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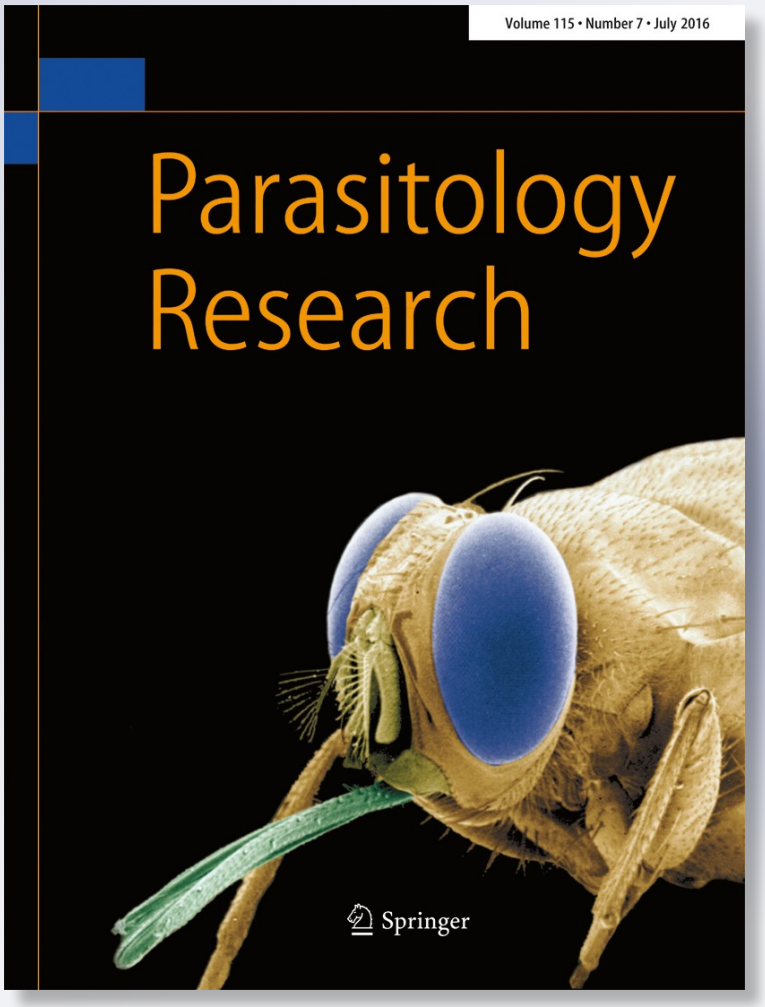

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ORIGINAL PAPER

Plasmodium delichoni n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission

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Abstract Malaria parasite *Plasmodium* (*Novyella*) *delichoni* n. sp. (Haemosporida, Plasmodiidae) was found in a widespread Eurasian songbird, the common house martin *Delichon urbicum* (Hirundinidae). It is described based on the morphology of its blood stages and segments of the mitochondrial cytochrome *b* and apicoplast genes, which can be used for molecular identification of this species. Erythrocytic meronts and gametocytes are strictly nucleophilic, and mature gametocytes possess pigment granules of markedly variable size, including large ones (1 μm in length). Due to these features, *P. delichoni* can be readily distinguished from all described species of avian malaria parasites belonging to subgenus *Novyella*. Additionally, mature erythrocytic merozoites contain a dense clump of chromatin, a rare character in avian malaria parasites. Erythrocytic merogony is asynchronous. Illustrations of blood stages of the new species are given, and phylogenetic analysis identifies DNA lineages closely related to this parasite. Domestic canary *Serinus canaria* and Eurasian siskin *Carduelis spinus* were infected after subinoculation of infected blood obtained from the house martin. Parasitemia was long lasting in both these hosts, but it was high (up to 70 %) in Eurasian siskins and low (up to 1 %) in canaries. Mortality was not observed, and histological examination and chromogenic in situ hybridisation did not reveal secondary exoerythrocytic meronts (phanerozoites) in the exposed birds. It is likely that persistence of this infection occurs due to long-lasting parasitemia in avian hosts. Sporogony was abortive in mosquitoes *Culex pipiens pipiens* form *molestus*, *Culex quinquefasciatus* and *Aedes aegypti* at gametogenesis or ookinete stages. The new species is absent from juvenile birds at breeding sites in Europe, indicating that transmission occurs at African wintering grounds.

Keywords *Plasmodium* · New species · Birds · *Delichon urbicum* · Molecular characterisation · Vectors · Persistence · Transmission

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Introduction

Malaria parasites of the genus *Plasmodium* (Plasmodiidae, Haemosporida) are widespread in birds, and many species cause severe diseases in avian hosts (Zehtindjiev et al. 2008; Braga et al. 2011; Palinauskas et al. 2011). Lethal outbreaks of avian malaria have been described in wildlife (Gabaldon and Ulloa 1980; Stone et al. 1971; Atkinson et al. 2008). However, the patterns and consequences of malaria infections remain insufficiently understood in wildlife populations (Valkiūnas 2005; Glaizot et al. 2012; Marzal 2012; Garcia-Longoria et al. 2015; Marinov et al. 2016). Over 50 species of *Plasmodium* infecting birds have been named and described (Garnham 1966; Valkiūnas et al. 2008; Mantilla et al. 2013; Ilgūnas et al. 2013; Walther et al. 2014). Genetic diversity of these parasites is remarkable, indicating that many more species exist (Bensch et al. 2009; Clark et al. 2014; Perkins 2014; Outlaw and Ricklefs 2014). Knowledge about the diversity of malaria parasites is crucial for better understanding evolutionary biology of *Plasmodium* parasites and epidemiology of avian malaria in wildlife.

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As part of an on-going study on the diversity of avian malaria parasites and other haemosporidians (Dimitrov et al. 2014; Valkiūnas et al. 2014; Palinauskas et al. 2015), blood samples from migrating birds were collected at the Ventės Ragas Ornithological Station in Lithuania. One previously undescribed species of avian *Plasmodium* was found in a widespread Eurasian songbird, the house martin *Delichon urbicum*. This is a migratory insectivorous bird belonging to the Hirundinidae. It breeds in Europe, North Africa and temperate Asia and winters in sub-Saharan Africa and tropical Asia. The house martin tends to breed colonially and is found in both open country with low vegetation and near human habitation. It uses similar habitats both on the breeding and wintering areas (Snow and Perrins 1998). There are numerous reports of haemosporidian and other blood parasites in house martins, but *Plasmodium* spp. have only been incidentally reported in this bird species. Malaria parasites of subgenus *Novyella* have not been found in house martins and other European species of the Hirundinidae (Bennett et al. 1982; Bishop and Bennett 1992; Valkiūnas 2005).

Here, we describe a new species of malaria parasite infecting the house martin using data on the morphology of its blood stages and partial sequences of the mitochondrial and apicoplast genomes. Experiments were carried out to better understand the development of this pathogen in different avian hosts and its potential vectors. The aims of this study were (1) to provide morphological description of a new *Plasmodium* species; (2) to determine DNA sequences, which can be used for molecular characterisation and identification of this parasite; (3) to compare genetic distances and phylogenetic relationships of the new species with closely related *Plasmodium* parasites; (4) to determine experimental avian hosts and patterns of this infection development in these hosts; and (5) to follow sporogonic development in experimentally infected widespread mosquito species.

Material and methods

Collection of blood samples

Fieldwork was carried out at the Ventės Ragas Ornithological Station, Lithuania in May, 2015. Twenty house martins and 29 barn swallows *Hirundo rustica* were caught with mist nets and large stationary traps. The blood was taken from birds by puncturing the brachial vein. About 30 μl of whole blood was taken in heparinised microcapillaries and stored in SET buffer (Hellgren et al. 2004) for molecular analysis. Blood films were prepared from each bird immediately after withdrawal of the blood. They were air-dried, fixed in absolute methanol and stained with Giemsa (Valkiūnas 2005).

To find malaria-infected birds and isolate parasite strains, blood films from each sampled bird were quickly examined microscopically in the field. For this purpose, one blood film was stained using 30 % Giemsa solution for 15 min, air-dried using an electric fan and examined at high magnification for 3–5 min. This provided opportunities to determine malaria infected birds, collect infected blood and release the birds at the study site within one hour after their capture. One common house martin infected with an undescribed *Plasmodium* sp. (parasitemia of 1.8 %) was used for the new parasite strain isolation during this study. Infected blood was collected in heparinised microcapillaries and used to expose two uninfected domestic canaries *Serinus canaria* and two juvenile Eurasian siskins *Carduelis spinus*. These birds were exposed by subinoculation of about 250 μl of freshly prepared mixture, containing infected blood, 3.7 % sodium citrate (anticoagulant) and 0.9 % saline (4:1:5) into their pectoral muscle (Iezhova et al. 2005). Before the subinoculation experiments, all recipient birds were proven to be uninfected with haemosporidian parasites by microscopic examination of blood films and later by PCR-based testing in the laboratory (see below). The donor house martin was released after obtaining infected blood.

The observation time for all experimental birds was 172 days post exposure (dpe). Blood was taken for microscopic examination and PCR-based testing as described above once per 3 or 4 days during the first month post exposure, once per a week during the second month post exposure and once per 1–2 weeks during the remaining experiment time. Additionally, blood of one Eurasian siskin was tested 241 dpe. All birds were kept indoors in a vector-free room under controlled conditions (20 ± 1 °C, 50–60 % relative humidity (RH), natural light-dark photoperiod (L/D)) and were fed a standard diet for seed-eating bird species. Parasitemia developed in all exposed canaries and Eurasian siskins. Blood samples with intense parasitemia (>10 %) were cryopreserved for future research, as described by Garnham (1966).

Histological examination of bird organs

Three exposed birds (two canaries and one Eurasian siskin) were euthanized 172 dpe. Parasitemia was 0.0001 and 0.04 % in the canaries and the Eurasian siskin, respectively. Organs (brain, heart, kidneys, liver, lungs, spleen, bone marrow) and pieces of the pectoral muscles of these birds were dissected, fixed in 10 % neutral formalin solution and embedded in paraffin. Histological sections of 4 μm were obtained and stained with haematoxylin-eosin (Valkiūnas 2005). Preparations were examined microscopically (see below).

Chromogenic in situ hybridisation

Chromogenic in situ hybridisation was performed using the same tissue samples, which were used for histological examination. We followed a previously published protocol by

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Dinhopl et al. (2011). In brief, 3 μm paraffin wax-embedded tissue sections were de-waxed and rehydrated. They were subjected to proteolytic treatment with proteinase K (Roche, Basel, Switzerland) 6 μg/ml in Tris-buffered saline at 37 °C for 50 min. Then, the slides were washed in distilled water and dehydrated in 96 % ethanol and 100 % isopropanol followed by air-drying. Afterwards, the slides were incubated overnight at 40 °C with hybridisation mixture, 100 μl of which was composed of 50 μl formamide (50 %), 20 μl 20× standard sodium citrate (SSC), 12 μl distilled water, 10 μl dextran sulphate (50 %, *w*/*v*), 5 μl herring sperm DNA (50 mg/ml), 2 μl Denhardt's solution and 1 μl *Plasmodium* sp. probe at a concentration of 100 ng/ml.

The oligonucleotide probe (sequence: 5′-TTTAAT AACTCGTTATATATATCAGTGTAGCAC-3′) was labelled with digoxigenin at the 3′ end (Eurofins MWG Operon, Ebersberg, Germany). It is specific to detect avian *Plasmodium* spp. (Dinhopl et al. 2015).

The next day, the slides were washed in decreasing concentrations of SSC (2× SSC, 1× SSC and 0.1× SSC, 10 min each at room temperature (RT)), for removal of unbound probe. Then, the slides were incubated with antidigoxigenin-AP Fab fragments (Roche) (1:200) for 1 h at RT. The hybridised probe was visualised subsequent to an additional washing step using the colour substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT) (Roche). After 1 h of incubation, the colour development was stopped using TE buffer (pH 8.0) for 10 min. Finally, the slides were counterstained with haematoxylin and mounted under coverslips with Aquatex (VWR International, Vienna, Austria).

Maintenance and experimental infection of laboratory-reared mosquitoes

To establish a colony of *Culex pipiens pipiens* f. *molestus*, we used the mosquito larvae, which were obtained from Dr. Roland Kuhn. The colony was originally started from larvae collected in Hesse Region (Germany). Colonies of *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes were established using eggs provided by Dr. Ana Rivero (France) and Dr. Hilary Ranson (UK), respectively. All mosquito colonies have been in continuous culture for many years. We colonised these insects, as described by Žiegytė et al. (2014). Briefly, mosquitoes were kept in cages (120 × 45 × 45 cm) under standard conditions (65–70 % RH and L/D photoperiod of 14/8 h). Because sporogonic development of *Plasmodium* parasites is sensitive to air temperature (Garnham 1966; Sherman 1998), two temperature conditions were used: one infected group of each species was maintained at 21 ± 1 °C and another infected group at 26 ± 1 °C. Adult mosquitoes were fed with 5–10 % saccharose solution. Cotton wool pads moistened with this solution were provided in mosquito cages.

Two days before exposure, approximately 30 unfed females of each species were haphazardly chosen and placed inside separate experimental cages of (45 × 45 × 45 cm). To increase favour of blood feeding, the experimental mosquitoes were deprived of saccharose. Eurasian siskins with gametocytemia ranging between 0.1 and 2 % were placed in mosquito cages and exposed, as described by Kazlauskienė et al. (2013). Briefly, infected birds were placed in plastic tubes (length 15 cm, diameter 5 cm) containing a rip, which was used to fix the bird legs. Both tube ends were covered with bolting silk. Only legs were exposed to mosquito bites. The birds were kept in insect cages for approximately 15–20 min once per 3–4 days. All mosquito species willingly took blood meals on bird legs. We evaluated parasitemia in all donor birds immediately after mosquito blood meals. Engorged females were taken from the experimental cages using an aspirator, placed in separate small insect cages (12 × 12 × 12 cm), maintained at the same conditions as their colonies to allow development of parasites and dissected in intervals (see below). Experiments with all mosquito species were carried out in parallel.

Ookinete preparations ($n = 20$) were made 1–3 dpe, oocyst preparations ($n = 39$) 6–14 dpe and sporozoite preparations ($n = 73$) 13–25 dpe. In total, we infected and dissected 132 mosquitoes. Among them were 37 females of *Cu. p. pipiens* f. *molestus* (20 individuals were maintained at 21 ± 1 °C and 17 individuals at 26 ± 1 °C), 46 females of *Cu. quinquefasciatus* (28 and 18 individuals, respectively) and 49 females of *A. aegypti* (26 and 23 individuals, respectively).

Dissection of mosquitoes and making preparations of ookinetes, oocysts and sporozoites

All mosquitoes were processed individually. Before dissection, insects were lightly anaesthetised by putting them into a tube closed with a cotton pad wetted in 96 % ethanol for several minutes. Wings and legs of the insects were removed before dissection, which was performed under a binocular stereoscopic microscope. To eliminate contamination of samples, we either used a new dissecting needle for each dissected insect or disinfected the needles in fire after each dissection.

Permanent preparations of the semi-digested midgut contents, entire midguts and salivary glands were prepared in order to observe ookinetes, oocysts and sporozoites, respectively. All preparations were prepared according to Kazlauskienė et al. (2013). Remnants of all dissected insects were also fixed in 96 % ethyl alcohol for PCR-based screening, which was used to confirm the presence of corresponding lineage of parasite.

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DNA extraction, PCR, sequencing and phylogenetic analysis

Total DNA was extracted from blood and insect samples using the standard ammonium-acetate protocol (Sambrook and Russell 2001) with minor modification: instead of 250 μl of fixed blood, we used 125 μl. Quantification of the DNA was performed by using a nanodrop spectrophotometer (Implen NanoPhotometer P330). All reactions were performed in 25 μl total volumes, including 50 ng of a total genomic DNA template (2 μl), 12.5 μl of DreamTag Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 μl nuclease-free water and 1 μl of each primer. One negative control (nuclease-free water) and one positive control (an infected sample, which was positive by microscopic examination of blood films) were used every 7 samples to control for false amplifications. No cases of false positive or negative amplifications were found.

The 479-bp length segments of parasite mitochondrial cyt *b* gene were amplified using nested PCR protocol (Bensch et al. 2000; Hellgren et al. 2004) and two pairs of primers: HaemNFI/HaemNR3, which amplify gene fragments of haemosporidians belonging to genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*; and the primers HAEMF/HAEMR2, which are specific to *Haemoproteus* and *Plasmodium* spp. The 'Basic Local Alignment Search Tool' (National Centre for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST) was used to determine cyt *b* lineages of detected DNA sequences. The primers ClpcF/R (outer) and ClpcF/R (nested) were used for the amplification of apicoplast gene fragments of 536 bp length (Martinsen et al. 2008).

Temperature profiles in all PCRs were the same as in the original protocol descriptions. All amplifications were evaluated by running 2 μl of the final PCR products on 2 % agarose gels. Fragments of DNA from all positive amplifications were sequenced with corresponding primers for both strands. The obtained sequences were aligned and analysed using BioEdit program (Hall 1999).

Visualisation of 'double bases' in electropherograms of sequences was used to estimate the presence of possible haemosporidian co-infections in wild-caught and experimentally exposed birds (Pèrez-Tris and Bensch 2005). Sequence divergence among lineages was calculated with MEGA version 5, using a Jukes-Cantor model of substitution in which all substitutions were weighted equally (Tamura et al. 2011).

For phylogenetic analysis, we downloaded haemosporidian parasite cyt *b* sequences from the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/). GenBank accession numbers of sequences and codes of the parasite lineages are given in Fig. 1. The phylogenetic tree was constructed using mrBayes version 3.1 (Ronquist and Huelsenbeck 2003). The appropriate model of sequence evolution was determined by the software mrModeltest version 2.3 (Nylander 2008) to be a General Time Reversible model including variable sites and gamma-shaped rate variation across sites (GTR + I + G). Phylogenetic analyses were conducted using PAUP version 4 (Swofford 2002). Visualisation of the phylogenetic tree was conducted using TreeView 1.6.6 (software available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Microscopic examination of blood, vector and histological preparations

Detailed microscopic analysis was carried out in the laboratory. An Olympus BX61 light microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera and imaging software AnalySIS FIVE (Olympus Soft Imaging Solution GmbH, Münster, Germany) was used to examine slides, to prepare illustrations and to take measurements. Approximately 100–150 fields were examined in blood films at low magnification (×400), and then at least 100 fields were studied at high magnification (×1000). Intensity of parasitemia was estimated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes if infections were light (<0.1 %). All vector and histological preparations were first examined at low magnification (×600) and then at high magnification (×1000). The morphometric features studied (Table 1) were those defined by Valkiūnas (2005). The analyses were carried out using the 'Statistica 7' package. Student's *t* test for independent samples was used to determine statistical significance between mean linear parameters of parasites. A *P* value of 0.05 or less was considered significant.

Ethical statement

Experimental procedures of this study were approved by the Ethical Commission of the Baltic Laboratory Animal Science Association, Lithuania; Lithuanian State Food and Veterinary Office (permit 2015-05-07, no. G2-27); Environmental Protection Agency, Vilnius (permits 2015-04-08, no. 21 and 2015-04-27, no. 25); and the International Research Co-operation Agreement between the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25-05-2010). All efforts were made to minimise handling time and potential suffering of animals.

Results

Description of parasite

Plasmodium (Novyella) delichoni n. sp.

Type host The house martin *D. urbicum* (Passeriformes, Hirundinidae).

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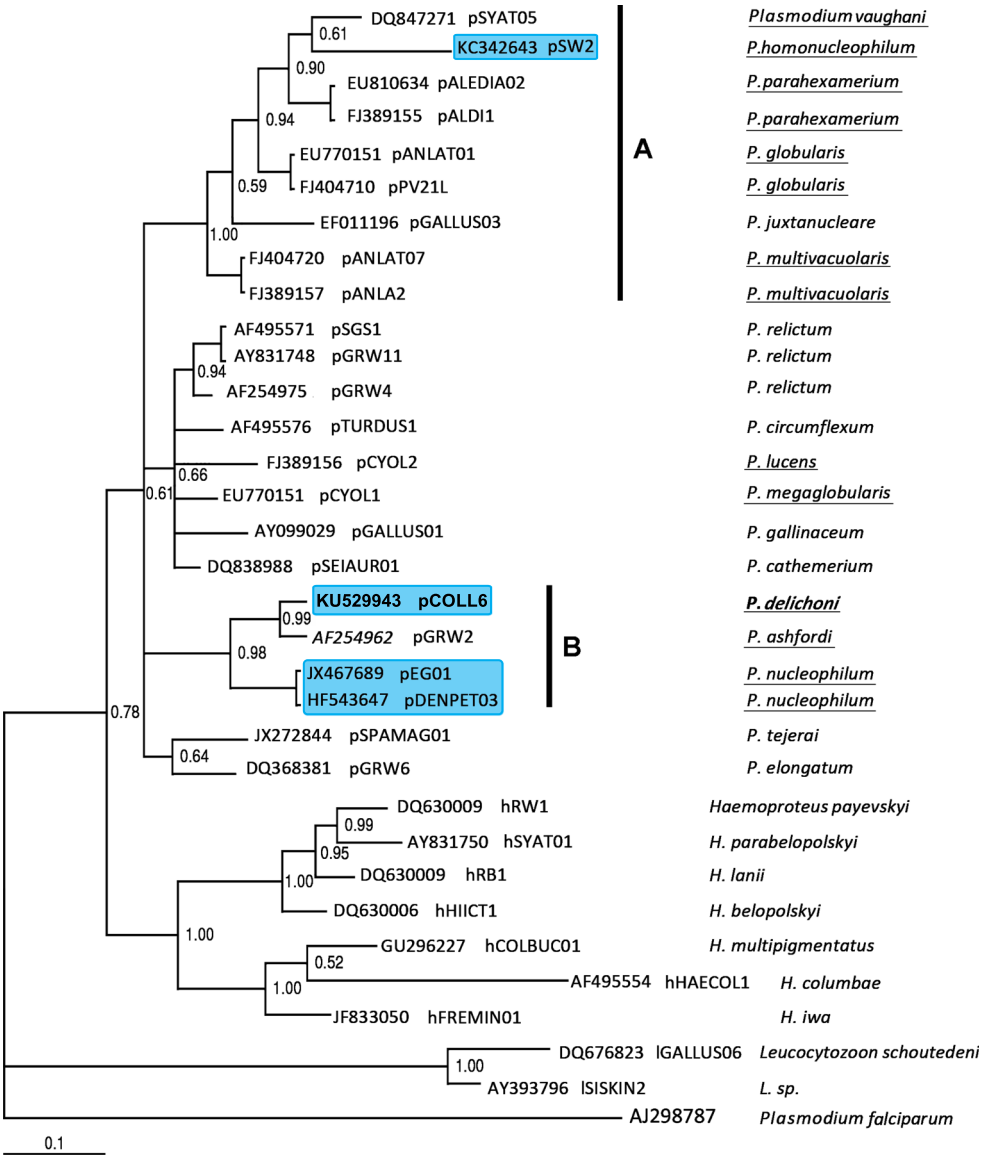

Fig. 1 Bayesian phylogeny of 33 mitochondrial cytochrome *b* gene lineages of avian haemosporidian parasites. GenBank accession numbers and codes of the lineages are given according to the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/index.html). Information about *Plasmodium* (*Novyella*) *delichoni* sp. nov. (lineage pCOLL6) is given in *bold*, and information related to *Plasmodium* parasites with nucleophilic blood stages is provided in *boxes*. Names of parasites belonging to subgenus *Novyella* are *underlined*. Nodal support values near branches show posterior clade probabilities. *Vertical bars* indicate clades (*A*, *B*), in which parasite species with nucleophilic blood stages are present

DNA sequences Partial mitochondrial cyt *b* and apicoplast gene sequences (GenBank accession nos. KU529943 and KU530116, respectively).

Additional hosts The barn swallow *H. rustica* (Hirundinidae) and collared flycatcher (*Ficedula albicollis*) (Table 2).

Vectors Unknown. Experimentally infected mosquitoes *Cu. p. pipiens* f. *molestus*, *Cu. quinquefasciatus* and *A. aegypti* were resistant. Numerous remnants of blood stages were seen in mosquito midgut contents of each species, and a few degrading ookinetes were observed in mosquitoes *A. aegypti* and *Cu. p. pipiens* f. *molestus* (Fig. 2). Oocysts did not develop and sporozoites were not seen in salivary glands. Sporogonic development is abortive on gametogenesis or ookinete stages in all exposed mosquito species.

Type locality Ventės Ragas, Šilutė District, Lithuania (55° 20′ 28.1″ N, 21° 11′ 25.3″ E).

Prevalence As reported by PCR-based testing and microscopic examination of blood films, the overall prevalence was 12.2 % in house martins and barn swallows. In all, 25.0 and 3.5 % of examined house martins and barn swallows were infected, respectively.

Distribution *P. delichoni* (pCOLL6) has been reported in adult house martins, barn swallows and collared flycatchers (*F. albicollis*) in Europe after arrival from African wintering grounds (Table 2), but is absent from juvenile birds of these species, indicating that transmission occurs away from the European breeding grounds.

Site of infection Mature red blood cells. No other data.

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Table 1 Morphometry of host cells, mature gametocytes and erythrocytic meronts of *Plasmodium delichoni* (lineage pCOLL6) ($n = 21$)

Feature	Measurements (μm)[a]
Uninfected erythrocyte	
Length	10.5–12.9 (11.8 ± 0.7)
Width	5.4–6.9 (6.2 ± 0.4)
Area	50.5–66.4 (58.7 ± 4.1)
Uninfected erythrocyte nucleus	
Length	4.9–6.1 (5.6 ± 0.4)
Width	1.8–2.4 (2.1 ± 0.2)
Area	8.0–12.0 (9.4 ± 1.1)
Macrogametocyte	
Infected erythrocyte	
Length	10.5–13.7 (12.3 ± 0.8)
Width	5.1–6.8 (5.7 ± 0.4)
Area	50.7–70.7 (58.1 ± 5.1)
Infected erythrocyte nucleus	
Length	4.8–6.3 (5.5 ± 0.5)
Width	1.8–2.3 (2.1 ± 0.1)
Area	7.8–11.0 (9.5 ± 0.9)
Gametocyte	
Length	10.0–12.7 (11.1 ± 0.7)
Width	1.4–2.3 (1.8 ± 0.2)
Area	16.6–27.8 (21.1 ± 2.8)
Gametocyte nucleus[b]	
Length	–
Width	–
Area	–
Pigment granules	4.0–10.0 (7.4 ± 1.5)
Microgametocyte	
Infected erythrocyte	
Length	11.3–14.0 (12.7 ± 0.7)
Width	5.0–6.8 (5.8 ± 0.4)
Area	49.7–68.7 (60.8 ± 5.7)
Infected erythrocyte nucleus	
Length	5.0–6.3 (5.6 ± 0.3)
Width	1.8–2.4 (2.0 ± 0.2)
Area	8.5–11.4 (9.8 ± 0.8)
Gametocyte	
Length	10.0–12.5 (10.9 ± 0.7)
Width	1.2–2.5 (1.8 ± 0.3)
Area	16.4–24.8 (19.8 ± 2.5)
Gametocyte nucleus	
Length	1.5–3.2 (2.2 ± 0.5)
Width	0.5–1.5 (0.9 ± 0.3)
Area	1.0–2.6 (1.8 ± 0.5)
Pigment granules	3.0–9.0 (6.5 ± 1.7)
Meront	
Length	2.4–4.7 (3.8 ± 0.6)
Width	1.3–2.7 (1.9 ± 0.4)
Area	3.9–8.3 (5.3 ± 0.9)
No. of pigment granules[c]	–
No. of merozoites	5.0–8.0 (6.1 ± 0.8)

[a] Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation

[b] Gametocyte nuclei are ill-defined and difficult to measure

[c] Pigment granules often are clumped and difficult to calculate

Type specimens Hapantotype (accession numbers 48920 NS and 48921 NS, parasitemia of 1.8 %, *D. urbicum*, Ventės Ragas, Lithuania, collected by G. Valkiūnas, 17 May 2015) was deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania. Parahapantotypes (the first blood passage from the type hosts *D. urbicum* to Eurasian siskin *C. spinus*, parasitemia of approximately 2 %, 16 June 2015) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania, and in the Queensland Museum, Queensland, Australia (accession nos. G466182 and G466183).

Additional material Blood films from experimentally exposed canary *S. canaria* (accession nos. 48926 and 48927 NS) and Eurasian siskin *C. spinus* (accession nos. 48928–48935 NS), cryopreserved infected blood (specimen accession nos. 10/15Rcc and 11/15Rcc) and whole blood samples fixed in SET buffer (specimen accession nos. 158/15R, 287/15V, 288/15V, 291/15V, 346/15V) were deposited in the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

Etymology The species name is derived from the genus name *Delichon* to which the type host of this parasite belongs.

Exoerythrocytic meronts Phanerozoites were not found both by histological and chromogenic in situ hybridisation methods in two canaries and one Eurasian siskin that were examined 172 dpe.

Parasitemia Prepatent period varied between 15 and 19 dpe in different individual avian hosts. All blood stages were seen in the peripheral circulation during the entire observation time (over 8 months).

Intense parasitemia developed in two exposed Eurasian siskins. Maximum parasitemia reached 26.4 and 70.0 % in different individual Eurasian siskins 36 and 46 dpe, respectively. Watery blood symptoms were readily visible during peak of parasitemia. Both exposed Eurasian siskins survived acute parasitemia, which lasted for approximately 2 weeks and then decreased to chronic levels. Light parasitemia (maximum 1 %) developed in two exposed canaries, and it remained at low level (≤0.1 %) during this study. Erythrocytic merogony is asynchronous, resulting in the presence of all blood stages at any stage of parasitemia. All exposed birds survived to the end of this study.

Trophozoites These develop in mature red blood cells. Outline is wavy or slightly amoeboid. Earliest trophozoites were seen anywhere in the cytoplasm of infected erythrocytes. As the parasite develops, trophozoites attach to the host cell nuclei and usually are seen in a polar or subpolar position in relation to the nuclei (Fig. 3a); they often are difficult to visualise and can be overlooked in blood films due to small size and tight attachment to the host cell nuclei. One or two tiny pigment granules are present. The influence of parasites on infected erythrocytes is not pronounced.

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Table 2 Avian hosts and countries where the pCOLL6 lineage of *Plasmodium delichoni* n. sp. has been reported

Order and family	Species	Country	Reference
Passeriformes			
Hirundinidae	*Delichon urbicum*[a]	Lithuania	This study
	D. urbicum[b]	Netherlands	Piesma and van der Velde (2012)
	Hirundo rustica[a]	Lithuania	This study
Muscicapidae	*Ficedula albicollis*[b]	Hungary	Szőllősi et al. (2009)
	F. albicollis[b]	Spain	Perez-Tris et al. (2007)

[a] Reports based both on microscopic and PCR-based diagnostics

[b] Reports based solely on PCR-based diagnostics

Erythrocytic meronts These develop in mature red blood cells and are closely appressed to the nuclei of infected erythrocytes from the stage of binuclear parasites to their complete maturity. Young meronts adhere to the nuclei of erythrocytes near one of their poles (Fig. 3b, c). Advanced meronts usually maintain the same position in host cells (Figs. 3d and 4a–c), but a few mature meronts were seen in sub-lateral position to the host cell nuclei (Fig. 3e). The cytoplasm is scanty, pale stained. Fully grown meronts are of variable form, and roundish, oval, irregular and even slightly elongate in shape parasites were seen. Nuclei usually distributed disorderly in fully grown meronts (Figs. 3d, e and 4a–c). One refractive globule was seen in many growing meronts (Fig. 3c), but the globules were not visible in completely mature meronts (Figs. 3e and 4a–c). The majority of meronts (over 60 %) produce 6 merozoites, but meronts containing 4, 5, 8 or even 10 merozoites are present. Pigment granules are of small size, dark, clumped in one or two small groups or aggregated in solid masses in mature meronts (Fig. 4b, c). Meronts usually do not influence infected erythrocytes, but the largest parasites can slightly displace the nuclei of erythrocytes laterally (Fig. 3e). Segmenting meronts are numerous in the peripheral blood (Figs. 3e and 4c). Mature merozoites are approximately 1 μm in diameter; they possess a readily visible prominent nucleus but poorly visible cytoplasm (Figs. 3e and 4a–c). Some merozoites remain attached to the host cell nuclei even after segmentation of meronts (Fig. 4c). The majority of merozoites contain a readily visible clump of chromatin (Figs. 3d and 4a–c), which is a distinctive feature of this parasite.

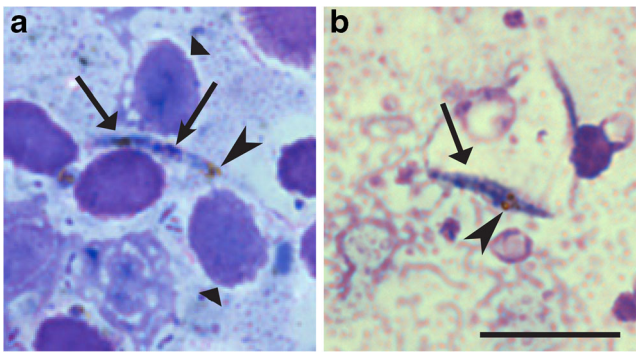

Fig. 2 Degrading ookinetes of *Plasmodium* (*Novyella*) *delichoni* sp. nov. (lineage pCOLL6) from the midgut contents of mosquitoes *Aedes aegypti* (**a**) and *Culex pipiens pipiens* form *molestus* (**b**). *Long triangle arrows*, ookinetes; *long simple arrow*, nucleus of parasite; *simple arrowheads*, pigment granules; *triangle wide arrowheads*, nuclei of semi-digested red blood cells. Giemsa-stained thin blood films. *Scale bar* = 10 μm

Macrogametocytes These develop in mature red blood cells. The cytoplasm stains pale-blue, slightly granular in appearance, and often contains several small vacuoles. Earliest gametocytes are elongated, slightly amoeboid in outline, usually seen in polar positions in erythrocytes (Fig. 3f). Growing gametocytes assume lateral positions to the nuclei of infected erythrocytes, and they are closely appressed to the nuclei (Fig. 3g, h). As parasite develops, gametocytes remain strictly nucleophilic (Fig. 3h–m), a characteristic feature of this species development. Growing gametocytes do not touch the envelop of erythrocytes along their entire margin (Fig. 3g–k), but many fully grown gametocytes are associated with the envelope of the host cells (Fig. 3m). Gametocyte outline varies from slightly irregular (Fig. 3h, i, m) to slightly amoeboid (Fig. 3g, j). Parasite nucleus is of variable form, pale stained, resulting in its unclear boundaries, usually subterminal in position (Fig. 3i, l); it contains a readily distinguishable roundish nucleolus (Fig. 3k, m). Pigment granules are dark, usually grouped (Fig. 3h), roundish and oval in form, markedly variable in size, a characteristic feature of this parasite species. Small and average size (<1 μm) pigment granules predominate, but large (1 μm and bigger) elongate granules are often present (Fig. 3j, k). The influence of parasites on infected erythrocytes usually is not pronounced.

Microgametocytes General configuration is as for macrogametocytes with the usual haemosporidian sexual dimorphic characters. Parasite nucleus is diffuse, centrally located, often ill-defined and difficult to measure (Fig. 3n–p).

Specificity Eurasian siskins and domestic canaries are susceptible and are good experimental hosts, in which long-lasting parasitemia develops. Reports of this parasite in naturally infected birds are given in Table 2.

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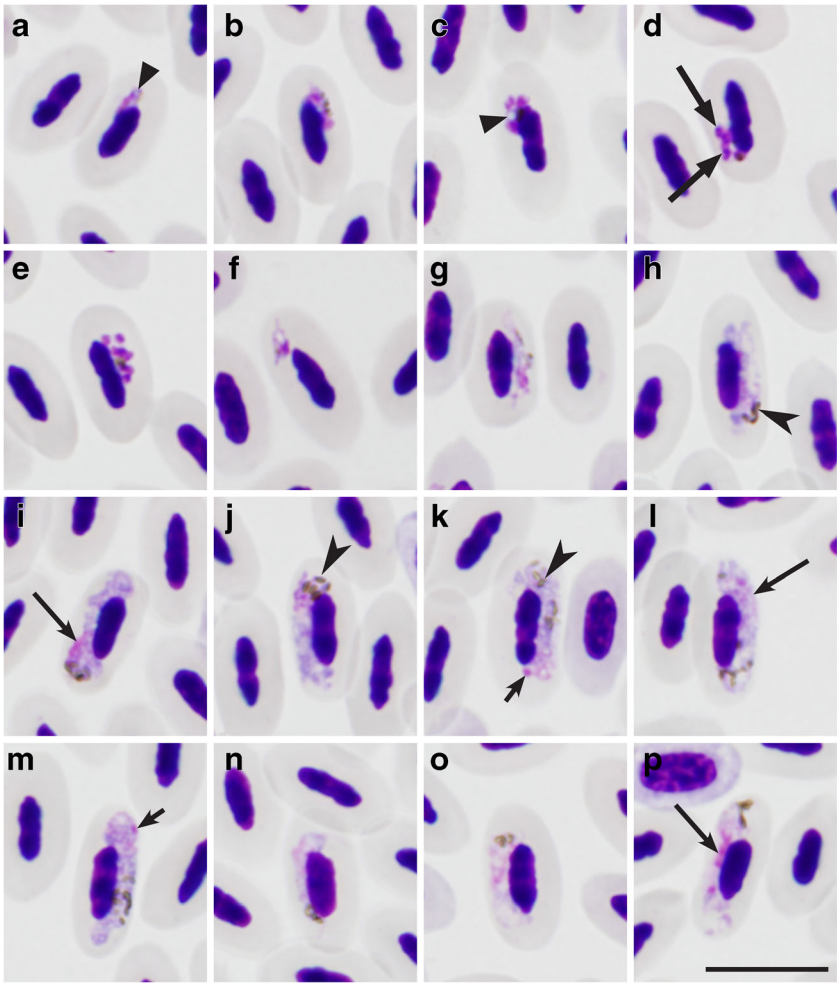

Fig. 3 *Plasmodium* (*Novyella*) *delichoni* sp. nov. (lineage pCOLL6) from the blood of house martin *Delichon urbicum*: **a** trophozoite, **b**–**e** erythrocytic meronts, **f** young gametocyte, **g**–**m** macrogametocytes, **n**–**p** microgametocytes. *Long simple arrows*, nuclei of parasites; *short simple arrows*, nucleoli; *simple arrowheads*, pigment granules; *triangle arrowheads*, blue, refractive globule; *long triangle arrowheads*, clumps of chromatin in nuclei of mature merozoites. Giemsa-stained thin blood films. *Scale bar* = 10 μm

Taxonomic remarks

P. delichoni sp. nov. belongs to subgenus *Novyella*, the species of which are characterised by the presence of (1) elongate gametocytes; 2) small fully grown erythrocytic meronts, whose size does not exceed or only slightly exceed that of the nuclei of infected erythrocytes; and (3) scanty cytoplasm in erythrocytic meronts (Garnham 1966; Valkiūnas 2005). Due to strict nucleophilicity of blood stages (Figs. 3 and 4), the new species can be readily distinguished from all described *Novyella* species, except *Plasmodium nucleophilum* (Manwell 1935; Chagas et al. 2013), *Plasmodium paranucleophilum* (Manwell and Sessler 1971b) and *Plasmodium homonucleophilum* (Ilgūnas et al. 2013). *P.*

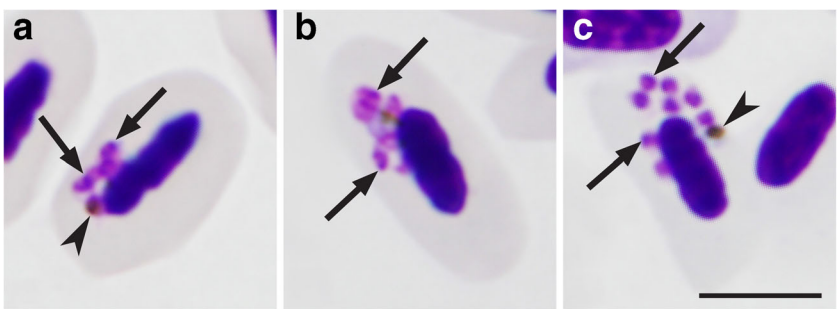

Fig. 4 Mature erythrocytic meronts of *Plasmodium* (*Novyella*) *delichoni* sp. nov. (lineage pCOLL6) from the blood of house martin *Delichon urbicum* (**a**), domestic canary *Serinus canaria* (**b**) and Eurasian siskin *Carduelis spinus* (**c**). *Simple arrowheads*, pigment granules; *long triangle arrowheads*, clumps of chromatin in nuclei of mature merozoites. Giemsa-stained thin blood films. *Scale bar* = 10 μm. Note that clumps of chromatin are readily visible in merozoites developed in different species of avian hosts, indicating that this is a useful taxonomic character of the new species

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delichoni can be distinguished from these parasites due to the presence of (1) large (≥1 μm in length) pigment granules in its gametocytes (Fig. 3j, k) and (2) distinct clumps of chromatin in nuclei of mature erythrocytic merozoites (Fig. 4a–c). Additionally, (1) gametocytes of *P. homonucleophilum* are not strictly nucleophilic, and the gametocytes, which do not adhere to the erythrocyte nuclei, are common (Ilgūnas et al. 2013); (2) mature erythrocytic meronts of *P. nucleophilum* often displace nuclei of infected erythrocytes (Manwell 1935); and (3) both mature gametocytes and erythrocytic meronts of *P. paranucleophilum* also displace nuclei of infected red blood cells (Manwell and Sessler 1971b). These features are not characteristics of *P. delichoni* (Figs. 3 and 4).

It is important to note that the main diagnostic morphological characters of blood stages observed in the type host, the house martin, maintained during the first passage of this parasite in domestic canaries and Eurasian siskins, but erythrocytic meronts producing eight merozoites (Fig. 4c) were more often seen in domestic canaries and Eurasian siskins than in the house martin.

Phylogenetic relationships of parasites

Partial DNA sequences of cyt *b* gene are available for three species of *Plasmodium* with nucleophilic blood stages, i.e. *P. homonucleophilum*, *P. nucleophilum* and *P. delichoni*. Genetic difference in cyt *b* gene among them is large (between 5.6 and 9.8 %), indicating that these species are valid. The three *Plasmodium* spp. appeared in different well-supported clades and are paraphyletic in our phylogenetic analysis (Fig. 1, clades A and B), indicating either the independent evolution of nucleophilicity of blood stages in these parasites or the insufficient sensitivity of phylogenies based on partial sequences of the cyt *b* gene in resolving the origin of this character in avian malaria parasites. In this relation, it is important to note that 10 species of subgenus *Novyella* were used in our phylogenetic analysis, and they also are paraphyletic in the phylogenetic tree (Fig. 1). Additionally, the new species appeared to be particularly closely related to *Plasmodium ashfordi* (Fig. 1, clade B), which both gametocytes and erythrocytic meronts are non-nucleophilic. These two parasites present in the same well-supported clade (Fig. 1, clade B), and genetic distance between their cyt *b* gene sequences is 2.8 %. The reported controversies between the morphological characters and the phylogeny based on partial cyt *b* gene sequences show that multigene phylogenetic analysis is needed for better understanding the evolutionary relationships among species of *Novyella* and other avian malaria parasite. However, such analysis is currently premature due to insufficient information about DNA sequences of other genes in avian *Plasmodium* spp.

P. delichoni appeared in the same clade with *P. nucleophilum* (Fig. 1, clade B), indicating possible close phylogenetic relationships between these two parasites, in which both gametocytes and erythrocytic meronts are strictly nucleophilic. The genetic distance in cyt *b* gene sequences between *P. delichoni* and *P. nucleophilum* is large (5.7 %).

Discussion

This study reports a new species of avian malaria parasites, whose blood stages (gametocytes, advanced trophozoites and erythrocytic meronts) are closely appressed to the nuclei of infected erythrocytes (Figs. 3 and 4). This finding deserves attention because the strict nucleophilicity is not characteristic of the majority of described avian *Plasmodium* species (Hewitt 1940; Garnham 1966; Valkiūnas 2005; Mantilla et al. 2013; Walther et al. 2014). However, this feature has been reported in many other species of related haemosporidian parasites. For example, the majority of bird *Haemoproteus* species (the sister genus to *Plasmodium*) possesses more or less nucleophilic gametocytes, which are appressed to the host cell nuclei from early stages of their development in red blood cells (Desser and Bennett 1993; Valkiūnas 2005; Atkinson et al. 2008; Mehlhorn 2015). Interestingly, all described species of haemosporidians belonging to the genus *Leucocytozoon* develop in close adherence to the nuclei of their host cells, which are often enlarged and/or deformed by developing both gametocytes and exoerythrocytic meronts (Desser and Bennett 1993; Forrester and Greiner 2008; Valkiūnas 2005; Lotta et al. 2015). Effects on the host cell nuclei (various modes of the nuclear displacement, atrophy, hypertrophy and even the host cell enucleation) are well expressed in many avian haemosporidian parasites, and such effects are particularly well-evident during development of *Leucocytozoon* sp. megalomeronts. The latter parasites induce remarkable increase in amounts of (1) the chromatin in nuclei of infected cells and (2) endoplasmic reticulum as well as the number of mitochondria in the cytoplasm of the cells (Desser 1970; Wong and Desser 1978; Desser and Bennett 1993; Valkiūnas 2005; Forrester and Greiner 2008). This indicates the active participation of the host cells in the metabolism process of the parasites. The biological meaning of nucleophilicity remains insufficiently investigated in haemosporidian parasites, and its molecular mechanisms remain unknown. *P. delichoni* and domestic canary offer a convenient study system for research on mechanisms of nucleophilicity in parasitic protists because this host-parasite association is easy and relatively inexpensive to maintain in laboratory conditions.

Only sporozoites induce exoerythrocytic merogony and formation of hypnozoites in malaria parasites of mammals (Sherman 1998). In avian malaria, merozoites from erythrocytic meronts can induce the development of the secondary exoerythrocytic meronts, which are known as phanerozoites (Garnham 1980; Valkiūnas 2005). Due to high and long-

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lasting parasitemia of *P. delichoni*, we expected to find phanerozoites in the exposed birds, but they were not seen in histological sections of internal organs in the canaries and Eurasian siskin. Chromogenic in situ hybridisation using *Plasmodium* spp.-specific oligonucleotide probes is a more sensitive method for the detection of tissue stages, providing opportunities to visualise exoerythrocytic meronts of malaria parasites even during light intensity in organs (Dinhopl et al. 2011, 2015). Because both the histological and chromogenic in situ hybridisation tools showed the same negative results, it is probable that phanerozoites do not develop in *P. delichoni*. Malaria parasites of subgenus *Novyella* have been insufficiently investigated from the point of view of exoerythrocytic merogony. Mainly blood stages have been described in the species of this subgenus (Garnham 1966; Valkiūnas 2005; Atkinson et al. 2008; Mantilla et al. 2013).

Phanerozoites were reported in *P.* (*Novyella*) *nucleophilum toucani* (Manwell and Sessler 1971a; Valkiūnas 2005), *P.* (*Novyella*) *paranucleophilum* (Manwell and Sessler 1971b), *Plasmodium* (*Novyella*) *bertii* (Gabaldon and Ulloa 1981) and *Plasmodium* (*Novyella*) *vaughani* (Nelson 1966; Garnham 1966; Valkiūnas 2005). However, phanerozoites were absent from birds subinoculated with blood infected by *Plasmodium* (*Novyella*) *columbae* (Gabaldon and Ulloa 1976), *Plasmodium* (*Novyella*) *rouxi* (Garnham 1966), *Plasmodium* (*Novyella*) *hexamerium* (Manwell 1951) and *Plasmodium* (*Novyella*) *kempi* (Christensen et al. 1983), which is in accord with this study. It seems probable that phanerozoites do not develop in some *Novyella* parasites.

Phanerozoites are responsible for multiplication of parasites and also are involved in the persistence of malaria infection in avian hosts (Garnham 1980; Valkiūnas 2005; Atkinson et al. 2008). Persistence of *P. delichoni* and some other *Plasmodium* spp. might occur due to long-lasting light parasitemia, as is the case in human malaria parasite *Plasmodium malaria* (Sherman 1998). Warm climates and the resulting long period of transmission in sub-Saharan Africa, which is the main wintering area of European species of the Hirundinidae, might contribute to evolution of such mode of avian malaria persistence.

Plasmodium relictum and related species of avian malaria parasites of subgenus *Haemamoeba* survive cold seasons of a year, which are unfavourable periods for mosquito transmission, mainly at the stage of persisting phanerozoites (Garnham 1980; Valkiūnas 2005; Atkinson et al. 2008). However, phanerozoites might markedly damage bird organs, and they often are the main reason of mortality in avian hosts (Garnham 1980; Dinhopl et al. 2015; Palinauskas et al. 2015). Because the lack of phanerozoites leads to less tissue damage and milder parasite virulence, which was the case in *P. delichoni*, the persistence by means of long-lasting light parasitemia should be evolutionarily advantageous due to survival of a greater number of infected avian hosts, which then can serve as donors of gametocytes to infect mosquitoes. It is worth mentioning that numerous *Novyella* species have been described from African and South American birds (Valkiūnas et al. 2009; Chagas et al. 2013; Ilgūnas et al. 2013; Mantilla et al. 2013; Walther et al. 2014), in which parasites of this subgenus predominate (Valkiūnas 2005; Loiseau et al. 2012), but exoerythrocytic merogony and modes of persistence remain unknown in these infections. This study shows that the mechanisms of persistence might vary in different species of avian malaria parasites. Comparative research of tissue merogony of *Plasmodium* species, which transmission occurs in countries with warm and cold climates, is needed for better understanding the patterns of exoerythrocytic merogony and mechanisms of persistence of avian malaria parasites.

Numerous studies reported *Plasmodium* and *Haemoproteus* parasites in house martins and barn swallows using both microscopic examination and PCR-based testing of blood samples in Europe (Dogiel and Navtsevich 1936; Peirce 1981; Valkiūnas 2005; Marzal et al. 2008; Piesma and van der Velde 2012). Interestingly, these infections have been detected only in adult swallows after their arrival from African wintering grounds. Species of *Plasmodium* and *Haemoproteus* have not been observed in over 500 individuals of juvenile house martins and barn swallows hatched in Europe, indicating solely African transmission of these infections (Peirce 1981; Valkiūnas 2005; Marzal et al. 2008; Piesma and van der Velde 2012). The obstacles for spreading of *P. delichoni* in European breeding grounds remain unclear. This study showed the lack of sporogonic development in mosquitoes, which are widespread in Europe (*Cu. p. pipiens*) and sub-Saharan Africa (*Cu. quinquefasciatus*, *A. aegypti*). Even gametogenesis and development of ookinetes were abortive in these mosquitoes, indicating that sexual process, ookinete formation and sporogony of *P. delichoni* require particular mosquito gut factors for successful development in vectors, as is the case in human malaria parasites (Sherman 1998). It is probable that the lack of specific mosquito vectors is an important factor preventing the spreading of *P. delichoni* infection in Europe. Endemic African mosquitoes should be tested for their vectorial capability. Sporogony of avian *Plasmodium* spp. completes and sporozoites develop in *Coquillettidia* mosquitoes transmitting avian malaria in Africa (Njabo et al. 2009). The species of this genus and related genera of bird-biting Culicidae should be considered in future experimental vector research in avian malaria studies. Such research is important for better understanding the mechanisms preventing this disease spreading by migrating birds from the tropics to areas with temperate climates. *P. delichoni* is a convenient model parasite for such research because it develops long-lasting parasitemia in domestic canaries. These birds are easy to breed, maintain and infect in captivity, and they can be used as donors of gametocytes for experimental exposure to different mosquito species.

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