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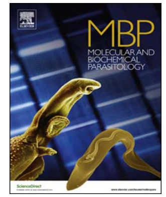

Experimental evidence for hybridization of closely related lineages in *Plasmodium relictum*

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ABSTRACT

Over 50 avian *Plasmodium* species have been described. However, PCR-based information shows much broader diversity of genetic lineages in these parasites. This discrepancy indicates insufficient knowledge about taxonomic diversity and boundaries of a single species in avian *Plasmodium* species. In recent taxonomy, most of genetically closely related lineages that share the same morphology and development patterns are attributed to the same biological species, but there is no information if these lineages are able to cross. This information is crucial to understand if these lineages form single or multiple evolutionary units.

Due to presence of sexual process and sporogonic development of *Plasmodium* parasites in mosquitoes, self and cross-fertilization can occur and be identified during the oocyst stage. We initiated *in vivo* hybridization experiments of two widespread *Plasmodium relictum* lineages (pSGS1 and pGRW11) in experimentally infected *Culex pipiens pipiens* form *molestus* mosquitoes. To study putative hybrid oocysts, we used a laser microdissection technique together with PCR-based analyses of mitochondrial and nuclear genes. We demonstrate that both pSGS1 and pGRW11 lineages develop in infected mosquitoes in parallel, but also form hybrid oocysts of these two lineages. Our results are in accord to a recent global phylogeographic study of *P. relictum* that suggested that cross-fertilization between pSGS1 and pGRW11 might occur. This information helps to understand population structure, gene flow and the evolutionary process of haemosporidian parasites.

1. Introduction

More than 50 avian *Plasmodium* species have been described [1,2]. Most of these descriptions were based on morphological features of erythrocytic stages and fragmented information about parasites' life cycle. According to recent molecular data, which are based on sequence information of the mitochondrial cytochrome *b* (cyt *b*) gene fragment, there are more than 700 different genetic lineages of avian *Plasmodium* spp. ([3]; MalAvi database 11/04/2016). That indicates limited knowledge about species diversity of haemosporidians and insufficient understanding the boundaries of a single biological species [4–6]. Based on various assumptions different authors defined genetic boundaries of haemosporidian parasite species starting from a single nucleotide difference in cyt *b* gene fragment up to 5% [6–10]. Human malaria studies have shown that *Plasmodium falciparum* obtained from different areas differ from one to 6 substitutions (up to 0.5%) in cyt *b* gene [11,12]. These results give a hint that some of avian malaria lineages with slight genetic differences in the mitochondrial genome should represent within species variation and some could represent cryptic species. The recently described avian *Plasmodium homocircumflexum* was attributed to a new cryptic species, because its morphology was identical to previously described *Plasmodium circumflexum*, but development in vertebrate hosts and position on the phylogenetic tree indicated two distantly related species [13]. Other cryptic species within some phylogenetically related clades of *Plasmodium* lineages likely exist. According to experimental *in vitro* studies on *Haemoproteus* parasites (sister genus to *Plasmodium*), microscopically defined possible hybrid ookinetes were observed after cross-fertilization of different lineages *in vitro* [14,15], however, it remains unclear if these traits reflect genetic recombination between investigated lineages. Compared to malarial parasites of mammals and squamates, the huge diversity and worldwide distribution of *Plasmodium* of birds is unique [1] thus, avian *Plasmodium* spp. are good model organisms to study species borders and possible development of between-lineage hybrids. This information is crucial to understand if these lineages exist as evolutionary independent entities or if they freely recombine.

Plasmodium relictum is the most prevalent avian malaria parasite infecting more than 300 avian hosts belonging to 10 orders [2]. Being

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widespread and prevalent in five continents and transmitted in tropical and temperate areas up to North Pole circle [16–18] this parasite is one of the biggest alien threats for exotic birds [19]. Four mitochondrial cyt *b* gene lineages (pSGS1, pGRW4, pGRW11, pLZFUS01) are morphologically characterized and attributed to *P. relictum* [20–22]. The sequences of this gene (479 bp) are grouped in a single clade with a genetic divergence < 3.5% among them [22]. However, it is unclear if these genetic lineages represent within species haplotype diversity or if they are cryptic species.

In a recent phylogeographic study [23], three of the most prevalent *P. relictum* lineages (pSGS1, pGRW11 and pGRW4) were investigated from 21 different locations using nuclear markers of partial sequences of the merozoite surface protein 1 (msp1) gene. The authors revealed five different alleles belonging to the lineage pGRW4. The lineage pSGS1 had four different alleles and three of them were shared with the lineage pGRW11. Based on the msp1 gene sequence analysis, it is probable that the lineage pGRW4 should be defined as a separate entity and described as a cryptic species. Because alleles of the msp1 gene were shared between pSGS1 and pGRW11 lineages it is likely that crosses between these two lineages occur frequently. However, because of the common mixed infections in naturally infected birds and the possible selective amplification of a single allele during such mixes, it is difficult to rule out that some of the samples could contain co-infections of these two lineages, but not their hybrids [24]. Experimental hybridization of these two closely related lineages would confirm their possibility to cross and form reciprocal hybrids.

Haemosporidian parasites, including agents of avian malaria are haploid throughout their life-cycle except the diploid zygote, which develops post fertilization in blood sucking dipterans [2]. Meiosis takes place during the early stage of ookinetes development but all haploid products of recombination are kept within this single cell [25,26]. During sporogonic development, self- and cross-fertilization have been recorded [26–28]. The ookinetes penetrate mosquito midgut lamina and form oocysts, in which thousands (up to 10 000) of haploid cells (sporozoites) are formed. In *Plasmodium falciparum* studies genetic analyses of oocysts were used to clarify population genetic structures and clonal reproduction events [25,27–30].

In this experimental study, we initiated *in vivo* hybridization of two widespread *P. relictum* lineages (pSGS1 and pGRW11) in *Culex pipiens pipiens* form *molestus* mosquitoes. Single oocysts were dissected and studied using markers for the mitochondrial cyt *b* gene and three nuclear genes available for genotyping *Plasmodium* parasites [31–36]. These are: msp1, apical membrane antigen-1 (ama1) and chitinase gene fragment (cht1). We show that both pSGS1 and pGRW11 lineages develop independently in infected mosquitoes, but also form hybrid oocysts in mosquitoes, which were fed on blood containing mixture of gametocytes of these two lineages. Our results confirm that cross-fertilization between pSGS1 and pGRW11 occurs, and these lineages could be attributed to the same *P. relictum* species.

2. Materials and methods

2.1. Experimental infection of canaries

We used *Plasmodium relictum* mitochondrial cyt *b* lineages pSGS1 and pGRW11 (GenBank accession numbers JX993045 and JX993047), which were isolated from naturally infected crossbill *Loxia curvirostra* and house sparrow *Passer domesticus,* respectively. The birds were caught at the Biological Station of the Zoological Institute of the Russian Academy of Science, on the Curonian Spit in the Baltic Sea in 2010 and 2011. Infections were multiplied and cryopreserved in liquid nitrogen as described in [13]. Samples of the infected blood are maintained at the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania.

The experimental work was carried out at the Nature Research Centre from October 2013 to May 2014. Domestic canaries *Serinus canaria* were used as donors of gametocytes to infect mosquitoes. The birds were commercially purchased and maintained in a vector-free room. They were kept in quarantine for one month before the experiments. All birds were tested for haemosporidian parasites both by microscopic and PCR-based examinations as described below. The cryopreserved strains of pSGS1 and pGRW11 were thawed and used to infect canaries as described in [13]. Briefly, a frozen tube with infected blood was thawed and mixed with 12% NaCl (1/3 of thawed sample amount). After equilibration for 5 min at room temperature, one volume of 1.6% NaCl was added following by centrifugation at 200*g* for 5 min. After centrifugation, the supernatant was removed and 1.6% NaCl (one third of original sample) was added and centrifuged again. After removing the supernatant, the same procedure was repeated 3 times with 0.9% NaCl solution. The final mixture was diluted with 0.9% NaCl and sub-inoculated into canaries, as described in [37]. Different individual birds were infected with single lineages and also both lineages in parallel. In all, four canaries were exposed to these infections and used as donors of gametocytes to infect mosquitoes. One bird was sub-inoculated with pSGS1 lineage, one bird with pGRW11 and two canaries were exposed to the lineages pSGS1 and pGRW11 (mixed infection).

To follow the development of parasitemia in exposed birds, the blood for microscopic and molecular examination was taken from all canaries every three or four days post inoculation (dpi) for approximately one month. The drop of blood was taken by puncturing the brachial vein in heparinized microcapillary and fixed in SET buffer (0.05 M tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analysis. At the same time three blood films were made for morphological analysis. Blood films were air-dried, fixed with absolute methanol, stained with Giemsa and examined microscopically as described in [38].

2.2. Maintenance and experimental infection of mosquitoes

Culex pipiens pipiens f. *molestus* mosquitoes were used as vectors of two *P. relictum* lineages [39]. The colony of this mosquito was established and maintained at the P. B. Šivickis Laboratory in Nature Research Centre. The insects were kept in mosquito cages (65 × 65 × 65 cm) under standard conditions (23 ± 1 °C, 60–65% relative humidity and 14:10 light–dark photoperiod). Mosquitoes were fed with 5–10% saccharose solution. Cotton wool pads moistened with this solution were provided in mosquito cages. A day before feeding, approximately 50 females were haphazardly chosen and placed inside an experimental cage. Canaries infected with pSGS1 (gametocytemia of 0.05%), pGRW11 (0.06%) and pSGS1 × pGRW11 (0.1% in both donors) were placed in mosquito cages and used to feed mosquitoes, as described in [40]. Birds were kept in the cage for 30 min and they were exposed to mosquito bites once per 3–4 days. 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. All engorged females were collected from the experimental cages using an aspirator, placed in separate small insect cages (12 × 12 × 12 cm) and maintained to allow development of oocysts.

2.3. Laser microdissection of single oocysts

Females of *C. p. pipiens* f. *molestus* were lightly anaesthetized by putting them into a tube with a cotton pad wetted with ethanol (96%) for several minutes. Wings and legs of the insect were removed before dissection under the binocular stereoscopic microscope Olympus SZX10. To eliminate contamination of samples, we disinfected the needles in fire after each dissection.

Single oocysts were dissected from the midguts of experimentally infected *C. p. pipiens* f. *molestus* mosquitoes 12–13 dpi. We dissected midguts from randomly chosen infected insect abdomens, placed them on membrane slides (Molecular Machines and Industries, Germany), cut

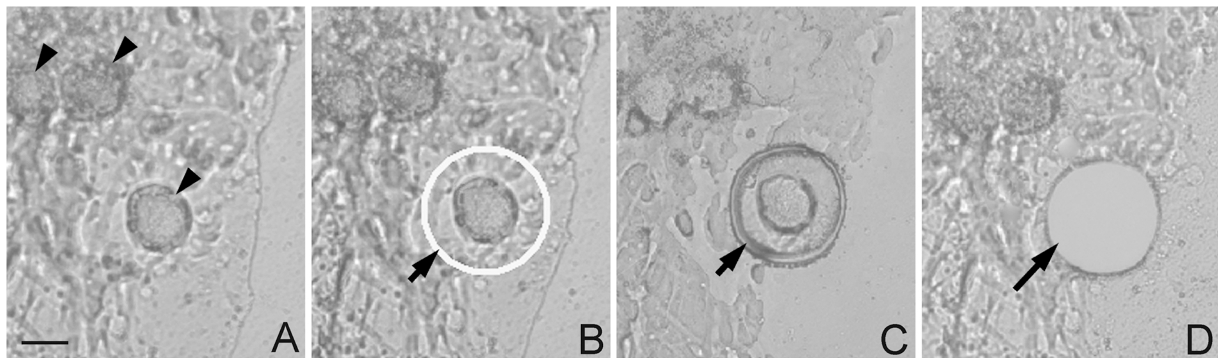

Fig. 1. Laser microdissection of single *Plasmodium relictum* (lineage pSGS1) oocysts from midgut of experimentally infected mosquito *Culex pipiens pipiens* form *molestus.* A – membrane slide with midgut of mosquito and oocysts of parasite 12 days post exposure, B – membrane slide with marked oocyst for laser microdissection, C – membrane slide after exposure to laser, D – membrane slide after replacement of a single oocyst to microtube. Arrowhead indicates oocysts; short arrow shows the place around oocyst, where the laser cuts the membrane; long arrow shows a hole in the membrane slide after replacement of one oocyst. Ethyl alcohol fixed mosquito mudgut. Scale bar = 30 µm.

Table 1
Observed genotypes of *Plasmodium relictum* oocysts in experimentally infected *Culex pipiens pipiens* form *molestus* mosquitoes. Genotypes are defined using partial sequences from the mitochondrial cytochrome *b* gene (cyt *b*) and the nuclear apical membrane antigen-1 (ama1) gene.

Observed genotype (cyt *b* × ama1)	Dissected oocysts (ooc) from mosquitoes (mos) experimentally infected with the cyt *b* lineages		
	pSGS1 (5 ooc/1 mos)	pGRW11 (5 ooc/1 mos)	Mix of the lineages (54 ooc/5 mos)
pSGS1 × pSGS1	5 (100)[a]	0	12 (22.2)
pGRW11 × pGRW11	0	5 (100)	36 (66.7)
pSGS1♀ × pGRW11♂	0	0	2 (3.7)[b]
pGRW11♀ × pSGS1♂	0	0	4 (7.4)[c]

♀ and ♂ symbols represent female and male gametes, respectively.
[a] Percentage of defined genotype is given in parentheses.
[b] pSGS1 macrogametes and pGRW11 microgametes.
[c] pGRW11 macrogametes and pSGS1 microgametes.

Table 2
The composition of *Plasmodium relictum* oocyst genotypes in 5 *Culex pipiens pipiens* form *molestus* mosquitoes experimentally infected with mixed infection (pSGS1 × pGRW11). Genotypes are defined using partial sequences from the mitochondrial cytochrome b gene (cyt *b*) and the nuclear apical membrane antigen-1 (ama1) gene.

Dissected mosquitoes[a] (donor)[b]	No of oocysts positive /analysed	Observed genotypes		
		pGRW11	pSGS1	Mix (pSGS1 × pGRW11)
A (a)	19/19	14 (73.7)[c]	3 (15.8)	2 (10.5)
B (a)	10/10	6 (60)	2 (20)	2 (20)
C (b)	10/10	7 (70)	2 (20)	1 (10)
D (b)	8/10	4 (50)	3 (37.5)	1 (12.5)
E (b)	7/10	5 (71.4)	2 (28.6)	0 (0)

[a] Each mosquito defined as capital letter.
[b] Two canaries used as donors for mixed infections are defined as letter in parentheses.
[c] Percentage of defined genotype is given in parentheses.

the midguts with a tiny razor along the gut length, and stretched to make a single gut layer. The midgut preparations were air dried, fixed, and used for the laser microdissection. The fixation was done by the straight application of 50, 70, and 96% ethyl alcohol on midguts. The Olympus/MMI CellCut Plus® laser system equipped with Predefined Target Position software (Molecular Machines and Industries, Germany) was used to cut single oocysts. The parasites were readily visible on the unstained membrane slides (Fig. 1A), and single oocysts (Fig. 1B, C) were captured on the silicon cap of microtubes (0.5 ml). The success of cell capture was confirmed by microscopic examination of the membrane slides (Fig. 1D) and tube caps. We dissected 5 oocysts from one mosquito with single pSGS1 infection, 5 oocysts from one mosquito with single pGRW11 infection and 59 oocysts from five mosquitoes with mixed infection of these lineages (Tables 1 and 2). Thirty oocysts were dissected from mosquitoes that took blood meal from first canary and 29 from mosquitoes that took blood from the second canary containing mixed infection (Table 2).

2.4. DNA extraction, PCR and genetic analysis

We used the standard ammonium-acetate method to extract genomic DNA from birds' whole blood [41]. Chelex® extraction method was applied for DNA extraction from a single oocyst as described by [42]. Briefly, we applied 0.2 g of Chelex suspended in 1000 µl of ddH_2O and kept for 1 h in a 56 °C water bath. A small drop of 25 µl of Chelex suspension was attached to the wall of the tube with dissected cells, which were on the silicon cap of the tube. Then, 0.7 µl of Proteinase K (10 mg/ml) was added and the tube was closed, then turned upside-down, and shaken down once so that the Chelex with Proteinase K was covering all the surface of the cap. The tube was kept in a water bath at 56 °C for 1 h and mixed every 20 min followed by boiling in water for 12 min to inactivate the Proteinase K. The sample was centrifuged briefly to recover the extraction mix at the bottom of the tube. The silicon caps were replaced with standard caps and the tubes were centrifuged at 13,400 *g* for 12 min. The supernatant (about 10–15 µl) was immediately transferred with a sterile micropipette into a new tube and was ready to use for molecular analysis.

For genetic analysis of parasites from infected bird blood we used the nested PCR protocol described in [7,43]: the 479 bp length segment of parasite mitochondrial cyt *b* gene was amplified using two pairs of primers: HaemNFI/HaemNR3, which amplify gene fragments of haemosporidians belonging to *Haemoproteus, Plasmodium* and *Leucocytozoon*, and HAEMF/HAEMR2, which are specific to *Haemoproteus* and *Plasmodium* spp. This PCR protocol helps to estimate double infection of pSGS1 and pGRW11 [24]. Presence of mixed infection of two genetic lineages in bird blood was estimated by visualising double-base calling in sequence electropherograms corresponding to two initial single sequences.

For genetic analysis of oocysts, we used one PCR protocol to amplify a fragment of mitochondrial DNA and three PCR protocols to amplify fragments of nuclear DNA. A nested PCR was used to amplify the mitochondrial cyt *b* gene fragment. The outer primers in the PCR were HAEMF and HAEMR2 [43] and the products from this reaction were amplified with the nested primers F144 and R368 [42] to obtain a 224 bp fragment.

Nuclear DNA fragments were amplified using a nested PCR protocol for amplification of the ama1 gene fragment using primers

Pg_AMA1F1/R1 and Pg_AMA1F2/R2 [35]; nested PCR protocol for amplification of the chitinase gene fragment with primers PgCHT1F/R and PgCHT2F2/R2 [33]; and PCR protocol for amplification of the msp1 gene fragment using primers OH1F2/R2, OH5F/R, OH3F/R [34]. Temperature profiles in all PCRs' were the same as in the original protocol descriptions. We performed PCR amplification in 25 μl total volumes including 25 ng of total genomic DNA template (1 μl), 0.1 μl of Platinum TaqDNA polymerase (Invitrogen), 0.5 μl 10 mM dNTP, 0.5 μl $MgCl_2$, 2.5 μl PCR buffer, 19.5 μl nuclease-free water and 0.5 μl of each primer.

All amplifications were evaluated by running 1.5 μl of the final PCR product on a 2% agarose gel. One negative control (nuclease-free water) and 1 positive control (microscopically proved positive sample with *Plasmodium relictum* infection) were used once every 7 samples to control for false amplifications. No case of false amplification was found. Fragments of DNA from all positive amplifications were sequenced with corresponding primers twice for both strands. The obtained sequences were aligned and analysed using Bioedit program [44].

2.5. Ethical statements

Care and handling of experimental birds was in accordance with the current laws of Lithuania (permit by the Lithuanian State Food and Veterinary Service, Ref. No. 2012/01/04-0221).

3. Results

In order to find differences between two genetic lineages in DNA fragments we amplified blood samples with a single *P. relictum* parasite lineage (pSGS1 or pGRW11). To identify possible hybrids of these two lineages we applied one mitochondrial and three nuclear DNA markers. We determined that two chitinase 1 (cht1) gene fragments (355 bp and 410 bp length; GenBank accessions KX601274-KX601277) and one gene fragment (976 bp length; GenBank accessions KX601272, KX601273) encoding merozoite surface protein 1 antigen being identical between the isolates of the lineages pSGS1 and pGRW11. Hence, these genes were not informative for identifying possible hybrids. However, we found a genetic polymorphism between these lineages in the apical membrane antigen-1 gene (ama1) (fragment length 477 bp; GenBank accessions KX601270, KX601271). Together with the already known difference in the mitochondrial DNA cytochrome *b* gene [3] (MalAvi database 11/04/2016), these two genes could be used to identify hybrid oocysts.

In total, from 69 oocysts isolated in 7 mosquito midguts, we received 64 positive PCR results. Five positive PCR results were obtained from oocysts isolated from midgut of mosquito infected with a single pSGS1 infection, five positive results from midgut of mosquito infected with a single pGRW11 infection, and 54 positive results from five midguts of mosquitoes infected with mixes of pSGS1 and pGRW11 (Tables 1 and 2). All obtained sequences (ama1 and cyt *b*) from oocysts in mosquitoes infected with a single lineage were the same as in single blood infection present in donor birds (Table 1). Meanwhile six cases of hybrids were recorded among the 54 oocysts dissected from mosquitoes fed on birds with mixed infection. In four cases, we obtained mitochondrial cyt *b* sequence corresponding to pGRW11, while ama1 sequences of these oocysts belonged to the pSGS1 isolate. As mitochondrial genome is maternally inherited [45], our results show that the crosses were between pGRW11 macrogametes and pSGS1 microgametes. The other two cases of hybrids showed evidence to be crosses between pSGS1 macrogametes and pGRW11 microgametes (Table 1).

4. Discussion

This is the first study experimentally showing that cross-mating takes place between two avian malaria lineages and the hybrid oocysts develop. We examined oocysts of the parasites, because this is the only sporogonic stage where numerous copies (sporozoites) of all products of meiosis are present. The large numbers of haploid copies of genetic material developing in each oocyst, serve as a good template for genetic analysis [2,27,46]. In former genetic studies of human malaria parasites using oocysts, the parasites were separated using heated and pulled out microcapillaries [27,28,47]. We used laser microdissection method, which allowed cutting single oocyst and directly placing it in separate microtube for genetic analysis (Fig. 1) [42,48]. The primers of mitochondrial cyt *b* and nuclear ama1 genes were suitable for cross-mating analysis of pSGS1 and pGRW11 lineages. We did not find genetic differences between these two *P. relictum* lineages using published msp1 and cht1 gene markers [33,34].

During analysis of oocysts of human malaria, it was shown that random mating (panmixia) is common or even more favored comparing to self-fertilization when two lines of *P. falciparum* are present in mosquito midgut [25,27–30,49–52]. However, other studies have indicated that self-fertilization may be frequent, especially in human populations at sites with low level of transmission [52,53]. Such information is absent for avian *Plasmodium* spp. parasites. In this study, we could not define the frequencies of cross-fertilization. Blood stages of these two lineages of *P. relictum* are morphologically identical [22] and it is not possible to separate gametocytes by microscopic examination during mixed infections. The quantification of genetic material using RT-qPCR assay is currently hardly possible because of high genetic similarity of these two lineages and lack of genetic markers. However, this study provides information that cross-fertilization certainly occurs between *P. relictum* pSGS1 and pGRW11 lineages *in vivo*. We show that pGRW11 lineage was amplified more often (66.7% of all cases) by both cyt *b* and ama1 primers than pSGS1 (Table 1), and that might be due to higher intensity of pGRW11 in donor bird or/and more successful development of this lineage in comparison to pSGS1 in this mosquito species. Our results are in accordance with a previous experimental study where the number of *P. relictum* pGRW11 oocysts was significantly higher in *C. p. pipiens* f. *molestus* comparing to those of pSGS1 even when the gametocytemia in donor birds was similar for both parasites [39]. More successful amplification of one parasite DNA could be also explained by a) favored self-fertilization of one lineage [25] or/and b) differences in multiclonality of infection [54]. According to Morlais et al. [54] the number of oocysts is higher in mosquitoes that took blood meal with monoclonal infection than in those with multiclonal infections. Our estimated percentage of hybrids was 11,1%, which is lower than would be expected, assuming equal gametocytemia of both lineages. Heterozygotes might be underestimated when DNA of a heterozygote oocyst fail to be amplified; that might cause poor representation of heterozygotes comparing to homozygotes [55]. However, in our study 92% amplifications were positive using the both primers. Using nuclear ama1 markers we expected to get double peaks on electropherograms of heterozygote oocysts sequences as they should contain both parental alleles. We obtained sequences with single peaks in all heterozygote samples. As was shown in previous studies [24,38], the general primers are selective in amplification during mixed infections, and this could be the reason why the mixes were not shown on electropherograms [24]. Ranford-Cartwright et al. [27] used msp1 gene fragment amplification and the fragment size differentiation to identify putative hybrids of two *P. falciparum* lines using single oocysts as a template. The authors found that cross-fertilization occurred in 50% of investigated oocysts, while self-fertilization of one strain (3D7A) occurred four times more often than the other (strain HB3A). Such studies on avian malaria parasites and other haemosporidians are absent, but are needed to clarify mating preferences and frequencies of crosses.

The lineages pSGS1 and pGRW11 recombine and develop heterozygote oocysts. The hybrids are viable because each oocyst development requires numerous morpho-functional changes occurring during transformation from zygote stage to ookinete stage and then to oocysts [2]. However, at this point we cannot ascertain if the sporozoites from

hybrid oocysts are capable of further development in salivary glands and infecting new hosts as several post-oocyst barriers still remain [56]. Mainly, sporozoites should reach the salivary glands and mature completely [2]. The losses of malaria parasites occur on each phase of sporogonic development, especially before ookinete stage [57], thus our results together with Hellgren's [23] findings provide strong evidence that these two lineages likely belong to a single *P. relictum* species. These results are similar to those in the human malaria agent *P. falciparum* lines 3D7 and HB3 that differ in a single nucleotide base pair in the mitochondrial cyt *b* gene. These lines are attributed to the same *P. falciparum* species and recombine during hybridization experiments [11,25]. Because of huge diversity of avian malaria parasites, it is unclear how many closely related lineages (morphologically identical and with cyt *b* genetic difference of < 5%) could be attributed to the same species and how many of them represent cryptic species [58–61]. From the present study, we assume that at least one bp difference in mitochondrial cyt *b* gene may present intraspecific variation. However, a recent study [61] shows that five genetically closely related mitochondrial lineages described as *Haemoproteus majoris* (sister genus) have unique alleles in four investigated nuclear genes, suggesting that they might represent five cryptic species. More data are needed for better understanding species boundaries in different groups of haemosporidian parasites.

It is important to note that the transmission areas and the broad range of vertebrate hosts of both the pSGS1 and pGRW11 lineages greatly overlap [18,23,62–67]. Moreover, vectors of these two lineages are the same mosquito species [39,64–66]. Based on available data it seems that transmission of the lineage pSGS1 takes place more up to north than of pGRW11 [18]. It remains unclear if these differences could have impact on lineage diversification or if the spread of the lineage pSGS1 to north is caused by more recent global climate change [16,17,68].

Despite common occurrence of haemosporidian mixed infections in wildlife, little is known about how avian haemosporidian parasites interact during such infections and what impact it has on development, transmission and distribution of the parasites. Additional experimental studies about crosses between haemosporidian parasite lineages and biological barriers for development of such crosses are needed for better understanding the epidemiology and diversification processes in malaria parasites.

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