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ORIGINAL ARTICLE

A quantitative PCR protocol for detecting specific *Haemoproteus* lineages: molecular characterization of blood parasites in a Sedge Warbler population from southern Poland

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Abstract In evolutionary studies, blood parasites in avian populations are commonly used as a model of host–parasite interactions. The effect of mixed infections on avian hosts has recently drawn more interest, but the effects of infection with multiple blood parasites and specific parasite lineages are poorly known. A protocol for reliable detection and quantification of lineages is essential to this type of research. Here, we present a newly developed quantitative PCR (qPCR) assay using SYBR Green I to assess the infection intensity of SW1 and SW3 *Haemoproteus belopolskyi* lineages in a Sedge Warbler (*Acrocephalus schoenobaenus*) population from southern Poland. These *Haemoproteus* lineages are not specific to the Sedge Warbler, so the proposed protocol should prove useful for many avian malaria studies. The assay is based on nucleotide primers designed to amplify a fragment of the cytochrome *b* gene, allowing the two avian malaria lineages to be differentiated. Using this assay, specific host–parasite interactions can be identified and the impact of mixed infections on a host population can be assessed. Most of the parasitized birds in our study were in a low-intensity, chronic phase of infection. In those with mixed SW1/SW3 infections, we detected significantly higher parasitemia caused by the SW3 lineage, whose prevalence was underestimated by the commonly used method, nested PCR. The prevalence of avian malaria parasites in the studied population as estimated by nested PCR was 61 % and did not differ between years, though the prevalence of the SW1 lineage showed significant annual variation. Altogether, two *Haemoproteus* and five *Plasmodium* lineages were detected. The two *Haemoproteus* lineages (SW1, SW3) were most prevalent in the population and comprised 93 % of all infections. We detected significantly higher haemoparasite prevalence and intensity in males, which were sampled immediately after arrival from wintering grounds, suggesting decreased immunoprotection as a result of adaptive resource allocation during migration.

Keywords Malaria parasites · qPCR · Nested PCR · Infection intensity · Prevalence · *Haemoproteus* · *Acrocephalus schoenobaenus*

Zusammenfassung

Eine quantitative PCR-Methode zum Nachweis spezifischer Haemoproteus-Stämme: Molekulare Charakterisierung von Blutparasiten bei einer Schilfrohrsänger-Population in Südpolen

In Studien zur Evolution finden Blutparasiten bei Vogelpopulationen häufig Verwendung als Modelle für Wirt-Parasiten-Beziehungen. Dabei hat zwar das Interesse an den Auswirkungen von Mischinfektionen auf die Wirtsvögel in letzter Zeit zugenommen, über die Folgen von Infektionen durch mehrere Blutparasiten und spezifische Parasitenstämme weiß man aber immer noch wenig. Für diesen Forschungsbereich sind Methoden zum sicheren Nachweis und zur Quantifizierung dieser Stämme unabdingbar. Hier stellen wir ein neu entwickeltes quantitatives PCR-Verfahren (qPCR) unter Verwendung von SYBR Green I zur Ermittlung der Infektionsintensität durch die beiden *Haemoproteus belopolskyi*-Stämme SW1 und SW3 in einer südpolnischen Population von Schilfrohrsängern

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(*Acrocephalus schoenobaenus*) vor. Diese Haemoproteus-Stämme sind nicht für Schilfrohrsänger spezifisch, so dass sich die vorgestellte Methode für eine breite Palette von Vogelmalaria-Untersuchungen als nützlich erweisen sollte. Das Verfahren basiert auf Nukleotid-Primern zur Vervielfältigung eines Fragments des Cytochrom b-Gens, anhand dessen die beiden Vogelmalaria-Stämme unterschieden werden können. Dieser Ansatz erlaubt es, spezifische Wirt-Parasiten-Wechselwirkungen zu identifizieren und die Auswirkungen von Mischinfektionen auf eine Wirtspopulation abzuschätzen. Die meisten der von Parasiten befallenen Vögel in unserer Studie befanden sich in einer chronischen Infektionsphase schwacher Intensität. Bei Vögeln mit gleichzeitiger SW1/SW3-Infektion fanden wir eine signifikant höhere Parasitenbelastung durch den SW3-Stamm, dessen Häufigkeit durch die normalerweise gebräuchliche Methode, die Nested PCR (geschachtelte PCR), als zu gering eingeschätzt wurde. Die durch die Nested PCR geschätzte Durchsetzung der Untersuchungspopulation mit Vogelmalariaerregern betrug 61 % und unterschied sich nicht von Jahr zu Jahr, obwohl die Verbreitung des SW1-Stamms eine signifikante jährliche Variation aufwies. Insgesamt fanden wir zwei Haemoproteus- und fünf Plasmodium-Stämme. Die beiden Haemoproteus-Stämme (SW1, SW3) waren in der Population am stärksten verbreitet und machten 93 % aller Infektionen aus. Wir fanden einen signifikant stärkeren Befall mit Blutparasiten und eine höhere Infektionsstärke bei Männchen, die unmittelbar nach der Rückkehr aus den Überwinterungsgebieten beprobt wurde, was auf einen verringerten Immunstatus infolge adaptiver Ressourcennutzung auf dem Zug schließen lässt.

Introduction

Avian malaria (sensu Pérez-Tris et al. 2005) is a vector-borne disease caused by parasites from the genera *Plasmodium* and *Haemoproteus*. Avian hemosporidian parasites are commonly used as a model system for studying host–parasite evolution and its consequences for population ecology. A number of studies have shown the influence of hemosporidian infection on certain life-history traits. They describe how the infection affects the host's condition, reproductive success and survival (e.g. Asghar et al. 2011; Knowles et al. 2011; Lachish et al. 2011; Asghar et al. 2012; van de Crommenacker et al. 2012; Marzal et al. 2013). To determine host–parasite interactions and their effects, the composition of the parasite communities across diverse habitats and populations needs to be known. Most ecological studies have examined fitness-associated traits as they relate to parasite prevalence (Merino et al. 2000; Marzal et al. 2005; Bensch et al. 2007), but few studies have examined parasitemia (Hasselquist et al. 2007; Stjernman et al. 2008; Knowles et al. 2010; Asghar et al. 2011). Parasitemia has usually been estimated from smear counts (Hasselquist et al. 2007; Stjernman et al. 2008), a procedure that requires a high level of expertise in parasite identification. It is especially difficult to detect avian malaria parasites during mild chronic infections, as they are frequently overlooked in blood films (Jarvi et al. 2003; Waldenström et al. 2004). Molecular methods are a quicker and more sensitive way of detecting infection (Jarvi et al. 2002; Richard et al. 2002; Fallon and Ricklefs 2008). They have revealed genetically differing lineages of these parasites, further increasing interest in this host–parasite system (Bensch et al. 2000; Pérez-Tris et al. 2005; Howe et al. 2012). The reason why molecular methods are rarely used for parasitemia quantification is that we lack good assays for quantitative scoring of parasite lineages.

Some work has suggested that mixed infections are especially severe (Graham et al. 2005; Davidar and Morton 2006). Coexisting parasites are often reported in birds (Valkiūnas et al. 2003; Losieau et al. 2010; Asghar et al. 2011; Zehtindjiev et al. 2011; Piersma and van der Velde 2012), but their effects are largely unknown. Some studies show aggravated effects of avian malaria co-infection (Marzal et al. 2008) but others do not (Sanz et al. 2002). The detectability of mixed infection may depend on the method used for analysis; results for the same species can vary considerably. For example, 43 % of birds determined by PCR to have single infections showed mixed infections by blood microscopy (Valkiūnas et al. 2006), prompting the suggestion that direct sequencing and scoring of mixed infections based on the presence of double-base calling is likely to underestimate the rate of mixed infections (see also Asghar et al. 2011). That is especially likely when the level of parasitemia differs considerably between coexisting lineages or if the primers differ in the parasite lineages they match (Pérez-Tris and Bensch 2005; Valkiūnas et al. 2006).

Our research used the Sedge Warbler *Acrocephalus schoenobaenus* population inhabiting natural wetlands of the Nida River Valley (south Poland). The Sedge Warbler is a trans-Saharan passerine migrant which breeds in Europe and winters in tropical Africa where it becomes infected with hemosporidian parasites (Waldenström et al. 2002). This infestation pattern makes the Sedge Warbler a good model for studying host–parasite interactions. The Nida wetlands population is the object of a long-term study on various aspects of species ecology and behavior (Król et al. 2002; Zając and Solarz 2004; Zając et al. 2006, 2008a, b, 2011). Here, we present a quantitative PCR (qPCR) assay that enabled us to accurately quantify parasitemia and detect mixed *Haemoproteus* infections in the

studied population. Our goal was to develop a simple protocol for use in studies of host–parasite interactions, one that can be employed by laboratories having little previous experience in running qPCR. We also applied a highly efficient nested PCR method (Waldenström et al. 2004) for identification of blood parasite species in the population and compared the performance of the two methods for assessing the prevalence of blood parasites.

Methods

The study was done between 2004 and 2008 in a large area of natural wetlands in the Nida River Valley in southern Poland (50°33′–50°35′N, 20°28′–20°32′E). Sedge Warbler males were mist-netted immediately after arriving from their winter quarters in late April and early May. The females were usually caught during late stages of incubation or during nestling feeding. A blood sample (<0.05 ml) was obtained by brachial vein puncture from adult birds (293 males, 149 females) and stored in 95 % ethanol for molecular analyses. Genomic DNA was extracted with the Nucleospin Tissue Kit (Macherey and Nagel, Germany). Total DNA concentration was measured using a Nanodrop 1,000 (Thermo Scientific). For a subset of 202 individuals, blood smears were prepared from the same blood sample to compare the performance of the qPCR assay with parasitemia assessment by light microscopy. Thin smears were made and then air-dried, fixed in 95 % methanol and stained with Hemacolor (Merck). The slides were examined for the presence of parasites of the genera *Plasmodium, Haemoproteus* and *Leucocytozoon*. For each slide, 100 fields at ×1,600 (Nikon 50i light microscope) were checked by the same person (W.B.) and parasitemia was expressed as the number of parasites per 100 fields. As no *Leucocytozoon* taxa were detected in the analyzed subset, only *Haemoproteus* and *Plasmodium* were screened by molecular methods.

Nested PCR

All samples were subjected to a highly efficient nested PCR procedure that amplifies the 480 bp fragment of cytochrome *b* (cytb) of *Plasmodium* and *Haemoproteus* (Waldenström et al. 2004). We used external primers HaemNFI and HaemNR3, and internal primers HaemF–HaemR2. Samples were amplified in 96-well reaction plates with negative and positive controls (DNA from individuals with known malarial infections) for every 15 samples to ensure that the outcome of each PCR run was not affected by contamination (Waldenström et al. 2004). Positive or negative infections were scored by separating PCR products on 2 % agarose gel. PCR products from positive samples were purified using ExoSAP. The products were then bidirectionally sequenced with an ABI 3130xl genetic analyzer (Applied Biosystems). Sequences were edited and aligned using BioEdit (Hall 1999). The obtained haplotypes were compared with avian malaria haplotypes deposited in the MalAvi database (Bensch et al. 2009).

qPCR

Primer design, protocol optimization, standard curve, and performance assessment

To design genus-specific primers, we amplified full-length cytb sequences for all *Plasmodium* and *Haemoproteus* lineages detected in this study. The nested PCR procedure described by Perkins and Schall (2002) was used. From the alignment of the obtained cytb haplotypes we designed nine pairs of specific primers to amplify the SW1 and SW3 *Haemoproteus* lineages, the ones most frequent in the studied population. All primer pairs were designed so that at least one primer of a pair was situated outside the region previously used for lineage identification. This was done to avoid any contamination by a product of nested PCR performed earlier in the laboratory. A range of annealing temperatures (52–60 °C) was tested to ensure optimal assay conditions for selective amplification of SW1 and SW3 lineages. Primers SW1F–SW1R and SW3F–SW3R, which respectively target 69 and 120 bp regions of this gene, were chosen for their efficiency and sensitivity. Table 1 lists the primer sequences and annealing temperatures. The specific amplification of each lineage was confirmed by amplifying samples bearing known *Haemoproteus* lineages. There were no primer-dimer artifacts present in the amplifications.

To create material for a standard curve, the full-length cytb gene of both *Haemoproteus* lineages was amplified using the protocol of Perkins and Schall (2002) and purified with a MinElute PCR Purification Kit (Qiagen). The total DNA concentration of this PCR product was determined using a Nanodrop 1000. Then, molecular conversion calculations based on the size and base composition of the DNA fragment were used to estimate DNA copy number in this solution. Five 5× serial dilutions of this PCR product were then used on each qPCR plate as a standard curve, covering 12,000–19 and 13,000–21 DNA copy number for SW1 and SW3, respectively.

To test for assay sensitivity, qPCR reactions were run on a dilution series of DNA from a host previously shown to be infected with either SW1 or SW3. We were able to obtain a reaction product for the lowest concentration of two copies of parasite DNA per 1 ng host DNA for both

Table 1 QPCR primer sequences and annealing temperatures for amplified targets

Primers	Primer sequence 5′–3′	Product size (bp)	Annealing temperature (°C)
SW1F	ATTATTAGCAACTTGCTATACC	69	60
SW1R	TCCAGTAGCATGCATATATCTG		
SW3F	ATGGTGTTTTAGATATATGCAC	120	60
SW3R	ACCCATAAAAGCAGTAACAATA		
SFSR/3Fb	ACTAGCCCTTTCAGCGTCATGT	114	55
SFSR/3Rb	CATGCTCGGGAACCAAAGG		

lineages. As hemosporidian parasites are single-celled organisms, one copy of PCR product is equal to one parasite.

Amplification efficiency was determined from the slope of the log-linear portion of the calibration curve. The efficiency of PCR reactions equals 100 % when the amount of template DNA doubles with each cycle. Following the guidelines of Bustin et al. (2009), we accepted the results from qPCR runs having standard curves with slopes between 3.3 and 3.8 (equal to 100–80 % reaction efficiency).

To assess the performance of the developed qPCR method, we compared parasitemia found from qPCR with that quantified by light microscopy. Light microscopy only identified parasites to genus level (*Haemoproteus* or *Plasmodium*), so we did not differentiate between SW1 and SW3 parasitemia detected by qPCR, and for mixed infections we summed the values for the two *Haemoproteus* lineages.

Reaction setup

Reactions were performed with an ABI 7500 Real-Time PCR system (Life Technologies) with SYBR-green-based detection. A UDG/dUTP-containing mix (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen) with ROX as reference dye was used. Two minutes of pre-incubation at 50 °C was performed to avoid PCR product contamination. Reactions for both *Haemoproteus* lineages were run in volumes of 25 µl containing 5 µl DNA, 12.5 µl SuperMix, 0.1 µl ROX and 5 µM of each primer. The amplification procedure consisted of 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

All samples were diluted to a standard working concentration of ca. 2 ng/µl prior to qPCR. To adjust host DNA concentration for every sample, we ran an additional reaction with host-specific primers SFSR/3Fb and SFSR/3Rb (Table 1). The primers amplify a portion of a single copy of the nuclear sequence of a host DNA region that is ultra-conserved across vertebrates (Bejerano et al. 2004). Standard curves were produced by diluting host DNA of a known concentration of 10 ng/µl in four 5× dilutions covering the range from 10 to 0.08 ng/µl DNA. To estimate the exact level of parasitemia we adjusted for the total DNA concentration in each reaction.

Each sample as well as the standard serial dilutions were run in triplicate. *Haemoproteus* DNA copy number and host DNA concentration were scored as the mean of three wells if the three replicates differed by less than one cycle threshold. If only one replicate deviated by more than one cycle threshold, copy number was scored as the mean of the other two scores. If all three scores differed by more than one cycle threshold the analysis was repeated. For each sample the product melt curve was inspected to confirm that only one specific product was amplified. All the reactions were run in 96 well plates. Six negative controls were included on each plate. Due to the high number of samples it was not possible to run all the samples in one experiment. To ensure repeatability of experiments run in different plates, one sample was run repeatedly in each plate.

Statistical analyses

Parasite prevalence was compared between groups using Fisher's exact test and the Chi squared test for multiple independent samples. For parasitemia analyses the values for infection intensity were $\log_{10}$-transformed to normalize the skewed distribution. One-way ANOVA was used to compare parasitemia between parasite lineages. Concordance between parasitemia obtained by qPCR and by light microscopy was analyzed by linear regression on $\log_{10}$-transformed and normalized data. The analyses used Statistica 10. Concordance between nested PCR (Waldenström et al. 2004) and qPCR in assessing the prevalence of *Haemoproteus* lineages was determined using the Kappa coefficient of agreement (Viera and Garrett 2005).

Results

Nested PCR and lineage identification

Of the 442 adult birds of both sexes analyzed for the presence of avian malaria parasites, 268 (61 %) gave positive results from PCR amplification. We obtained readable

Table 2 The prevalence of detected avian malaria parasites detected with nested PCR ($n = 255$)

Lineage	Species	GenBank acc. number	% of lineage detected among infected individuals
SW1	*Haemoproteus belopolskyi*	AF254967	59.2
SW3	*Haemoproteus belopolskyi*	AF495573	34.1
GRW04	*Plasmodium relictum*	AF254975	2.7
SW2	*Plasmodium homonucleophilum*	AF495572	1.6
GRW02	*Plasmodium ashfordi*	AF254962	1.2
RTSR1	*Plasmodium* sp.	AF495568	0.8
SW5	*Plasmodium* sp.	AF495574	0.4

sequences for 255 samples, among which we identified two *Haemoproteus* and five *Plasmodium* lineages. Table 2 gives the lineages and their prevalence among infected individuals in the studied population. As 93 % ($n = 238$) of the infected individuals bore SW1 or SW3 lineages of *Haemoproteus*, all subsequent analyses were done for those two lineages only. SW1 showed 34 % prevalence, and SW3 19 %. Prevalence differed significantly between seasons for SW1 ($\chi^2 = 9.89$; $p = 0.04$) but not for SW3 ($\chi^2 = 3.67$; $p = 0.45$). Infections were significantly more frequent in males ($n = 180$) than in females ($n = 71$; Fisher's exact test; $p = 0.007$) but not when the comparison was made within SW1 and SW3 separately (SW1: $\chi^2 = 2.42$; $p = 0.119$; SW3: $\chi^2 = 3.68$; $p = 0.06$). Mixed infections, detectable as double-base calling in chromatograms, were detected in only 4 % of the infected individuals (the level of mixed infections expected from the percentages of single infections was 6.5 %). Due to the low quality of those chromatograms it was not possible to distinguish between the detected lineages.

qPCR

qPCR revealed that 53 % of the 442 analyzed individuals were infected with *Haemoproteus*. Prevalence was 36 % for SW1 and 28 % for SW3. Maximum intensity of infection, expressed as parasite copy number per 1 ng host DNA, was 87,635 (mean 1,083 ± 7,034) for SW1 and 183,127 (mean 4,536 ± 22,409) for SW3. Most of the infected birds bore chronic infections, with infection intensity of 1–50 for SW1 and 50–100 for SW3. For both parasite lineages, there were only a few individuals with very high parasitemia. Such high numbers reflect the acute phase of infection (Fig. 1). Mean parasitemia was significantly higher in SW3 ($F_{(1,308)} = 59.60$; $p < 0.0001$). Parasitemia for both lineages was higher in males (SW1: $F_{(1,172)} = 3.88$, $p = 0.05$; SW3: $F_{(1,133)} = 3.77$, $p = 0.05$). Among infected individuals, 49 (19 %) bore a mixed infection, almost double the level expected from the percentages of single infections (10 %). The detected level of mixed infections differed significantly between nested PCR (4 %) and qPCR (19 %) ($\chi^2 = 11.8$; $p < 0.0001$). In analyses restricted to individuals with mixed infections of SW1 and SW3, the intensity of SW3 infection was significantly higher ($F_{(1,96)} = 15.65$; $p < 0.0001$).

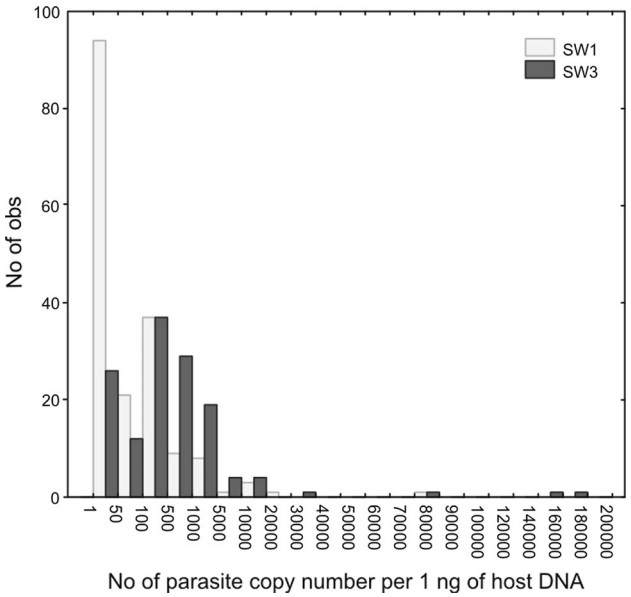

Fig. 1 Infection intensity of SW1 and SW3 *Haemoproteus* lineages detected in the Sedge Warbler (*Acrocephalus schoenobaenus*) population of Nida River Valley wetlands

In our analysis of agreement on parasite detection between the nested PCR method (Waldenström et al. 2004) and the newly developed qPCR assay, we obtained a Kappa coefficient of 0.824, indicating almost perfect agreement (Viera and Garrett 2005), for the two lineages taken together. In separate analyses of the lineages the coefficient was 0.644 (substantial agreement) for SW1 and 0.394 (fair agreement) for SW3. QPCR and light microscopy showed a strong positive correlation in efficiency of assessing *Haemoproteus* infection intensity (Fig. 2; $r = 0.711$; $n = 202$; $p < 0.0001$). QPCR detected *Haemoproteus* infection in all the samples used for comparison ($n = 202$), while light microscopy detected *Haemoproteus* infection in only 70 % of those samples. The samples in which light microscopy failed to detect infection were scored lowest by qPCR.

Discussion

The new qPCR assay developed in this study proved to be sensitive and efficient. The high and strongly significant correlation between the levels of *Haemoproteus*

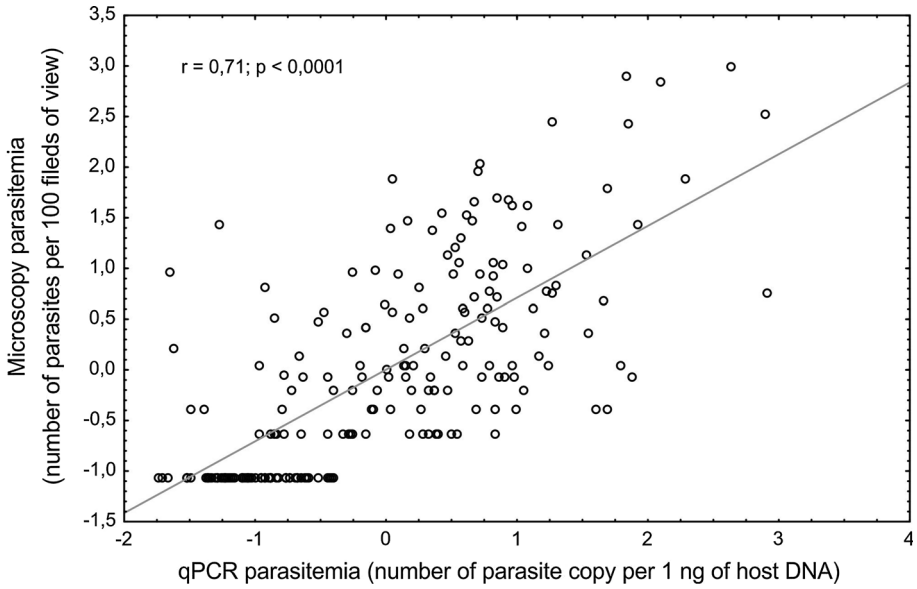

Fig. 2 Number of *Haemoproteus* parasites assessed by light microscopy plotted against *Haemoproteus* qPCR scores. Analysis performed on $\log_{10}$-transformed and normalized values

parasitemia detected by qPCR and light microscopy means that both approaches are suitable for parasite quantification, but qPCR gave clearly better resolution. Its main advantage is superior specificity, as it amplifies distinct avian malaria lineages. The effects of coexisting parasite lineages have drawn increasing interest (Valkiūnas et al. 2003; Losieau et al. 2010; Asghar et al. 2011; Zehtindjiev et al. 2011; Piersma and van der Velde 2012). Mixed infections might be expected to be especially severe (Graham et al. 2005; Davidar and Morton 2006), hence the need for a protocol such as ours in future bird studies.

The qPCR assay revealed five times more mixed infections than the nested PCR approach (Waldenström et al. 2004) in the same samples. Valkiūnas et al. (2006) argued that methods employing cytb fragment amplification underestimate the number of mixed infections, and that the amplified haplotype does not always match the one present at higher intensity in the host. Underestimation of mixed infections by the protocol of Waldenström et al. (2004) might be due to differences in copy number between parasite lineages or to differences in primer matching to lineages. Low performance in identifying mixed infections by nested PCR was the reason for the imperfect agreement between the two methods in our study, especially evident for the SW3 lineage: many individuals scored as having SW1 by nested PCR were identified as having mixed infections of SW1 and SW3 by qPCR. As the mean intensity of SW3 infection was higher both overall and in mixed infections, the reason for the low level of mixed infections detected by nested PCR was low affinity of the primers to the target sequence.

The SW1 *Haemoproteus* lineage has been found in the Sedge Warbler both in Europe (Bensch et al. 2000; Dimitrov et al. 2010) and in its wintering grounds in Nigeria (Waldenström et al. 2002), and also in other species (African Reed Warbler *A. baeticatus*, Waldenström et al. 2002; Marsh Warbler *A. palustris*, Hellgren et al. 2007; Eurasian Reed Warbler *A. scirpaceus*, Dimitrov et al. 2010). So far, the SW3 lineage has been found only in the Sedge Warbler (Waldenström et al. 2002; Hellgren et al. 2007; Dimitrov et al. 2010), but it may have been overlooked in different species due to problems with primer-target sequence compatibility.

In our work we characterized avian malaria parasites in a Sedge Warbler population studied over a long period. Five of the seven lineages we identified have been found before in this species (*Haemoproteus belopolskyi*: SW1, SW3; *Plasmodium* sp.: SW2, SW5, GRW04), and the other two haplotypes (*Plasmodium* sp.: GRW02, RTSR1) had been found in the Great Reed Warbler *A. arundinaceus* (MalAvi database; Bensch et al. 2009). As compared with different Sedge Warbler populations, in which 2–6 malaria parasite lineages have been found (Bensch et al. 2000; Waldenström et al. 2002; Pérez-Tris et al. 2007; Dimitrov et al. 2010; Fernández et al. 2010), our population exhibited high diversity of hemoparasites. The number of birds we sampled is four times higher than the largest sample studied in other research (442 vs. 122 in Pérez-Tris et al. 2007). Five of the seven identified lineages were only detected in a a few birds, suggesting that those were sporadic infections by lineages that depend mainly on other host species (Bensch et al. 2007).

The prevalence of the SW1 lineage differed significantly between years. Annual variation in hemosporidian parasites is often reported (Bensch et al. 2007; Lachish et al. 2011) and may reflect patterns of parasite-driven selection (Westerdahl et al. 2004), parasite-mediated population

cycles, or vector abundance in the environment (Hudson et al. 1998). We cannot state reasons for this variation in the studied population, but for future research the present results will be combined with the large body of data obtained in other work on this population.

The total prevalence of malaria parasite infection in the studied population was relatively high (61 %) on the scale of values from other Sedge Warbler studies, which ranges from 7 % (Bensch et al. 2007) to 100 % (Dimitrov et al. 2010). It was higher and more intense in males than in females. The males in our study were sampled immediately after arriving from their wintering grounds. Migration is an extremely energy-costly behavior (Wikelski et al. 2003; Newton 2006) which might reduce immunoprotection as a result of adaptive resource allocation during migration (Weber and Stilianakis 2007). Birds sampled shortly after arrival may carry a higher parasite load, more easily detected by nested PCR. The females, on the other hand, were usually sampled either during late stages of incubation or during nestling feeding, that is, almost a month after arrival (Król et al. 2002). Thus the observed sex differences in parasite prevalence and intensity could be an artifact of the differences in sampling time.

Our qPCR assay should prove broadly useful in host–parasite interaction studies and hemosporidian parasitemia analyses, not necessarily restricted to single populations or single avian species.

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