

# Two new species of *Haemoproteus* Kruse, 1890 (Haemosporida, Haemoproteidae) from European birds, with emphasis on DNA barcoding for detection of haemosporidians in wildlife

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**Abstract** Two new species of *Haemoproteus* Kruse, 1890 (Haemosporida, Haemoproteidae) are described: *Haemoproteus* (*Parahaemoproteus*) *homovelans* n. sp. from Grey-faced Woodpecker, *Picus canus* Gmelin, and *Haemoproteus* (*Parahaemoproteus*) *concoventralis* n. sp. recorded in Hawfinch, *Coccothraustes coccothraustes* (Linnaeus), both sampled in Bulgaria. The morphology of the gametocytes and their host-cells are described and mitochondrial cytochrome *b* (cyt *b*) gene sequences are generated. *Haemoproteus homovelans* possesses circumnuclear gametocytes lacking volutin granules. This parasite is particularly similar to *Haemoproteus velans* Coatney & Roudabush, 1937 also possessing circumnuclear gametocytes that are, however, overfilled with volutin. *Haemoproteus concavocentralis* can be readily distinguished from all described avian haemoproteids due to the presence of an unfilled concave space between the central part of advanced gametocytes and

erythrocyte nucleus. Bayesian phylogenetic analyses of 40 haemosporidian cyt *b* lineages showed close relationships of *H. concavocentralis* (hHAWF2) with a group of *Haemoproteus* spp. possessing gametocytes that are pale-stained with Giemsa. The lineage hPICAN02 of *H. homovelans* clustered with parasites infecting non-passerine birds. Phylogenetic analyses support the current subgeneric classification of the avian haemoproteids and suggest that cyt *b* lineage hPI PUB01 (GenBank EU254552) has been incorrectly assigned to *Haemoproteus picae* Coatney & Roudabush, 1937, a common parasite of corvid birds (Passeriformes). This study emphasises the importance of combining molecular techniques and light microscopy in the identification and field studies of avian haemosporidian parasites. Future development of barcodes for molecular identification of haemoproteids will allow better diagnostics of these infections, particularly in veterinary studies addressing insufficiently investigated tissue pathology caused by these parasites.

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## Introduction

Haemoproteids (Haemosporida, Haemoproteidae) represent the most diverse group among avian blood parasites, with over 140 species described to date. In the last decade, knowledge about the diversity of this group of parasites has increased rapidly due to the implementation of molecular methods for

identification and more intensive research being carried out in tropical areas (Valkiūnas et al., 2008c; Bensch et al., 2009; Iezhova et al., 2010, 2011; Levin et al., 2011, 2012; Merino et al., 2012). The great genetic diversity of haemosporidian parasites has encouraged taxonomists to look more closely at parasites with similar morphology and, as a result, several new species have recently been described from temperate regions of Europe (Valkiūnas et al., 2007; Križanauskienė et al., 2010, 2012).

Species of the genus *Haemoproteus* Kruse, 1890 are closely related to the agents of malaria belonging to the genus *Plasmodium* Marchiafava & Celli, 1885 (Haemosporida, Plasmodiidae), which cause diseases in animals and humans. Sexual process and sporogony of *Haemoproteus* spp. take place in biting midges (Ceratopogonidae) and louse flies (Hippoboscidae) which transmit these parasites (Valkiūnas, 2005). Haemoproteid infections can cause direct and indirect pathologies affecting avian host fitness, breeding success, moulting, predation risks, and sometimes can be even lethal for the birds (Atkinson et al., 1988; Marzal et al., 2005, 2013; Ferrell et al., 2007; Møller & Nielsen, 2007). Several outbreaks associated with mortalities due to haemoproteid infections were recently described as an abortive development in non-adapted avian host (Olias et al., 2011; Cannell et al., 2013). Such abortive infections are difficult to diagnose because gametocytes are not present in the blood stream, but tissue stages can be determined using histological sections and PCR-based methods. However, identification of *Haemoproteus* spp. is hardly possible based only on the morphology of tissue stages (exoerythrocytic meronts) (Valkiūnas, 2005; Atkinson, 2008). Molecular approaches can help distinguish different *Haemoproteus* lineages (sequences), but PCR-based identification of the majority of described haemoproteid species still needs development e.g. it would be helpful for studies addressing distribution, vectors, areas of transmission and many other issues. Morphological characterisation of *Haemoproteus* spp. lineages is essential for better understanding of their molecular epidemiology, particularly during abortive development in avian hosts (Olias et al., 2011; Cannell et al., 2013; Levin et al., 2013). Studies addressing the development of *Haemoproteus* spp. barcodes are still insufficient (Valkiūnas et al., 2008a).

Haemoproteids are characterised by a long-lasting chronic stage of infection, with relatively high

intensity of parasitemia that usually facilitates morphological identification of the species of *Haemoproteus* in wild-caught birds, which is rare in natural *Plasmodium* spp. infections (Valkiūnas, 2005). On the other hand, the lack of erythrocytic merogony makes the experimental transmission of haemoproteids by subinoculation of infected blood in susceptible hosts impossible. This is an obstacle for experimental research with *Haemoproteus* spp. Additionally, difficulties in infecting and maintaining vectors of *Haemoproteus* spp. in the laboratory also complicate experimental studies of haemoproteids (Valkiūnas et al., 2002; Garvin et al., 2003). Nevertheless, the enhanced possibility for molecular characterisation of *Haemoproteus* spp. from natural infections is essential for taxonomic research of these parasites.

Current systematics and taxonomy of haemoproteid parasites is based mainly on morphological characters of their blood stages (gametocytes) and the information available about vertebrate-host specificity of the parasites (Valkiūnas, 2005). Molecular methods have revealed great genetic diversity, with over 470 mitochondrial cytochrome *b* (cyt *b*) lineages of *Haemoproteus* spp. currently deposited in MalAvi database (<http://mbio-serv2.mbioekol.lu.se/Malavi/index.html>, see Bensch et al., 2009). Many of these lineages might represent different parasite species (Bensch et al., 2004, 2009). However, it should be noted that the rapidly increasing number of molecular studies result not only in marked accumulation of genetic information, but also in accumulation of incorrect parasite identifications in GenBank (Valkiūnas et al., 2008a). Furthermore, the majority of DNA sequences in GenBank, especially those obtained from wildlife, have been identified only to the generic level, and sometimes at even higher levels of classification. Currently, it is still unclear if closely related but distinct cyt *b* lineages of haemosporidians represent biological species or different levels of intraspecific variation (Iezhova et al., 2010).

Hellgren et al. (2007) suggested that cyt *b* lineages of *Haemoproteus* spp. with a genetic divergence higher than 5% are expected to be morphologically differentiated in most cases. However, this does not mean that lineages with divergence lower than 5% in this gene do not differ morphologically. There are examples of morphologically readily distinguishable species with cyt *b* divergences of 1% or even less (Valkiūnas et al., 2008a; Križanauskienė et al., 2010;

Levin et al., 2012). Additional studies on the morphology of haemoproteids in complementary blood slides should be conducted. Development of DNA barcodes of haemosporidian species would bring new opportunities for better understanding parasite taxonomy, life history, distribution and phylogeny.

This study aims to provide morphological description and molecular characterisation of two new haemoproteid species found in wild birds from Southeast Europe. We obtained data on the morphology of the parasites and submitted their *cyt b* sequences to GenBank and MalAvi databases. We also (i) compare the novel morphological and sequence information with the data available for other avian haemosporidian parasites, and (ii) discuss obstacles and advantages of using PCR-based information in molecular detection and identification of haemosporidian parasites in wildlife.

## Materials and methods

### *Study site and collection of the blood samples*

Among the complementary blood slides from 12 new *Haemoproteus* spp. *cyt b* lineages found by Dimitrov et al. (2010), we identified two undescribed parasites and their *cyt b* lineages (hPICAN02 and hHAWF2), which exhibited readily distinguishable novel morphology. *Cyt b* lineage hPICAN02 was found in the blood of a single sample from the Grey-faced Woodpecker, *Picus canus* Gmelin, captured in Brodilovo, Southeast Bulgaria (n = 1; 42°04'N, 27°51'E). The lineage hHAWF2 was identified in six of nine examined Hawfinches, *Coccothraustes coccothraustes* (Linnaeus), at Kalimok Biological Station, Northeast Bulgaria (n = 7; 44°00'N, 26°26'E); River Kamchia, East Bulgaria (n = 1; 43°07'N, 26°47'E); and Brodilovo, Southeast Bulgaria (n = 1; 42°04'N, 27°51'E).

Birds were captured by mist nets in deciduous forest habitats during spring–summer in 2007–2009. From each bird, several blood drops were taken and immediately used for preparation of two or three blood smears. Approximately 25 µl of blood was collected for DNA analysis in 500 µl SET buffer (0.15 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) and stored at ambient temperature in the field and later at –20°C in the laboratory. The blood slides were air-dried, fixed in absolute methanol for one minute in the field and

stained with Giemsa solution in the laboratory, as described by Valkiūnas et al. (2008b).

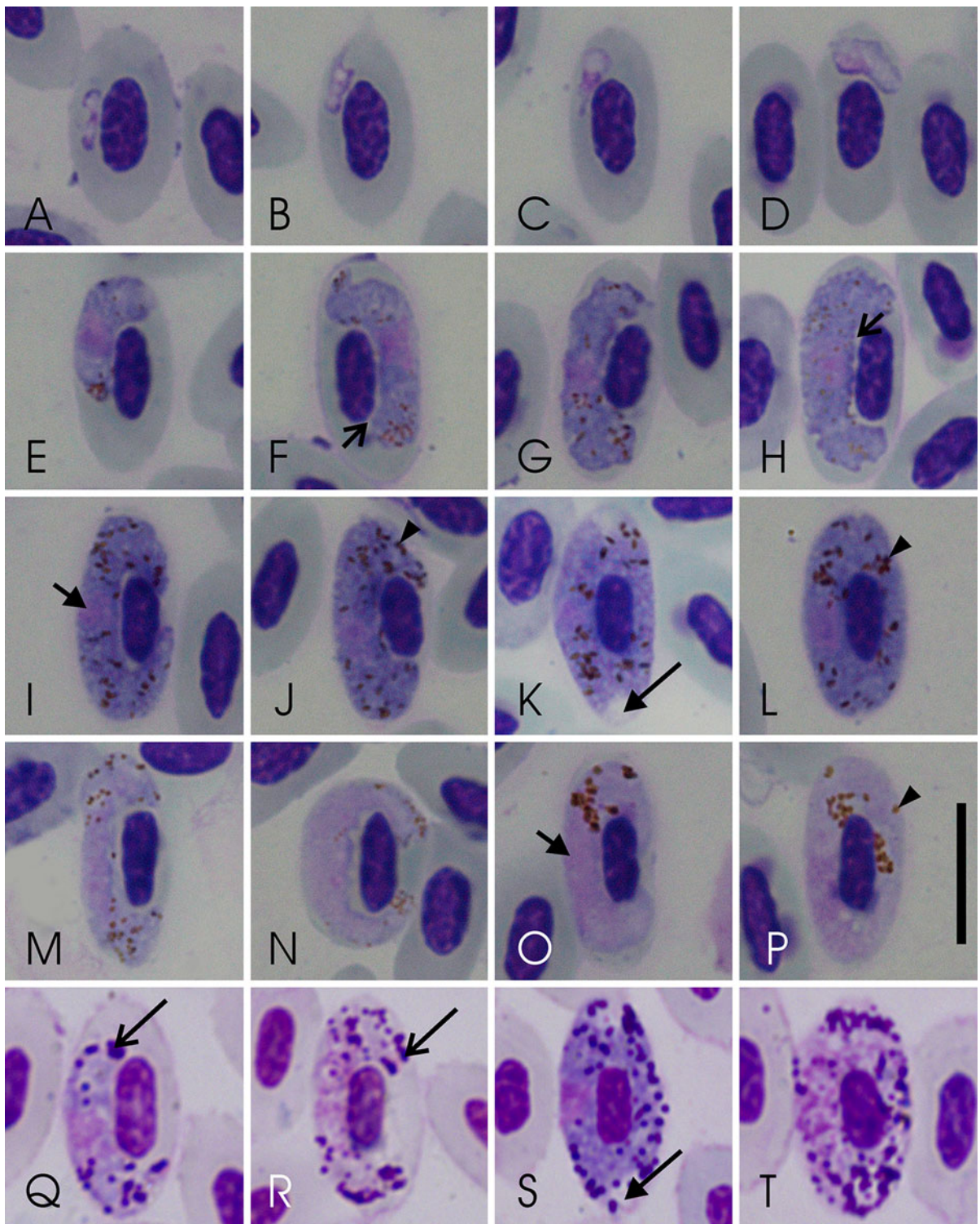
### *Examination of the blood films and parasite morphology*

Blood films were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1000), as described by Valkiūnas et al. (2008b). Olympus BX51 and Zeiss Axio Imager M2 light microscopes equipped with Olympus DP12 and ProgRes c10 plus digital cameras and imaging software DPSOFT and ProgRes CapturePro v2.8.0 were used to examine the blood slides and prepare illustrations. We used calibrated Image-Pro Plus software to take measurements. To identify the parasites, we used morphological and morphometric features according to Valkiūnas (2005). Additionally, to confirm the morphological identification of the new species, we compared our material with type-specimens of morphologically or genetically similar parasites, i.e. *Haemoproteus velans* Coatney & Rudabush, 1937 deposited at Queensland Museum, Queensland, Australia (accession number 45241) (Fig. 1Q–T) and of *Haemoproteus vacuolatus* Valkiūnas, Iezhova, Loiseau, Chasar, Smith & Sehgal, 2008 deposited at the Nature Research Centre, Vilnius, Lithuania (accession number 42415 NS) (Fig. 2Q–T).

Intensity of infection was estimated as a percentage by actual counting the number of parasites per 1,000 red blood cells or per 10,000 red blood cells in light infections (i.e. <0.1%), as recommended by Godfrey et al. (1987).

### *Extraction of DNA, PCR and sequencing*

DNA was extracted by a standard phenol-chloroform protocol (Sambrook et al., 2002) and quantified by spectrophotometer (Spectromer Genesis 10 Bio). Diluted to 25 ng/µl, the total DNA was used as a template in PCR assays for detection of the parasites. We used primers and temperature profiles as described in Hellgren et al. (2004). The method consists of a nested PCR assay that amplifies a part of the parasite mitochondrial *cyt b* gene in two steps, first an initial PCR (primers HaemNFI/HaemNR3; 570 bp excluding primers) that amplifies *cyt b* lineages of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* Berestneff, 1904, followed by a second step that amplifies lineages of the genera *Plasmodium* and *Haemoproteus* (primers HaemF/HaemR2; 479 bp excluding primers). By





◀ **Fig. 1** Gametocytes of *Haemoproteus homovelans* n. sp. from the blood of Grey-faced Woodpecker *Picus canus* (A–P) and *Haemoproteus velans* from the blood of Northern Flicker *Colaptes auratus* (Q–T): A–D, young gametocytes; E–L, Q, S, macrogametocytes; M–P, R, T, microgametocytes. Short simple arrows indicate unfilled spaces between gametocyte and erythrocyte nucleus; long simple arrows indicate volutin granules; short triangle arrows indicate parasite nuclei; long triangle arrows indicate unfilled space resembling vacuoles at erythrocyte poles; triangle arrowheads indicate pigment granules. Giemsa-stained thin blood films. Scale-bar: 10 µm

amplifying the parasite DNA in two PCRs the sensitivity of the screening is increased (Hellgren et al., 2004; Waldenström et al., 2004). Samples that showed positive amplification on a 2% agarose gel were sequenced using the procedures described by Bensch et al. (2000). Amplified fragments were sequenced in both directions with primers HaemF and HaemR2. We used a dye terminator cycle sequencing kit (Big Dye Terminator Ready Reaction Mix, Applied Biosystems Inc., Foster City, CA, USA) and the samples were loaded on an ABI PRISM™ 3100 sequencing robot (Applied Biosystems, Florida, USA).

Sequences were edited and aligned using BioEdit (Hall, 1999). The appearance of double peaks in the sequence was considered a co-infection. Co-infection was only detected in one Hawfinch; parasites from this bird were excluded from the morphological description.

#### Phylogenetic analysis

In total, 40 cyt *b* lineages were used in the final alignment for Bayesian analysis. We used 36 sequences of haemosporidian parasites (30 for *Haemoproteus* spp., five for *Plasmodium* spp. and one for *Leucocytozoon* spp.), where species were microscopically identified and the sequences deposited in GenBank and MalAvi. Additionally, we used four cyt *b* lineages of *Haemoproteus* spp. which were reported in birds from order Piciformes in MalAvi database. The cyt *b* lineage of *Leucocytozoon schootedeni* Rodhain, Pons, Vandenbranden & Bequaert, 1913, was used as an outgroup.

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) selected by the software MrModeltest 2.3 (Nylander, 2004; software available from [http://](http://www.abc.se/~nylander/)

[www.abc.se/~nylander/](http://www.abc.se/~nylander/)). Gaps and missing data in the alignment were discarded prior to analyses. Two simultaneous runs were conducted with a sample frequency of every 100th generation over 10 million generations; 25% of the trees were discarded as ‘burn-in’ period. The remaining trees were used to construct a majority rule consensus tree. The phylogenies were visualised using Tree View 1.6.6. (software available from <http://evolution.genetics.washington.edu/phylip/software.html>).

The sequence divergence between different lineages was calculated with Jukes-Cantor model of substitution, with all substitutions weighted equally, implemented in the programme MEGA version 5 (Tamura et al., 2011) in which pairwise deletion was selected. The pairwise deletion option removed from the analysis sites containing missing data or alignment gaps.

MalAvi reference codes (see Bensch et al., 2009) and GenBank accession numbers of all cyt *b* lineages are given in Fig. 3.

#### *Haemoproteus (Parahaemoproteus) homovelans* n. sp.

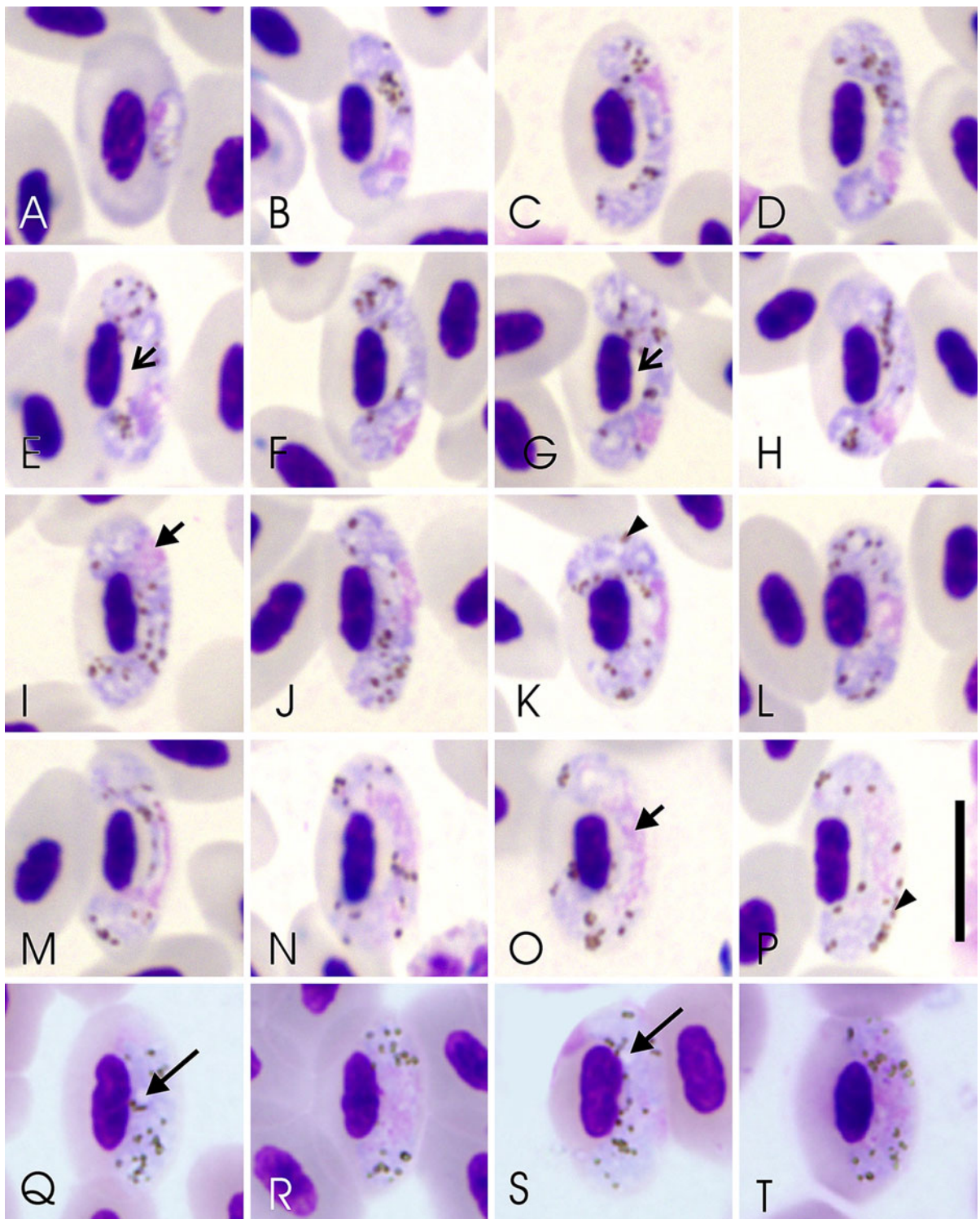
*Type-host*: Grey-faced Woodpecker *Picus canus* Gmelin (Piciformes, Picidae). Female, adult bird caught on 13 July 2008.

*Type-locality*: Mixed deciduous forests of beech and oak, shrubs and grasslands near River Veleka, Strandja Nature Park, in the region of village of Brodilovo, Burgas District, Bulgaria (42°04′40″N, 27°51′28″E; 21 m above sea level).

*Site of infection*: Mature erythrocytes; no other data.

*Prevalence*: One of one bird.

*Type-specimens*: Hapantotype (accession number 48757NS, ex *Picus canus*; parasitemia intensity approximately 0.1 %, 13 July 2008, Strandja Nature Park, collected by M. Ilieva) is deposited in the Institute of Ecology of Nature Research Centre, Vilnius, Lithuania. Parahapantotypes are deposited in the U. S. National Parasite Collection, Beltsville, Maryland, USA (accession number USNPC 107271.00) and the Queensland Museum, Queensland, Australia (accession number G465688). In the hapantotype one trypomastigote of *Trypanosoma* sp. (Kinetoplastida: Trypanosomatidae Doflein, 1901) was recorded.



◀ **Fig. 2** Gametocytes of *Haemoproteus concavocentralis* n. sp. from the blood of Hawfinch *Coccothraustes coccothraustes* (A–P) and of *Haemoproteus vacuolatus* from the blood of Yellow-whiskered Greenbul *Andropadus latirostris* (Q–T): A, young gametocyte; B–L, Q, S, mature macrogametocytes; M–P, R, T, microgametocytes. Short simple arrows indicate unfilled spaces between gametocyte and erythrocyte nucleus; short triangle arrows indicate parasite nuclei; long triangle arrows indicate prominent single vacuoles; triangle arrowheads indicate pigment granules. Giemsa-stained thin blood films. Scale-bar: 10 µm

**Distribution:** Based on MalAvi database this parasite and its lineage hPICAN02 have been recorded only in the type-host in Bulgaria.

**Representative DNA sequence:** Mitochondrial *cyt b* gene, lineage hPICAN02 (479 bp, GenBank accession no. GU085195).

**Etymology:** The species name refers to the morphological and morphometric similarity of the new species with *Haemoproteus velans* Coatney & Roudabush, 1937, a parasite infecting birds of the order Piciformes.

#### Description (Fig. 1A–P)

**Young gametocytes** (Fig. 1A–D). Develop in mature erythrocytes. Earliest forms seen anywhere in infected erythrocytes, predominantly occupy sub-central or polar position in infected erythrocytes; often rod-like or comma-like shaped (Fig. 1A, D). Volutin granules absent. Pigment granules small (<0.5 µm), poorly visible. Outline even or amoeboid (Fig. 1A–D). Young gametocytes do not displace erythrocyte nuclei laterally (Fig. 1A–D).

**Macrogametocytes** (Fig. 1E–L). Grow along the erythrocyte nucleus, enclosing the nucleus with ends; gametocyte extends longitudinally along the nucleus, with one end often surrounding nucleus asymmetrically in relation to erythrocyte poles (Fig. 1E, F). Growing forms often (in approximately 80% of gametocytes) do not touch erythrocyte nucleus, resulting in presence of more or less evident unfilled space (a cleft), which is usually seen between parasite and erythrocyte nucleus (Fig. 1F–I); this cleft disappears in fully-grown gametocytes. Outline becomes more amoeboid as gametocyte grows (Fig. 1G, H). Some mature gametocytes (approximately 5%) do not encircle erythrocyte nucleus, but markedly displace it laterally (Fig. 1J). Fully-grown gametocytes encircle nucleus completely and occupy all cytoplasmic space in erythrocyte. Unfilled space resembling vacuole

sometimes seen between circumnuclear gametocytes and poles of infected erythrocyte (Fig. 1K). Cytoplasm stains dark blue, heterogeneous in appearance. Parasite nucleus compact, oval or sometimes of irregular shape, central or sub-central in position. Nucleolus not seen. Pigment granules small, <0.5 µm, usually roundish in growing gametocytes (Fig. 1E–H); as gametocyte develops, they assume a rod-like shape and reach medium size, 0.5–1.0 µm. Pigment granules randomly scattered throughout cytoplasm. Volutin granules absent. Infected erythrocytes hypertrophied in length, width and area (Table 1).

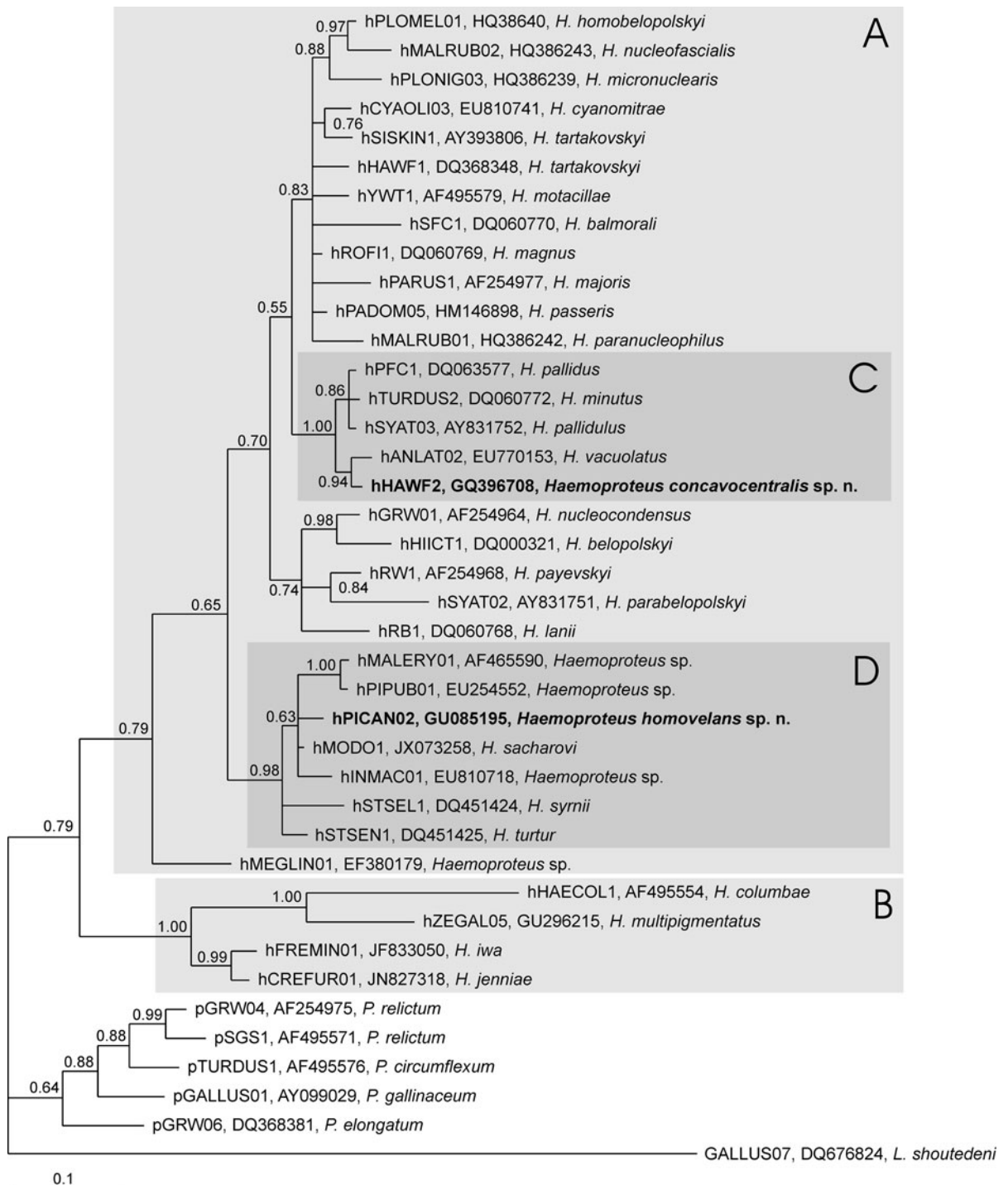
**Microgametocytes** (Fig. 1M–P). General configuration as for macrogametocytes with the usual haemsporidian sexual dimorphic characters. Parasite nucleus stains pink, diffuse, of variable form and often with unclear margins. Volutin granules absent. Number of pigment granules lower than in macrogametocytes (Table 1).

#### Remarks

Among parasites of birds belonging to the Piciformes, gametocytes of *H. homovelans* n. sp. are particularly similar to those of *H. velans* and *H. cornuata* Bennett & Nandi, 1981 (Valkiūnas 2005). The new species is most similar to *H. velans* due to patterns of growth and predominant circumnuclear fully-grown gametocytes; this is reflected in the species name. *Haemoproteus velans* can be readily distinguished from *H. homovelans* due presence of numerous prominent volutin granules and their clearly distinct clumps both in macro- and microgametocytes (compare Fig. 1E–P with Fig. 1Q–T). Additionally, pigment granules are markedly masked and poorly visible in gametocytes of *H. velans*, but they are readily visible in *H. homovelans* (see Fig. 1E–P). Patterns of growth of gametocytes of *H. cornuata* are also similar to *H. homovelans*, but circumnuclear gametocytes do not develop in the former species.

#### ***Haemoproteus (Parahaemoproteus) concavocentralis* n. sp.**

**Type-host:** Hawfinch *Coccothraustes coccothraustes* (Linnaeus) (Passeriformes, Fringillidae). The type-specimen is from a male juvenile bird caught on 20



June 2007 at Kalimok Biological Station. Parahapantotype material came from a male adult bird sampled on 18 May 2009 at Strandja Nature Park, near the village of Brodilovo, Burgas District, Bulgaria

(42°04'40"N, 27°51'28"E; 21 m above sea level), collected by D. Dimitrov.

*Type-locality:* Planted forest of black locust (*Robinia pseudoacacia*) and ash trees (*Fraxinus* spp.) at the



◀ **Fig. 3** Bayesian phylogeny of 40 cytochrome *b* (*cyt b*) gene lineages of positively identified species of avian pigment-forming haemosporidian parasites. *Cyt b* lineage of *Leucocytozoon schoutedeni* was used as an outgroup. New species are given in bold. Codes of the lineages (according MalAvi database, <http://mbio-serv2.mbioekol.lu.se/Malavi/index.html>) are given before GenBank accession numbers and species names of parasites. Nodal support values near branches indicate posterior clade probabilities. Light grey boxes indicate groups of closely related species of haemoproteids: A, *Parahaemoproteus*, B, *Haemoproteus*. Dark grey boxes indicate groups of closely related species within subgenus *Parahaemoproteus*: C, pale-stained parasites, D, non-passeriform parasites

territory of the Kalimok Biological Station, region of the village of Nova Cherna, Silistra District, Bulgaria (44°00'40"N, 26°26'14"E, 18 m above sea level).

*Site of infection:* Mature erythrocytes; no other data.

*Prevalence:* In the type-locality, the prevalence was 5 out of 7 birds (71.4%); overall prevalence was 6 out of 9 birds (66.7%) in all samples collected in Bulgaria.

*Type-specimens:* Hapantotype (accession number 48756NS, ex *Coccothraustes coccothraustes*, 20 June 2007, Kalimok Biological Station, collected by D. Dimitrov, intensity of parasitemia approximately 0.3 %) is deposited in the Institute of Ecology of Nature Research Centre, Vilnius, Lithuania. Parahapantotypes are deposited in the U. S. National Parasite Collection, Beltsville, Maryland, USA (accession number USNPC 107272.00), Queensland Museum, Queensland, Australia (accession number G465689) and Institute of Biodiversity and Ecosystem Research, Sofia (accession number Protozoa-2013-0003). Co-infection with *Leucocytozoon* spp. (similar to *L. fringillinarum* Woodcock, 1910 and *L. dubreulli* Mathis & Léger, 1911) is present in the hapantotype material. Co-infection with *Haemoproteus tartakovskiyi* Valkiūnas, 1986 is present in parahapantotypes.

*Distribution:* Based on MalAvi database this parasite and its lineage hHAWF2 have been recorded only in Bulgaria. It is probably restricted to the Hawfinch populations in their range in Southeast Europe.

*Representative DNA sequence:* Mitochondrial *cyt b* gene lineage hHAWF2 (479 bp, GenBank accession no. GQ396708).

*Etymology:* The species name reflects the concave shape of the central part of advanced gametocytes (Fig. 2B–G, M–N), the most distinctive feature of *H. concavocentralis*.

Description (Fig. 2A–P)

*Young gametocytes* (Fig. 2A). Only one young gametocyte was found in a mature erythrocyte in the type-material (Fig. 2A).

*Macrogametocytes* (Fig. 2B–L). Develop in mature erythrocytes. Cytoplasm pale-blue, heterogeneous in appearance, volutin granules absent. Outline even or slightly irregular at the terminal gametocyte edges. Several vacuoles or vacuole-like spaces of different size and position present in the majority of gametocytes. Gametocyte grows along the nucleus of infected erythrocyte; encloses the nucleus with its ends, but does not encircle it completely. Growing gametocyte usually adheres to erythrocyte envelope but does not touch erythrocyte nucleus resulting in presence of a broad cleft between gametocyte and erythrocyte nucleus. Cleft broader in gametocyte centre and narrower at gametocyte edges (Fig. 2B–G) resulting in a concave shape of the growing parasite (Fig. 2D–G). As parasite develops, gametocyte fills up space on the poles of erythrocyte and then gradually adheres to erythrocyte nucleus close to its poles (Fig. 2D–G). A clear unfilled space between gametocyte and erythrocyte nucleus is maintained at this development stage (Fig. 2F, G). Thin portion of cytoplasm appears close to erythrocyte nucleus; it moves along the nucleus (Fig. 2C, G), gradually increases in size and finally the cleft between the parasite and the nucleus disappears. Fully-grown gametocyte completely fills up the space between erythrocyte nucleus and envelope closely appressing erythrocyte nucleus and markedly displacing it laterally (Fig. 2L). Parasite nucleus relatively small (Table 1), of variable form and position, usually sub-central and often located close to erythrocyte envelope. Nucleolus not seen. Pigment granules roundish or oval, small (<0.5 μm) and medium (0.5–1.0 μm) in size, usually randomly scattered throughout cytoplasm. Influence of growing gametocyte on infected erythrocyte not pronounced. Fully-grown gametocyte markedly displaces erythrocyte nucleus laterally and hypertrophies infected cell in length and area (Table 1).

*Microgametocytes* (Fig. 2M–P): General configuration and pattern of growth as macrogametocyte with usual haemosporidian sexually dimorphic characters. Parasite nucleus stains pale-pink, diffuse, of variable form and often with unclear margins.

**Table 1** Morphometry of host cells and mature gametocytes of *Haemoproteus homovelans* n. sp. (cytochrome *b* lineage hPI-CAN02) and *Haemoproteus concavocentralis* n. sp. (cytochrome *b* lineage hHAWF2) from their avian type-hosts. Minimum and maximum values are provided (in micrometres), followed by the mean and standard deviation in parentheses

Feature	Measurements (n = 21)	
	<i>H. homovelans</i> n. sp.	<i>H. concavocentralis</i> n. sp.
Uninfected erythrocyte		
Length	12.6–15.4 (14.1 ± 0.6)	11.4–13.1 (12.1 ± 0.6)
Width	6.0–7.7 (7.0 ± 0.4)	6.0–7.2 (6.7 ± 0.3)
Area	63.5–92.7 (79.7 ± 6.6)	57.4–77.6 (66.0 ± 4.8)
Uninfected erythrocyte nucleus		
Length	6.0–7.4 (6.5 ± 0.4)	5.3–7.3 (5.9 ± 0.4)
Width	2.3–3.2 (2.8 ± 0.2)	2.3–3.1 (2.7 ± 0.2)
Area	12.4–18.8 (15.1 ± 1.7)	11.2–16.1 (13.5 ± 1.2)
Macrogametocyte infected erythrocyte		
Length	11.6–16.8 (14.9 ± 1.3)	12.4–14.8 (13.6 ± 0.7)
Width	6.5–9.1 (7.4 ± 0.7)	5.7–9.2 (6.7 ± 0.8)
Area	77.0–100.3 (89.0 ± 6.3)	67.5–88.5 (74.7 ± 4.9)
Infected erythrocyte nucleus		
Length	4.9–6.6 (5.9 ± 0.5)	5.0–6.5 (5.7 ± 0.4)
Width	2.0–3.1 (2.5 ± 0.3)	2.0–2.7 (2.4 ± 0.2)
Area	9.4–15.1 (11.7 ± 1.6)	10.4–13.7 (11.6 ± 0.8)
Gametocyte		
Length	17.9–26.2 (23.6 ± 2.3)	13.8–19.1 (16.6 ± 1.3)
Width	2.4–4.6 (3.3 ± 0.6)	2.1–4.1 (2.9 ± 0.5)
Area	58.4–80.9 (70.1 ± 6.2)	43.7–61.2 (49.0 ± 4.4)
Gametocyte nucleus		
Length	2.0–4.8 (2.9 ± 0.6)	1.4–5.0 (3.0 ± 0.9)
Width	1.3–3.3 (2.2 ± 0.4)	1.0–2.3 (1.6 ± 0.4)
Area	2.7–6.7 (4.62 ± 1.1)	1.3–4.9 (3.2 ± 0.9)
Pigment granules		
NDR <sup>a</sup>	0.2–0.9 (0.6 ± 0.2)	0.3–0.9 (0.6 ± 0.2)
Microgametocyte infected erythrocyte		
Length	12.3–16.7 (14.9 ± 1.4)	11.4–15.2 (13.5 ± 0.8)
Width	6.5–9.1 (7.3 ± 0.7)	5.5–8.3 (6.7 ± 0.6)
Area	65.4–114.8 (89.5 ± 11.9)	61.2–88.2 (75.9 ± 6.4)
Infected erythrocyte nucleus		
Length	4.6–7.0 (6.0 ± 0.7)	4.6–6.0 (5.6 ± 0.3)
Width	2.3–3.3 (2.7 ± 0.2)	2.1–2.7 (2.4 ± 0.1)
Area	9.6–15.7 (13.1 ± 1.8)	9.8–12.6 (11.4 ± 0.8)
Gametocyte		
Length	15.5–26.9 (20.1 ± 3.1)	14.3–20.2 (17.3 ± 1.5)
Width	2.6–4.7 (3.5 ± 0.6)	1.9–4.1 (3.1 ± 0.5)
Area	45.5–82.2 (63.7 ± 8.5)	45.5–58.3 (51.9 ± 3.9)
Gametocyte nucleus		
Length	6.4–12.6 (9.4 ± 1.6)	4.3–10.2 (7.3 ± 1.5)
Width	2.1–5.0 (3.6 ± 0.9)	1.6–5.0 (2.9 ± 0.9)
Area	15.0–34.0 (23.9 ± 5.8)	8.9–19.6 (14.2 ± 3.4)

**Table 1** continued

Feature	Measurements (n = 21)	
	<i>H. homovelans</i> n. sp.	<i>H. concavocentralis</i> n. sp.
Pigment granules	13.0–36.0 (26.8 ± 6.1)	13.0–23.0 (16.1 ± 2.3)
NDR <sup>a</sup>	0.1–0.9 (0.5 ± 0.2)	0.3–0.9 (0.6 ± 0.2)

<sup>a</sup> NDR, nucleus displacement ratio according to Bennett & Campbell (1972)

## Remarks

Data on the pattern of development and the position of gametocytes in infected erythrocytes are essential for identification of this species. A readily distinguishable space between the growing gametocyte and the erythrocyte nucleus is a unique character for *H. concavocentralis* n. sp. This space gives gametocytes a concave shape (Fig. 2D–G) and this is reflected in the species name. Due to this character *H. concavocentralis* can be readily distinguished from all described species of haemoproteids of passeriform birds.

Based on the phylogenetic analysis (Fig. 3, clade C) *H. concavocentralis* is closely related to *H. vacuolatus*. The existence of one clear circular vacuole, which is present in the cytoplasm of each advanced macrogametocyte is a characteristic feature of *H. vacuolatus* (Fig. 2Q–T). Such vacuoles were not seen in *H. concavocentralis*. Additionally, *H. concavocentralis* can be readily distinguished from *H. vacuolatus* due to (i) unique patterns of growth (see above), and (ii) marked vacuolisation of the cytoplasm.

During identification of *H. concavocentralis*, attention also should be paid to the pale-blue staining of the cytoplasm in macrogametocytes. This character helps to distinguish this parasite from *H. tartakovskyi* and *Haemoproteus fringillae* (Labbé, 1894), common parasites of the Hawfinch.

## Morphological and molecular examination of the blood samples and phylogenetic analysis

Morphological and molecular examination of the samples used for designation of the type-specimens of *H. homovelans* (hPICAN02) and *H. concavocentralis* (hHAWF2) showed the presence of single infections. This was confirmed by screening of entire blood smears and sequencing of the PCR products from both directions. The hapantotype material used

for the morphological description of the lineage hHAWF2 was from a bird with co-infection of *H. concavocentralis* and *Leucocytozoon* sp. There are different primers used for molecular identification of parasites belonging to *Leucocytozoon* spp. that were not applied in this study and in the present case only the lineage hHAWF2 was detected.

A second bird was co-infected with *H. concavocentralis* and *H. tartakovskyi*; blood films from this bird were used for taking measurements and were deposited as parahapantotype material. The haemoproteid infection in this bird comprised mainly fully grown mature gametocytes of *H. concavocentralis* but also light parasitemia of *H. tartakovskyi* (0.02%). PCR amplification did not detect the infection with *H. tartakovskyi*; only the lineage hHAWF2 of *H. concavocentralis* was present on the electrophoregrams. However, both parasite species were readily distinguishable under light microscope and we easily selected mature gametocytes of *H. concavocentralis* (hHAWF2) to take measurements.

We confirmed *Haemoproteus* spp. infections by light microscopy examination in four of six Hawfinches infected with lineage hHAWF2. In two of these birds, we recorded only *Leucocytozoon* sp. and *Plasmodium* sp. on the blood slides. Of the remaining four birds, three were co-infected with *H. concavocentralis* and *H. tartakovskyi* and one carried an additional unidentified *Plasmodium* infection. However, no co-infections in these birds were detected by PCR.

The genetic divergence between cyt *b* lineages of *H. concavocentralis* (hHAWF2) and *H. tartakovskyi* (lineages hHAWF1 and hSISKIN1) was relatively high, i.e. 4.5 and 4.8% respectively; both parasites were located rather far from each other in the phylogenetic tree (Fig. 3). Although the lineage hHAWF2 clustered together with the lineage hANLA1 of *H. vacuolatus* (Fig. 3, clade C), the genetic divergence between them (1.8%) was the same as the divergence between the lineages hPFC1 of *H. pallidus*

Valkiūnas & Iezhova, 1991 and hSYAT03 of *H. pallidulus* Križanauskienė, Pérez-Tris, Palinauskas, Hellgren, Bensch & Valkiūnas, 2010. However, the morphological features of *H. vacuolatus* were more similar to *H. concavocentralis* (hHAWF2) than to both *H. pallidus* (hPFC1) and *H. pallidulus* (hSYAT03).

The intensity of haemoproteid infection was approximately 0.1% in the type-preparations of *H. homovelans*; all stages of gametocyte development were present. Cyt *b* lineage hPICAN02 formed a well-supported clade (with nodal support value of 0.98) with other lineages parasitising birds of the Columbiformes, Strigiformes and Piciformes (Fig. 3, clade D). The most closely related cyt *b* lineage was hMOD01 of *H. sacharovi* Novy & MacNeal, 1904 with genetic divergence of 2.3% between these lineages. However, both parasites exhibit significant morphological differences.

## Discussion

Species identification of avian haemosporidian parasites is a difficult task in wildlife due to the following main reasons: (i) common light natural infections that might be a consequence of the methods of bird sampling (mist-netting and trapping) that predominantly select actively moving, healthy individuals with light parasitemia; (ii) common co-infections of parasites belonging to the same or different genera; (iii) insufficient training in taxonomy of researchers to identify species; and (iv) application of different protocols for molecular identification and labelling of the parasite lineages by different researchers. These difficulties should be addressed because studies on parasite diversity and species richness are of great importance for further analyses on host–parasite interactions such as host specificity, pathogenicity, distribution and co-evolution.

Traditional taxonomy and systematics of haemosporidian parasites are predominantly based on morphological features of the blood stages in the vertebrate hosts observed under light microscopy (Garnham, 1966; Valkiūnas, 2005). The implementation of molecular methods for detection and identification of haemosporidian parasites (Bensch et al., 2000, 2004; Perkins & Schall, 2002; Ricklefs & Fallon, 2002; Beadell et al., 2004; Hellgren et al., 2004; Waldenström et al., 2004; Martinsen et al., 2008) has revealed remarkable genetic diversity

among these hematozoans and has markedly supplemented traditional taxonomy. Further taxonomic research, focused on the linkages between different parasite species and their genetic lineages is essential for parasite barcoding (Martinsen et al., 2006, 2008; Hellgren et al., 2007; Valkiūnas et al., 2007; Križanauskienė et al., 2006, 2010, 2012).

Recent morphological descriptions of new haemosporidian species are usually accompanied by DNA sequence information and phylogenetic hypotheses about the relationships of the novel species with already described parasites. In this study, *H. homovelans* n. sp. and *H. concavocentralis* n. sp. were attributed to the subgenus *Parahaemoproteus* because cyt *b* lineages of these parasites cluster (Fig. 3, clade A) with the lineages transmitted by biting midges (Ceratopogonidae) (Križanauskienė et al., 2013). The lineages of the hippoboscid-transmitted species *Haemoproteus columbae* Kruse, 1890, *Haemoproteus multipigmentatus* Valkiūnas, Santiago-Alarcon, Levin, Iezhova, & Parker, 2010, *Haemoproteus iwa* Work & Rameyer, 1996 and *Haemoproteus jenniae* Levin, Valkiūnas, Iezhova, O'Brien & Parker, 2012 which belong to the subgenus *Haemoproteus*, are positioned in a separate clade with high nodal support (clade B in Fig. 3). This group appears to be a sister clade to the species of subgenus *Parahaemoproteus*, as also revealed by Iezhova et al. (2011) and Križanauskienė et al. (2013). According to recent phylogenetic studies (Santiago-Alarcon et al., 2010; Iezhova et al., 2011; Levin et al., 2012; Križanauskienė et al., 2013), *Parahaemoproteus* and *Haemoproteus* are closely related sister groups of avian haemoproteids, so the traditional subgeneric classification of avian haemoproteids (Levine & Campbell, 1971; Valkiūnas, 2005) remains valid.

In our samples, we reported three individual birds with co-infection with *H. concavocentralis* and *H. tartakovskiyi*, but none with infection of *H. fringillae*. It is important to note that co-infection was not detected in the same birds by PCR. Hence, we confirm the results from previous studies that have applied general PCR protocols and often underestimated co-infections in the wild birds (Valkiūnas et al., 2006; Martínez et al., 2009). This is an obstacle for studies that rely solely on PCR-based methods for assessment of haemosporidian diversity. PCR screening alone is not advised in studies of haemosporidian parasites, particularly in such species as the Hawfinch because co-infections were reported in over 80% of infected



individuals of this species in Europe (Valkiūnas et al., 2003).

Important advantages of the PCR-based diagnostics in comparison to light microscopy diagnostics of haemosporidian parasites are: (i) high sensitivity to detect light infections; (ii) accurate identification of light chronic infections; (iii) opportunities to identify species lineages based on tissue stages; (iv) opportunities to construct phylogenetic hypotheses; and (v) possibilities to apply molecular identification which can be used easily by non-specialists in taxonomy of haemosporidians (Bensch et al., 2000, 2004; Hellgren et al., 2004; Waldenström et al., 2004). However, all currently used general PCR protocols (Perkins & Schall, 2002; Hellgren et al., 2004; Waldenström et al., 2004; Martinsen et al., 2008) usually markedly underestimate co-infections which are common in wildlife and even predominate in some bird populations (Valkiūnas et al., 2003, 2006; Martínez et al., 2009). It is worth mentioning that PCR-based methods also detect circulating haemosporidian sporozoites, whose fate is unclear in the birds (Valkiūnas et al., 2009). Because PCR may detect sporozoites and also abortive haemosporidian infections (Cannell et al., 2013; Levin et al., 2013), conclusions regarding the specificity and distribution of haemosporidian lineages in wildlife should be made with caution. In order to be accepted as a successfully developing lineage of haemosporidians, such PCR-based information should be supported with the detection of blood stages (gametocytes) of the parasites. There is a crucial need of a synthesis of the information provided by the tools of traditional parasitology and molecular biology, particularly in the field research of blood parasites.

Vector species of *H. homovelans* and *H. concavocentralis* need to be identified, but the close phylogenetic relationships of their *cyt b* lineages with the lineages of ceratopogonid-transmitted parasites (Fig. 3, clade A) show that the biting midges (*Culicoides* spp.) should be suspected as possible vectors. It is worth mentioning that the recent molecular survey of whole bodies of biting midges recorded the lineage hHAWF2 in *Culicoides circumscriptus* (Kieffer) indicating that this midge could be a vector of *H. concavocentralis* (see Bobeva et al., 2013). The genetic distances among the *cyt b* lineages of the two new species described here and the lineages of hippoboscid-transmitted parasites are more than 10%; it is unlikely that these parasites could be transmitted by hippoboscid flies.

Based on gametocyte morphology, *H. homovelans* n. sp. is especially similar to *H. velans* which has been recorded in the same avian host, the Grey-faced Woodpecker, but at a distant geographical location, Southeast Asia (Thailand) (Greiner et al., 1977). The new species can be readily distinguished from *H. velans* due to several morphological features, which are summarised in the Remarks. It is worth mentioning that *H. velans* has also been reported from the Syrian Woodpecker, *Dendrocopus syriacus* (Hemprich & Ehrenberg), in South Bulgaria (Shurulinkov & Golemansky, 2002). However, the latter study was not accompanied by illustrations or sequence information, so the report of *H. velans* in Syrian Woodpecker requires confirmation. In the notes to the description of *H. velans*, Shurulinkov & Golemansky (2002) mentioned that gametocytes observed in their material lack volutin granules and differ from the original description of *H. velans*. From this point of view, the haemoproteid species from the Syrian Woodpecker is more similar to *H. homovelans*. Collection of additional material for molecular characterisation of *H. velans* from its type-host, the Northern Flicker, *Colaptes auratus* (Linnaeus), and from the type-locality (North America, Nebraska, USA) is required for further phylogenetic comparison between *H. homovelans* and *H. velans*.

This study provides the first information for molecular characterisation of haemoproteid parasites of birds belonging to the Piciformes; *cyt b* lineage hPICAN02 can be used for molecular diagnostics of *H. homovelans*. Martinsen et al. (2008) identified one *Haemoproteus cyt b* lineage (hPIPUB01, GenBank accession no. EU254552) isolated from the Downy Woodpecker, *Picoides pubescens* (Linnaeus), as *Haemoproteus picae* Coatney & Roudabush, 1937, a widespread parasite of birds from the family Corvidae of the Passeriformes (Valkiūnas, 2005). According to our phylogeny, *cyt b* lineage hPIPUB01 appeared in clade D (Fig. 3), with genetic divergence of 3.2% between the lineage hPICAN02 of *H. homovelans*, a parasite of non-passeriform birds. It is probable that this is an incorrectly developed DNA barcode because the authors did not provide morphological evidence for the identification of this lineage. Further studies are needed for identification of haemoproteids of the Downy Woodpecker.

The clearly visible unfilled concave space between the growing gametocytes and the erythrocyte nuclei (Fig. 2B–G) is a unique morphological feature of *H. concavocentralis*; it can be used for distinguishing this

parasite from all other described species of haemoproteids parasitising birds (Valkiūnas, 2005). It was unexpected to find a new species of *Haemoproteus* in a well-studied avian host such as the Hawfinch (Peirce, 1981; Valkiūnas et al., 2003; Palinauskas et al., 2007), particularly at as high prevalence and with so readily distinguishable morphology. This is probably due to the restricted area of distribution of this parasite in Southeast Europe, a suggestion that needs confirmation from larger spatial study of different European populations of Hawfinches. *Haemoproteus tartakovskiyi* and *H. fringillae* are the most common haemoproteid parasites in *C. coccothraustes* (see Valkiūnas et al., 2003) but gametocytes of both species are markedly different from those of *H. concavocentralis* (see Valkiūnas, 2005) (see Remarks). Furthermore, the type-host of *H. concavocentralis*, the Hawfinch is mainly sedentary widespread bird on Balkan Peninsula (BWPi 2.0, 2006) suggesting that this infection is endemic in the region.

Lineage hHAWF2 clustered together with four so-called pale-stained parasites: *Haemoproteus pallidus*, *H. pallidulus*, *H. minutus* Valkiūnas & Iezhova, 1992 and *H. vacuolatus* (Fig. 3, clade C). In comparison with other *Haemoproteus* spp., the cytoplasm of macrogametocytes of these species stains pale with Giemsa, probably due to the relatively low density of some cytoplasmic structures in the macrogametocytes (endoplasmic reticulum, ribosomes, osmiophilic bodies) (Valkiūnas, 2005). Interestingly, all these parasites are members of the same strongly-supported clade (Fig. 3, clade C) with a small mean genetic divergence (1.3%) among their cyt *b* lineages. However, blood stages of these parasites are readily distinguishable from each other (Valkiūnas, 2005; Valkiūnas et al., 2008a; Križanauskienė et al., 2010; Palinauskas et al., 2013) and possess one common feature, i.e. the cytoplasm of both macro- and microgametocytes assume similar pale-blue staining. We observed this character clearly on the slides with co-infection of *H. concavocentralis* and *H. tartakovskiyi*. This is a characteristic feature of all parasites from clade C (Fig. 3) but is a rare feature in avian haemoproteids.

Parasites of the genus *Haemoproteus* have been often considered as relatively benign, with minimal or no effect on avian hosts (Bennett et al., 1993; Gilman et al., 2007). This is probably true in evolutionary adapted host–parasite systems (Valkiūnas, 2005).

However, mortality and severe pathologies were recorded during abortive *Haemoproteus* spp. development at tissue stage in non-adapted hosts, for instance, captive parrots in Europe and Little Penguins, *Eudyptula minor* (J. R. Forster), in Australia (Olias et al., 2011; Cannell et al., 2013). It remains unclear how often avian haemosporidians cause lethal disease in wildlife (Levin et al., 2013). Unfortunately, it is difficult or even impossible to use the morphology of the exoerythrocytic tissue stages alone for accurate diagnosis of *Haemoproteus* spp. infections (Valkiūnas, 2005; Atkinson, 2008). Due to difficulties in identification of haemosporidian species from histological preparations of tissues, recent studies (Olias et al., 2011; Cannell et al., 2013) applied PCR-based techniques to tissue samples and reported cyt *b* lineages of *Haemoproteus* spp. in dead birds.

In conclusion, the present study describes two new species, develops DNA barcodes for their molecular diagnostics, and identifies their phylogenetic relationships within subgenus *Parahaemoproteus*. This study also emphasises the value of molecular and microscopy methods in the identification of avian haemosporidians and descriptions of new species. The development of barcodes for molecular diagnostics of avian haemosporidian parasites is an urgent task, particularly because of the huge biodiversity of these avian pathogens, the common co-infections in the wildlife, and the severe pathology caused by some species. Importantly, the molecular diagnostics of haemoproteid parasites is becoming an essential tool in veterinary studies, particularly in determining parasites in tissue samples from necropsies of birds (Olias et al., 2011; Cannell et al., 2013). Identification of haemosporidian parasites, which have abortive development in avian hosts, but cause lethal diseases, is a new field of interest that reveals the pathogenic potential of some haemoproteid species that have been traditionally considered to be relatively benign.

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