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A COMPARATIVE ANALYSIS OF MICROSCOPY AND PCR-BASED DETECTION METHODS FOR BLOOD PARASITES

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ABSTRACT: We compared information obtained by both microscopy and nested mitochondrial cytochrome *b* PCR in determining prevalence of haemosporidian infections in naturally infected birds. Blood samples from 472 birds of 11 species belonging to 7 families and 4 orders were collected in Europe, Africa, and North America. Skilled investigators investigated them using the PCR-based screening and microscopic examination of stained blood films. The overall prevalence of haemosporidian infections, which was determined by combining results of both these methods, was 60%. Both methods slightly underestimated the overall prevalence of infection, which was 54.2% after the PCR diagnostics and 53.6% after microscopic examination. Importantly, both these tools showed similar prevalence for *Haemoproteus* spp. (21% by PCR and 22% by microscopy), *Plasmodium* spp. (17% and 22%), and *Leucocytozoon* spp. (30% and 25%), verifying that microscopy is a reliable tool in determining patterns of distribution of blood haemosporidian parasites in naturally infected birds. We encourage using optical microscopy in studies of blood parasites in parallel to the now widely employed molecular methods. Microscopy is unlikely to result in false positives, which is a major concern in large-scale PCR studies. Moreover, it is relatively inexpensive and provides valuable information regarding the ways in which molecular methods can be further improved and most effectively applied, especially in the field studies of parasites. Importantly, blood films, which are used for microscopic examination, should be of good quality; they should be examined properly by skilled investigators. In spite of the substantial time investments associated with microscopy, such examination provides opportunities for simultaneous determination and verification of taxonomically different parasites. Presently, different PCR protocols must be used for the detection of parasites belonging to different genera; this is expensive and time consuming.

Species of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* (Haemosporida) are common dipteran-borne haemosporidian blood parasites, which have been reported in birds all over the world (Greiner et al., 1975; McClure et al., 1978; Atkinson and van Riper, 1991; Bishop and Bennett, 1992). For more than 100 yr, the haemosporidians have been studied predominantly by microscopic examination of Giemsa-stained blood films. The current knowledge on the basic life history strategies of haemosporidians, their geographical distribution and distribution by hosts, vertebrate hosts and vector specificity, seasonal changes of infection, and other aspects of ecology of these parasites has been accumulated primarily by microscopy (Garnham, 1966; Atkinson and van Riper, 1991; Forrester and Spalding, 2003; Valkiūnas, 2005). General conclusions of these studies have been supported by recent molecular investigation, which added new and innovative aspects to the knowledge of the biology of haemosporidians, especially their genetic diversity, phylogeography, phylogeny, and vertebrate-host specificity (Perkins and Schall, 2002; Ricklefs et al., 2004; Szymanski and Lovette, 2005; Kimura et al., 2006; Križanauskienė et al., 2006; Martinsen et al., 2006; Sehgal, Hull et al., 2006; Bensch et al., 2007; Palinauskas et al., 2007; Perkins et al., 2007; Krone et al., 2008).

In some recent molecular studies, microscopy was shown to be significantly less sensitive than PCR-based methods in determining prevalence of haemosporidian infections in birds. Richards et al. (2002) compared several PCR assays and microscopy for detection of avian haemosporidians; they concluded that the PCR is faster, cheaper, and more reliable than microscopic blood smear examination for large-scale screening. According to Jarvi et al. (2002), PCR tests were 3- to 4-fold better than microscopy for detecting chronic blood parasite infections. Durrant et al. (2006) reported that specimens examined using both PCR-based techniques and blood smears showed an approximately 10-fold difference in prevalence of haematozoa, with PCR-based techniques detecting many more infections. This raised a question about the value of microscopy in field studies and the reliability of conclusions, which have been based on the microscopy data, regarding the ecology of haemosporidian parasites.

The aim of the present study was to verify the sensitivity of microscopy in determining prevalence of haemosporidian infections in birds. We compared results of screening a large number of blood samples by 3 independent groups of skilled researchers who used 2 nested mitochondrial cytochrome *b* (cyt *b*) PCR assays and microscopy in determining the prevalence of haemosporidian infections in naturally infected birds.

MATERIAL AND METHODS

Study sites and collection of blood samples

Blood samples from 472 birds of 11 species belonging to 7 families and 4 orders were collected in Europe, Africa, and North America (Table I). All birds were ringed, bled, and released after collecting the blood samples. None of them was recaptured. Study sites included (1) Lithuania (Baltoji Vokė, Vilnius district; June–July 2002); (2) Russia (the Curonian Spit in the Baltic Sea; May–July 2003 and 2004); (3) California (environs of San Francisco; September–November 2003 and 2004); (4) Uganda (villages adjacent to Bwindi Impenetrable National Park, Kibale National Park and Mabira Forest Reserve; July 2003); (5) Cameroon (sites near the villages Ndibi, Nkwouak, Zoebefame, Mengong, Nkouembpoer, Beh, Koto, Mokoko, Bitye, Djaposten, Mvono and Douni; July–August 2005 and 2006). Detailed description of the study sites were published by Valkiūnas et al. (2005), Venskutė et al. (2005), Križanauskienė et al. (2006), Sehgal, Hull et al. (2006), and Sehgal, Valkiūnas et al. (2006).

The blood was taken by puncturing the brachial vein. From each bird, 2 or 3 blood films were prepared on ready-to-use glass slides. Blood films were air-dried within 5–15 sec after their preparation. In humid climates we used a battery-operated fan to aid in the drying of the blood films. Smears were fixed in absolute methanol for 1 min on the day of

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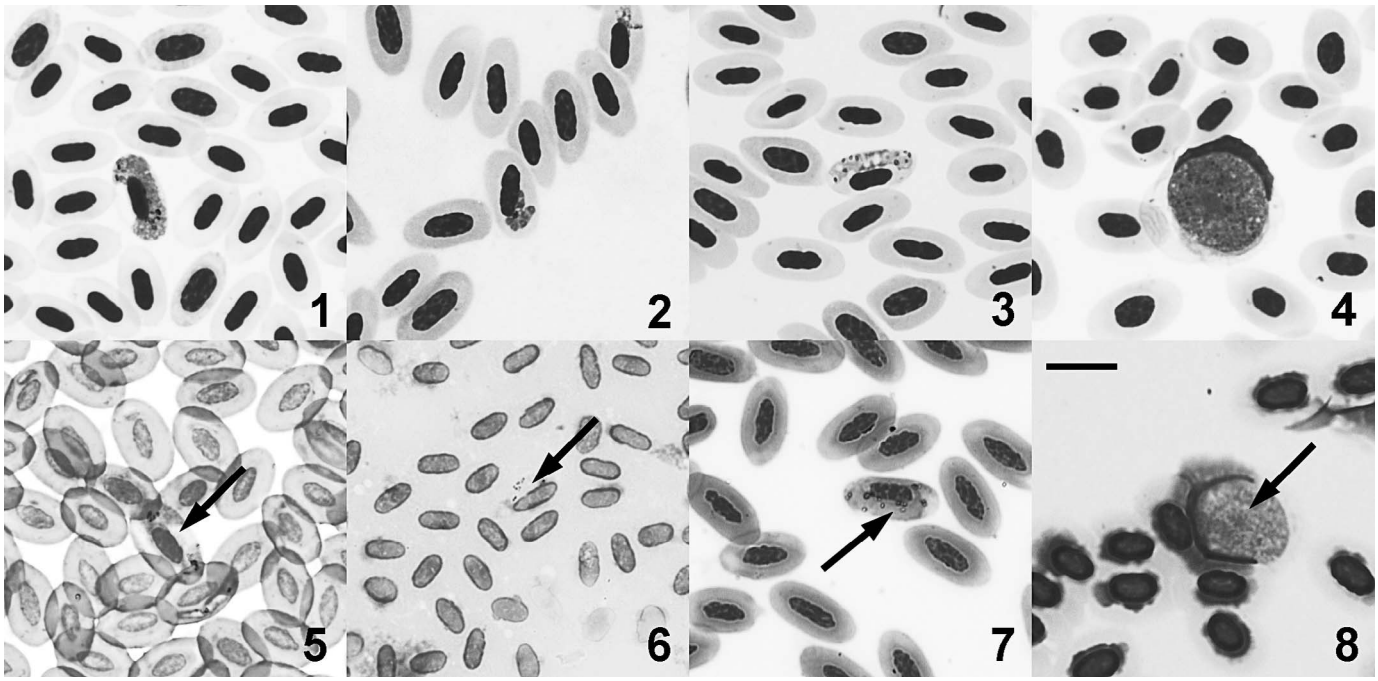
TABLE I. Birds tested and outcome of microscopic examination of blood films and the PCR screening of the same samples. (— data are absent).

Study site and bird species	No. tested	No. positive											
		Microscopic examination				PCR screening				Both methods combined			
		H*	P†	L‡	Total	H	P	L	Total	H	P	L	Total
Cameroon													
Cyanomitra olivacea	193	39	84	71	137	31	64	92	135	46	102	104	160
Gallus gallus	71	0	0	5	5	0	0	5	5	0	0	5	5
Total	264	39	84	76	142	31	64	97	140	46	102	109	165
		(14.8)§	(31.8)	(28.8)	(53.8)	(11.7)	(24.2)	(36.7)	(53.0)	(17.4)	(38.6)	(41.3)	(62.5)
Uganda													
Gallus gallus	77	0	0	22	22	0	0	22	22	0	0	22	22
				(28.6)	(28.6)			(28.6)	(28.6)			(28.6)	(28.6)
Lithuania													
Acrocephalus palustris	12	0	0	—	0	0	1	—	1	0	1	—	1
Sylvia communis	17	8	2	—	10	10	5	—	15	10	5	—	15
Phylloscopus trochilus	28	6	0	—	6	6	0	—	6	6	0	—	6
Total	57	14	2	—	16	16	6	—	22	16	6	—	22
		(24.6)	(3.5)		(28.1)	(28.1)	(10.5)		(38.6)	(28.1)	(10.5)		(38.6)
Russia													
Ficedula hypoleuca	27	23	5	—	27	22	4	—	26	23	5	—	27
Parus major	21	20	3	—	21	20	1	—	21	20	3	—	21
Total	48	43	8	—	48	42	5	—	47	43	8	—	48
		(89.6)	(16.7)		(100)	(87.5)	(10.4)		(97.9)	(89.6)	(16.7)		(100)
California													
Bubo virginianus	5	0	1	5	5	0	1	5	5	0	1	5	5
Buteo jamaicensis	8	7	5	5	8	8	0	5	8	8	5	6	8
Megascops kennicottii	6	0	2	6	6	0	4	6	6	0	4	6	6
Tyto alba	7	0	1	5	6	0	1	6	6	0	1	6	7
Total	26	7	9	21	25	8	6	22	25	8	11	23	26
		(26.9)	(34.6)	(80.8)	(96.2)	(30.8)	(23.1)	(84.6)	(96.2)	(30.8)	(42.3)	(88.5)	(100)
Grand total	472	103	103	119	253	97	81	141	256	113	127	154	283
		(21.8)	(21.8)	(25.2)	(53.6)	(20.6)	(17.2)	(29.9)	(54.2)	(23.9)	(26.9)	(32.6)	(60.0)

* *Haemoproteus* sp.
† *Plasmodium* sp.
‡ *Leucocytozoon* sp.
§ Percentage of birds positive.



FIGURES 1–8. Blood stages of hemosporidian parasites as they are seen in good-quality (**1–4**) and bad-quality (**5–8**) blood films. (**1, 5**) Gametocytes of *Haemoproteus* spp. (**2, 6**) Erythrocytic meronts and (**3, 7**) gametocytes of *Plasmodium* spp. (**4, 8**) Gametocytes of *Leucocytozoon* spp. (**5, 7**) Blood films with good fixation and bad staining; parasites and their nuclei are hardly distinguishable. (**6, 8**) Bad fixation and staining of blood films; blood cells are destroyed because of haemolysis, and nuclei of parasites are hardly seen. Arrows: parasites in bad-quality preparations. Giemsa-stained thin blood films. Bar = 10 μm.

their preparation. Fixed smears were air dried and packed into paper bands so that they did not touch each other. The blocks of slides were then wrapped in paper and kept in sealed plastic packs. In the laboratory the blood films were stained in a 10% working solution of a commercially purchased stock solution of Giemsa's stain, pH 7.0–7.2, at 18–20 C for 1 hr. All blood films were stained between 5 and 30 days after their fixation. Details of preparation and staining of blood films were described by Valkiūnas (2005).

Approximately 50 μl of whole blood were drawn from each bird. For subsequent molecular analysis, the samples from European birds were fixed in SET-buffer (Hellgren et al., 2004), and those from Africa and California were fixed in lysis buffer (Sehgal et al., 2001). The samples were held at ambient temperature in the field and later at −20 C in the laboratory. The fixed samples were analyzed between 1 and 4 mo after their collection.

Examination of blood films and parasite morphology

A skilled parasitologist examined 1 blood film from each infected bird. Approximately 100 fields were examined at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000). The microscopy of each sample took 20–25 min. In total, the approximate number of screened red blood cells was 5×10^5 in each blood film. Intensity of infection was estimated as a percentage by counting the number of parasites per 10,000 erythrocytes examined, as recommended by Godfrey et al. (1987).

An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used for microscopic examination of blood films and preparation of illustrations. Only good quality slides—without any features of lysis of cells and well-stained blood cells and parasites—were used for microscopic examination (Figs. 1–4). To show differences in detectability of parasites in good and bad quality blood films, illustrations of the latter are provided from material that was deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania (Figs. 5–8).

The representative blood slides were deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania (accession nos. 45623–45626NS, 7272NS, 7445NS).

Extraction of DNA, PCR, sequencing, and statistical analysis

Processing of samples from Europe: The European samples were screened for species of *Haemoproteus* and *Plasmodium*. For total DNA extraction from blood, we used standard phenol-chloroform or ammonium-acetate protocols. For genetic analysis, a nested-PCR protocol was used (Waldenström et al., 2004). We amplified a segment of the parasite mitochondrial cyt *b* gene using the initial primers HaemFNI [5′-CAT ATATTAAGAGAAITATGGAG-3′] and HaemNR3 [5′-ATAGAAAG ATAAGAAATACCATTC-3′], which are general for species of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* and amplify a fragment, including primers, of 619 bp (Hellgren et al., 2004). For the second PCR, we used primers specific to *Haemoproteus* and *Plasmodium* spp., HaemF [5′-ATGGTGCTTTCGATATATGCATG-3′] and HaemR2 [5′-GCATTATCTGGATGTGATAATGGT-3′] (Bensch et al., 2000) that amplify a fragment of 525 bp. These primers are placed in conserved regions of the cytochrome *b* gene and appear to amplify approximately 95% of all *Haemoproteus* and *Plasmodium* lineages (data not shown). In Waldenström et al. (2004), it was shown that 3 repeated runs with these primers gave a repeatability of 99.85%; in experimental 10-fold dilution series, the detection limit was between 1 of 10^5–10^6 infected erythrocytes. Because the amplified fragments are rather long, the protocol requires good quality DNA (Freed and Cann, 2006). It also is worth noting that good quality DNA is essential for accurate estimates of prevalence when primers for shorter regions are used because the level of DNA degradation cannot be controlled and will always bias prevalence estimates downwards.

Both the first and the second PCR were carried out in a 25 μl volume and included 50 ng of total genomic DNA (first PCR) or 1-μl from the reaction with the primers HaemFNI/ HaemNR3 (second PCR), 1.5 mM $MgCl_2$, 1× PCR buffer, 1.25 mM of each deoxynucleoside triphosphate, 0.6 mM of each primer, and 0.5 units *Taq* DNA polymerase. The cycling profile consisted of an initial denaturation for 3 min at 94 C, 30 sec at 94 C, 30 sec at 50 C, 45 sec at 72 C for 20 (first PCR) or 35 (second PCR) cycles, followed by final extension at 72 C for 10 min. One negative control was used for every 8 samples to control for false amplification due to the high sensitivity of the nested PCR. In no case were any false positives obtained because all positives were sequenced.

For sequencing, we used procedures as described by Bensch et al. (2000). Fragments were sequenced from the 5′ end with the primer HaemF, and new lineages were sequenced from the 3′ end with the primer HaemR2. We used dye terminator cycling sequencing (big dye), and the samples where loaded on an ABI PRISM™ 3100 sequencing robot (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using the software BioEdit (Hall, 1999). We used the software MEGA, version 3.0 (Kumar et al., 2004), to generate a Neighbor-Joining tree with a Kimura 2-parameter distance matrix. Sequences were identified to the generic level of haemosporidians according to their clustering on the phylogenetic tree.

Processing of samples from Africa and California: To obtain total DNA, the blood was extracted following a DNeasy kit protocol (Qiagen®, Valencia, California). We used molecular protocols to confirm presence or absence of parasites for each of the 3 genera, *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*.

To test for *Leucocytozoon* spp., extracted DNA was used in a nested PCR reaction that amplifies the cyt *b* region of the mtDNA. The first round of amplification used the following primers developed by Perkins and Schall (2002): DW2: 5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′, and DW4: 5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′. The first PCR reaction was performed using the following conditions: 25 μl reaction mixtures contained 10–100 ng of genomic DNA (2 ml of template DNA), 0.5 units of Qiagen Taq DNA Polymerase (Qiagen), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl2, 0.4 mM of each primer, 0.4 mM of each dNTP, and 5 ml of Q buffer (Qiagen). The cycling profile consisted of an initial denaturation at 94 C for 3 min, followed by 35 cycles of 94 C denaturation for 30 sec, 52 C annealing for 30 sec, and 72 C extension for 1 min. The samples went through a final extension at 72 C for 10 min. The second PCR reaction used the first PCR product to seed the reaction instead of DNA template. The following primers for the second round were developed by Sehgal, Hull et al. (2006): Leuco Cyt F: 5′-TCT TAC TGG TGT ATT ATT AGC AAC-3′, and Leuco Cyt R: 5′-AGC ATA GAA TGT GCA AAT AAA CC-3′. The reaction conditions using the second primer set were identical to the first round and used a similar cycling profile with a 50 C annealing temperature.

For *Plasmodium* and *Haemoproteus* spp., we used the PCR method that amplifies the cyt *b* region of the mtDNA. This PCR reaction uses the same reaction conditions as Sehgal, Hull et al. (2006) with the following primers: L15183: 5′-GTG CAA CYG TTA TTA CTA ATT TAT A-3′ and H15730: 5′-CAT CCA ATC CAT AAT AAA GCA T-3′ (Fallon, Bermingham, et al., 2003; Szymanski and Lovette, 2005). The cycling profile consisted of an initial denaturing at 94 C for 3 min, followed by 35 cycles of 94 C for 50 sec, 53 C annealing for 50 sec, and 72 C extension for 60 sec, and then a final extension at 72 C for 5 min.

For the detection of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* spp., positive and negative controls were used. Positive controls were from birds with known infections evident from microscopy results, and the negative controls used purified water in place of DNA template. The PCR products were run out on a 1.8% agarose gel using 1×TBE and visualized by an ethidium bromide stain under ultraviolet light, and positive samples were sequenced as described above.

All of the DNA sequences were edited using Sequencher 3.1 (GeneCodes, Ann Arbor, Michigan) and MacClade 3.0 and 4.03 PPC (Maddison and Maddison, 2002). For distinguishing between *Plasmodium* and *Haemoproteus* spp., the sequences were compared to their closest sequence matches in GenBank using the NCBI nucleotide blast search and by confirmation through microscopy slides.

Obtained sequences were deposited in GenBank (Bensch et al., 2004; Križanauskienė et al., 2006; Sehgal, Hull et al., 2006; Sehgal, Valkiūnas et al., 2006; Valkiūnas et al., 2006; Bensch et al., 2007). Prevalences were compared by a Yates corrected chi-square test. A *P*-value of 0.05 or less was considered significant.

RESULTS

Haemosporidian parasites of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* were recorded (Figs. 1–4; Table I). Species of *Trypanosoma* (the overall prevalence of infection was 36.4%), *Hepatozoon* (0.6%), and microfilariae (0.4%) were also found during microscopic examination of blood films; they were not considered in this study because we used only PCR protocols that detect haemosporidians. Over 80% of recorded infections were light (<0.01%) at all study sites and can be classified as chronic.

There was no significant difference in the prevalence of *Haemoproteus* spp., *Plasmodium* spp., and *Leucocytozoon* spp. infections in the same samples tested by microscopy and PCR at each study site, and in the overall sample (Table I). The overall prevalence of infection determined by combining results of both these methods was higher than the overall prevalence determined by each of these methods separately, but the differences were insignificant. Importantly, both microscopy and PCR diagnostics showed similar prevalences for *Haemoproteus*, *Plasmosdium*, and *Leucocytozoon* spp. infections in the same samples at each study site and in the overall data set (Table I).

Compared to microscopic examination, the sensitivities of 2 nested PCR protocols for the detection of haemosporidian infections were similar.

DISCUSSION

The present study does not support the conclusions by Jarvi et al. (2002), Richards et al. (2002), or Durrant et al. (2006) regarding a much lower sensitivity of microscopy in comparison to the PCR-based methods in determining the prevalence of haemosporidian infections. These discrepancies are likely due to the following main shortcomings of microscopy methods, which were used in those studies.

Richards et al. (2002) used poor quality slides for microscopic examination in their study. The slides were stained several years after their preparation (R. N. M. Sehgal, pers. comm.). It is difficult to obtain a good quality of cell staining by Giemsa in such old material using traditional techniques (Garnham, 1966). The staining of blood films was pale, and corpuscles stained too blue, so the nuclei of parasites are poorly seen or even invisible. Additionally, numerous slides from this study contain hemolyzed blood cells, which testify to the insufficient desiccation, or fixation of blood films, or both, in the field. The conclusion of this paper, that PCR is more reliable than blood smear analysis for large-scale screening, reflects not the insensitivity of microscopy as such, but shows that microscopy of poor quality blood films is an unreliable method and should be discouraged. It is much easier to see parasites on good quality slides (compare Figs. 1–4 and 5–8).

To determine the prevalence of infection, Jarvi et al. (2002) screened approximately 50,000 red blood cells in each blood film with chronic parasitemia. This is enough to determine the intensity of infection (Godfrey et al., 1987) but is too small a number of the cells to determine the prevalence of infection, especially in light chronic infections (Garnham, 1966; Valkiūnas, 2005). Their conclusion that the PCR tests are 3- to 4-fold better than microscopy for detecting chronic infections can be explained by this shortcoming in their microscopy protocol.

The protocol for preparation, fixation, and staining of blood films was not described by Durrant et al. (2006). The quality of the slides was not good (R. C. Fleischer, pers. comm.). Furthermore, the authors examined only 20,000 red blood cells during this study, which was certainly not enough to determine

prevalence of infections, especially chronic light ones. In their study PCR-based techniques showed an approximately 10-fold difference in prevalence of *Haemoproteus* and *Plasmodium* spp. infections, with PCR-based techniques being much better. This discrepancy is in part due to shortcomings of their microscopy protocol.

We agree with Jarvi et al. (2002) that both microscopy and PCR screening slightly underestimate prevalence of infection of blood parasites in naturally infected birds. Combined results of both these methods usually show higher prevalence data (Table I). In our study the overall difference in prevalence of haemosporidian blood parasites, as determined by microscopy (53.6%) and PCR diagnostics (54.2%), was marginal. In some of our samples, microscopy was slightly more sensitive than PCR, and, in some samples, the opposite was true (Table I). However, in all samples, both these methods showed the same trends of prevalence; this is essential information for the investigation of patterns of the distribution of blood parasites in wildlife.

According to the present study, both microscopy and PCR-based detection methods underestimate approximately the same number of the patent infections of haemosporidian parasites (Table I). The main shortcoming of microscopic examination of blood films is the low sensitivity in determining exceptionally light infections (<0.001%) when just a few parasites are present in blood films. Such light parasitemias would be easily overlooked even with increased observation time. All PCR protocols need optimization and may not work similarly well across laboratories depending on DNA extraction methods, taq-DNA supplier, PCR instrumentation, etc. (Freed and Cann, 2003, 2006). Also, applying more than 1 PCR protocol, targeting shorter fragments and more conserved gene regions, might further increase the sensitivity (Fallon, Ricklefs et al., 2003). The main shortcoming of the current nested PCR-based protocol as implemented in our laboratories is a limited sensitivity in reading simultaneous infections of haemosporidian parasites, especially parasites belonging to the same genus or closely related genera, *Haemoproteus* spp. and *Plasmodium* spp. This is mainly because amplification is often highly selective during simultaneous infections; it is sometimes not related to the intensity of parasitemia with a particular parasite (Valkiūnas et al., 2006). To determine the true species composition of haemosporidian parasites in each individual host, PCR diagnostics will require improvement, such as using lineage specific primers and quantitative PCR (Zethindjiev et al., 2008). A combination of microscopy approaches and PCR-based methods is recommended for development of such protocols. A better understanding of this group of parasites currently requires a comparison and synthesis of microscopy and molecular data.

PCR-based methods are particularly attractive because they provide sequence information for phylogenetic and epidemiologic studies of parasites and for the diagnosis of parasitic disease (Jarvi et al., 2002; Perkins and Schall, 2002; Bensch et al., 2004; Ricklefs et al., 2004; Kimura et al., 2006; Križanauskienė et al. 2006; Martinsen et al., 2006; Sehgal, Valkiūnas et al., 2006; Palinauskas et al., 2007; Perkins et al., 2007). A problem with PCR, but unlikely with microscopy, is the risk of false positives. We tried to minimize this by running 1 blank per 7 samples; however, rare false positives can still go undetected. Hence, microscopy will provide a hard lower limit for prevalence estimates. Microscopy also has a number of advantages in comparison to the current PCR-based techniques in detecting infections of blood parasites in naturally infected birds. First, microscopy is an inexpensive investigation method, which is generally available in any laboratory; moreover, technical staff can be easily trained for the routine screening of blood films. Second, microscopy provides an opportunity to determine or verify the identity and intensity of infections, and with PCR this is especially difficult to do with simultaneous infections of closely related species of parasites. Presently, this is particularly important with the rapid accumulation of sequence data that should be linked with data of traditional taxonomy (Hellgren et al., 2007; Palinauskas et al., 2007). It is important to note that simultaneous infections of haemosporidians belonging to the same and different genera and subgenera are common and, in some bird species, have been recorded in over 80% of all samples that were positive by microscopic examination of blood films (Valkiūnas et al., 2003). Third, despite the relatively long time requirement for microscopy of each sample (20–25 min during this study), such examination provides opportunities for simultaneous determination and verification of taxonomically different parasites: numerous species of *Haemoproteus*, *Plasmodium*, *Leucocytozoon*, *Trypanosoma*, *Atoxoplasma*, *Hepatozoon*, *Babesia*, microfilariae, and others (Bishop and Bennett, 1992). Presently, different PCR protocols must be used for the detection of parasites belonging to different genera, and even some different species of the same genus (Valkiūnas et al., 2006); this is expensive and also time consuming.

It is important to note that blood films, which are used for microscopic examination, should be of good quality (see Figs. 1–4): they should be thin, clean from artifacts, properly fixed, and adequately stained. Unfortunately, the quality of blood films that are used for microscopy has never been addressed in recent molecular studies of avian blood parasites, but it is essential for reliable prevalence results obtained using this method (Cooper and Anwar, 2001).

Preparation of good quality blood films is cheap and easy. However, even small errors in standard techniques can render films entirely unsuitable for examination. Several errors in the preparation of blood films are common (Hewitt, 1940; Shute and Maryon, 1960; Garnham, 1966; Cooper and Anwar, 2001; Valkiūnas, 2005). First, contamination by dust or flies, exposure to strong sunlight, and excessive heat or humidity frequently spoil blood films. Second, slow drying of blood films (>30 sec), which usually is the case in humid climates, leads to rapid changes of morphology of mature gametocytes of haemosporidians due to the onset of gametogenesis. Identification of haemosporidian species usually is difficult, or even impossible, in such material. Battery-operated fans can be used to aid in the drying of the blood films during field studies. Third, the use of nonabsolute methanol for fixation leads to hemolysis of blood cells, usually during the subsequent staining of blood films. Methanol should be kept in hermetically sealed bottles and not reused if frequently exposed to air in humid environments. Fourth, delay in staining of blood films after their preparation can lead to problems. Ideally, blood films should be stained soon after their fixation. However, this is often difficult to do during field studies. It is possible to achieve the satisfactory staining if the slides are stained 1–2 mo after their fixation. After that time, the blood films become alkaline due to exposure of air and humidity, and, as a result, the cells stain too blue

with Giemsa. Our experience shows that good staining quality cannot be achieved if staining of fixed slides is carried out later than 3 mo after their preparation (Valkiūnas, 2005); it is usually unsatisfactory after 6 mo (G. Valkiūnas, unpubl. obs.). If there is no opportunity to stain blood films soon after fixation, they can be stored by placing them in plastic hermetic boxes with a desiccating material, and keeping them in a refrigerator. Such blood films stained well even after approximately a year or more.

It is important to note that the length of time for scanning blood films for scientific research hardly can be standardized because different examiners have different experience, skills, and commitment to quality work. Microscopic examination of blood films for approximately 20–25 min, as described above, gives good results in detecting the prevalence of avian haemosporidians in comparison to the PCR-based methods (Table I). We have been using this microscopy protocol for a long time (Valkiūnas and Iezhova, 2001); we recommended its use during microscopic screening of blood films for blood parasites of vertebrates, especially in wildlife, where the diversity of haematozoa on the species level frequently is unknown, making microscopy as a tool especially valuable.

Jarvi et al. (2002) recommended the use of a combination of approaches for the reliable diagnostics of blood parasites in wildlife. These would ideally include examination of blood smears, PCR tests, and suitable serological methods. We agree. It is important to note that both microscopy and PCR diagnostics are insensitive in detecting latent infections, when blood parasites are absent from the peripheral circulation (Jarvi et al., 2002). These tools provide better results in the detection of blood parasites when the majority of infections are patent. That takes place in different seasons of the year at different latitudes but usually coincides with the maximum breeding period of birds (Valkiūnas, 2005) when parasitemias are relatively high and the transmission of infection to juvenile birds takes place. For a better understanding of the distribution of haemosporidians in wildlife using microscopy and PCR tools, we recommend collecting blood samples during the period of the active transmission of blood parasites.

In conclusion, both microscopic examination of blood films and nested PCR-based diagnostics show similar level of prevalence of infection of blood parasites in naturally infected birds. Thus, microscopy is a reliable method in field studies if blood films are of good quality and are examined properly by skilled investigators. In each study using PCR for prevalence estimates, it is essential to verify the quality of the DNA and optimize the protocols (Freed and Cann, 2003, 2006), and checking the PCR results for false negatives and positives is best done in concert with microscopy on the same samples. We thus recommend the continued use of optical microscopy in the research of haemosporidian parasites of vertebrates. This tool provides important information how molecular methods can be further improved and most effectively applied, especially in the field studies of parasites. This conclusion is particularly important for researchers in developing countries, where the use of molecular techniques in field studies has been limited because of the high costs of the molecular reagents and equipment.

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