;20:308–18.
[37] Simpson VR. *Leucocytozoon*-like infection in parakeets, budgerigars and common buzzard. Veterinary Record 1991;13:30–3.
[38] Earlé RA, Bastianello SS, Bennett GF, Krecek RC. Histopathology and morphology of the tissue stages of *Haemoproteus columbae* causing mortality in Columbiformes. Avian Pathology 1993;22:67–80.

Please cite this article as: Palinauskas V, et al, Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): A pathogenic avian parasite, Parasitology International (2013), http://dx.doi.org/10.1016/j.parint.2013.03.006