Haemoparasites of the pied flycatcher: inter-population variation in the prevalence and community composition

Anna Dubiec[1], Edyta Podmokła[2], Iga Harnist[1] and Tomasz D. Mazgajski[1]

[1]Museum and Institute of Zoology, Polish Academy of Sciences, Wilcza 64, 00-679 Warszawa, Poland and [2]Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

Abstract

The prevalence and community composition of haemoparasites can substantially differ among avian host populations, which may lead to different selection pressures. Therefore, information about these parameters is crucial for understanding, e.g. the inter-population variation in host life history traits. Here, we molecularly screened a population of a long-distance migrant, the pied flycatcher *Ficedula hypoleuca*, from central Poland for the presence of three genera of blood parasites: *Haemoproteus*, *Plasmodium* and *Trypanosoma*. The infection rate in this population was the highest for haemosporidians (86·8%) and one of the highest for trypanosomes (39·7%) among the thus far screened breeding populations of this species. The haemosporidian community was composed of six *Haemoproteus/Plasmodium* lineages, and the trypanosome community – 4 species and a parasite assigned to genus level. Trypanosomes were dominated by *T. culicavium*, a recently described species, corroborating the prediction that insectivorous songbirds are vertebrate hosts of this parasite. Host sex and age did not explain variation in infection incidence except for the higher trypanosome infection rates in males. A comparison of the study population with three other breeding populations previously screened molecularly for haemosporidians showed some geographic differences. This study confirms the importance of examining local parasite communities across a host distribution range.

Introduction

Parasites are one of the most important factors exerting selection pressures in animal populations (Haldane, 1949). Generally, each species may act as a competent host for a very versatile and large community of parasites. However, only rarely do these communities show high similarity in terms of prevalence and diversity across the distribution range of the host (Freeman-Gallant *et al.* 2001; Poulin, 2003). Differences in parasite communities should translate into diverse selection pressures acting upon the host, and this in turn could result in variations of life-history traits among host populations. Consequently, in order to understand the role of parasites in shaping inter-population differences in fitness-associated traits, it is necessary to examine local parasite communities and investigate the patterns of variation in infection/infestation rates.

In birds, among the commonest parasites are vector-transmitted protozoans, which complete some stages of the life cycle in the blood of the vertebrate host (therefore, they are generally referred to as blood parasites or haemoparasites). This group includes, among others, parasites from the genera *Plasmodium*, *Haemoproteus* and *Trypanosoma* (Clark *et al.* 2009). Avian haemosporidians and trypanosomes are transmitted by blood-sucking arthropods. Specifically, *Plasmodium* is transmitted by mosquitoes (Culicidae), *Haemoproteus* by hippoboscid flies (Hippoboscidae) and biting midges (Ceratopogonidae) and *Trypanosoma* by black flies (Simuliidae), hippoboscid flies (Hippoboscidae), biting midges (Ceratopogonidae), *Culex* spp. mosquitoes (Culicidae) and dermanyssid mites (Dermanyssidae) (Baker, 1976; Molyneux, 1977; Votýpka *et al.* 2012). Despite being transmitted by the same type of vectors (blood-sucking arthropods) haemosporidians and trypanosomes differ in their transmission modes: haemosporidians enter the host organism through the bite of the vector, and trypansomes – through ingestion of the vector or contamination of abraded skin or conjunctiva with material containing infective parasite stages (Desser *et al.* 1975; Molyneux, 1977; Votýpka and Svobodová, 2004; Votýpka *et al.* 2012). Parasites from all three genera can negatively affect components of fitness in birds (Merino *et al.* 2000; Knowles *et al.* 2010; Martínez-de la Puente *et al.* 2010), although a number of studies failed to find such associations (Dufva, 1996; Siikamäki *et al.* 1997). The introduction at the beginning of the XXI century of molecular techniques for detection and identification of these parasites not only improved the detectability of infection (Hellgren *et al.* 2004; Waldenström *et al.* 2004), but also uncovered their extreme genetic diversity (Bensch *et al.* 2009). Importantly, by using a molecular approach, it was possible to show that genetic lineages identified as the same morphospecies may, in fact, represent separate biological entities (e.g. Bensch *et al.* 2004; Nilsson *et al.* 2016).

Examination of different populations of the same host species in the context of infection rates and diversity of blood parasites has shown that both parameters can vary geographically (Bensch and Åkesson, 2003; Pagenkopp *et al.* 2008; Szöllősi *et al.* 2011; Spurgin *et al.* 2012), even within a short distance (Sol *et al.* 2000; Ferrer *et al.* 2012). However, the geographic variation of these parameters is not a universal phenomenon. In some host species, it has not been found or was restricted to a subgroup of individuals (Swanson *et al.* 2014; Svoboda *et al.* 2015). For example, in the breeding populations of the migratory lark sparrow (*Chondestes grammacus*) located across the USA, geographic differences in the overall prevalence were present only in juvenile birds, but not in the adults (Swanson *et al.* 2014). Similarly, adult bluethroats (*Luscinia svecica*) sampled in seven populations ranging in location from central to northern Europe did not differ in overall infection rates (Svoboda *et al.* 2015).

In the present study, we molecularly examine the prevalence and community composition of parasites from the *Haemoproteus*, *Plasmodium* and *Trypanosoma* genera in a population of the pied flycatcher (*Ficedula hypoleuca*) located in central Poland. The pied flycatcher is a long-distance migrant which breeds in most of Europe and western Asia and winters in West Africa (Cramp and Perrins, 1993; Ouwehand *et al.* 2016). It is a model species in the evolutionary ecology of cavity-nesting long-distance migrants with several closely monitored populations located across its breeding range (e.g. Laaksonen *et al.* 2015; Ouwehand *et al.* 2016). To date, several studies looked into the community of blood parasites in this species, but most of them were based on blood smear screening (Bennett *et al.* 1994; Dale *et al.* 1996; Siikamäki *et al.* 1997; Sanz *et al.* 2001*a*) and consequently may have underestimated infection rates and the diversity of these parasites (Fallon *et al.* 2003; Garamszegi, 2010; Nilsson *et al.* 2016). Importantly, this is one of the few studies molecularly examining avian trypanosomes. In contrast to haemosporidian parasites, infections with *Trypanosoma* have been largely understudied in birds, although such infections may have serious consequences for a host's fitness, including the study species (Rätti *et al.* 1993; Sanz *et al.* 2001*a*; Dyrcz *et al.* 2005, also see Siikamäki *et al.* 1997; Sanz *et al.* 2001*b*), and are known to widely occur in birds (Sehgal *et al.* 2001; Oakgrove *et al.* 2014). Additionally, we: (i) studied the relationship between infection incidence and two host traits commonly considered in the analyses of the presence of infection, namely sex and age (McCurdy *et al.* 1998; Deviche *et al.* 2001; van Oers *et al.* 2010), and (ii) investigated geographic variation in the prevalence and diversity of haemosporidian parasites (*Haemoproteus* and *Plasmodium*) among four molecularly screened breeding populations of the pied flycatcher using data from the current and previously published studies (Wiersch *et al.* 2007; Kulma *et al.* 2013).

Materials and methods

Data were collected from a small population (up to 17 pairs per year) of nest box-breeding pied flycatchers in 2011–2014. The study site is located in Sękocin Stary, 10 km south-west of Warsaw's city borders, Poland (52°05′N, 20°52′E). All study plots except for one are located in a pine (*Pinus sylvestris*) forest with an admixture of deciduous trees [birch (*Betula* spp.), sessile (*Quercus petraea*) and pedunculate (*Quercus robur*) oaks and common hornbeam (*Carpinus betulus*)] with a moderate to dense understory. Nest boxes (for their dimensions and distribution see Dubiec and Mazgajski, 2013) are primarily occupied by great tits (*Parus major*), blue tits (*Cyanistes caeruleus*) and pied flycatchers, and occasionally by coal tits (*Periparus ater*). The number of nest boxes varied among breeding seasons, from 156 in 2011 to 259 in 2014. Pied flycatchers bred only in pine-dominated plots. We additionally monitored over 35 nest boxes, differing in size and the height of attachment, located in close vicinity to the study plots. Each year at least one pied flycatcher breeding attempt was recorded in these boxes.

Before each breeding season nest boxes were cleaned of remaining nest material and feces of roosting birds. Regular controls of boxes, in order to record phenological data and breeding parameters, started in the beginning of April. Adult birds were caught when the nestlings were 13 days old (hatching day = day 1) or older, either by mist-netting or using traps installed inside the box. Birds were ringed, sexed, aged, measured (data not shown) and sampled for blood. Sexing and ageing (as yearlings or at least 2 years old) were based on plumage characteristics following Svensson (1994). In the case of re-trapped birds, the assigned age was later verified based on ringing records from previous seasons. Blood, obtained by puncturing the brachial vein, was collected into non-heparinized capillaries placed in 96% ethanol. Until the molecular analyses were performed, blood samples were stored at ambient temperature. Because of the brood mortality at the early post-hatching stage or unsuccessful catching trials (mostly because the birds were not present in the vicinity of the box during a catching trial), a few individuals were not caught.

DNA was extracted using the standard ammonium acetate precipitation method (Bruford *et al.* 1998). DNA isolates were checked for concentration and purity (absorbance ratios: A260/A280 and A260/A230) using a NanoDrop spectrophotometer. Samples were screened for the presence of blood parasites: (1) from the *Haemoproteus* and *Plasmodium* genera by amplifying a 478 bp fragment of the mitochondrial cyt *b* gene, using the nested polymerase chain reaction (Waldenström *et al.* 2004; Cosgrove *et al.* 2008); (2) from the *Trypanosoma* genus by amplifying a 326 bp fragment of the small subunit ribosomal RNA (SSU rRNA) gene as described in Sehgal *et al.* (2001). Four μL of final PCR products were run on 2% agarose gels stained with GelRed (Biotium, Hayward, CA, USA) and visualized under ultraviolet light. Each plate contained a positive (DNA from individuals with confirmed infection based on blood smear screening) and negative (ddH$_2$O) control, to monitor for possible contamination or failures during PCRs. The quality of the DNA isolate of all samples yielding no PCR product was checked with sex-specific primers (Griffiths *et al.* 1998). PCR products of positive samples were purified with FastAP (Fermentas) and then sequenced directly (mostly unidirectionally, except for a subset of trypanosome PCR products, which were sequenced bidirectionally) with an automated ABI 3130 DNA analyser (Applied Biosystems) using BigDye terminator ver. 3.1 (Applied Biosystems). The obtained sequences were aligned using BioEdit software (Hall, 1999) and compared with the MalAvi database (Bensch *et al.* 2009) in the case of *Plasmodium* and *Haemoproteus* and with the GenBank database in the case of *Trypanosoma*.

In total, we screened 97 samples from 68 individuals. Twenty-one individuals were sampled more than once. To avoid the issue of pseudoreplication, only one record per multiply sampled individuals was selected for further analyses. The record was selected either randomly (most cases) or non-randomly when information was lacking (age of the individual) in the datasets collected in different seasons. In such cases, the record with the full set of data was chosen for statistical analyses. The final data set included 43 females and 25 males.

The association between occurrences of parasites representing different genera within a host was analysed with Fisher's exact test. To assess the association between the probability of infection (separately for each parasite genera) and sex and age of the host generalized linear mixed models (GLMM) with a binomial error distribution and a logit link function were used. The individual

age and sex were defined as fixed categorical variables, a year as a random variable and infection status (0 for absence and 1 for presence) as a response variable. The interaction between sex and age was introduced into the model, but removed if not significant. The model was run using a dataset reduced by four females, because these individuals were not aged (final data set: females – 18 yearlings and 21 older birds, males – 12 yearlings and 13 older birds). Because *Plasmodium* infections occurred at a low rate (see 'Results' section), the analyses of haemoparasite co-occurrence and age- and sex-related variation were limited to *Haemoproteus* and *Trypanosoma* infections. GLMM models were run using the glmer function in the package 'lme4' (Bates *et al.* 2015) in R (R Development Core Team, 2014) with the Laplace approximation of maximum likelihood (Bolker *et al.* 2009).

Geographic variation in the prevalence and ecological diversity of haemosporidian parasites among the breeding populations of the pied flycatcher was estimated for three previously molecularly screened populations – Lingen and Bahrdorf in Germany (sampling period: 1993 for Bahrdorf and 1996 for Lingen; Wiersch *et al.* 2007) and the island of Öland in Sweden (sampling period: 2004–2010, except 2008; Kulma *et al.* 2013) – and the population examined in the present study (Fig. 1). In the case of four mitochondrial sequences from German populations (one out of six sequences in Lingen and three out of four sequences in

Bahrdorf), it was not possible to assign them to specific lineages from the MalAvi database because only information about the GenBank entry most closely matching each of these sequences (the similarity was over 98% in all cases) was provided. These entries were used in the current study as a reference to assign the sequence to a parasite genus. Two ecological diversity indices were used: the species richness (S – the total number of detected lineages) and the Shannon index (H; combining information on species richness and relative abundance). The diversity indices were calculated in the 'vegan' package (Oksanen *et al.* 2016) in R, while the 95% confidence intervals (CIs) of the Shannon index and differences in those indexes between populations were calculated in the 'boot' package (Canty and Ripley, 2015) according to the recommendations of Gardener (2014). CIs were estimated based on 1000 bootstrap resamples (Custom R Command: 'H_boot' from Gardener, 2014). The statistical significance of the difference in diversity index between populations was estimated using a randomization method based on 2000 bootstrap resamples (Custom R Command: 'H_bss' from Gardener, 2014).

The phylogenetic analysis includes sequences of *Haemoproteus* and *Plasmodium* lineages from the present study and two other European breeding populations of pied flycatchers [Lingen (five sequences that could be matched with specific lineages from the MalAvi database) and Öland] as well as additional lineages found previously in this species (birds caught on breeding
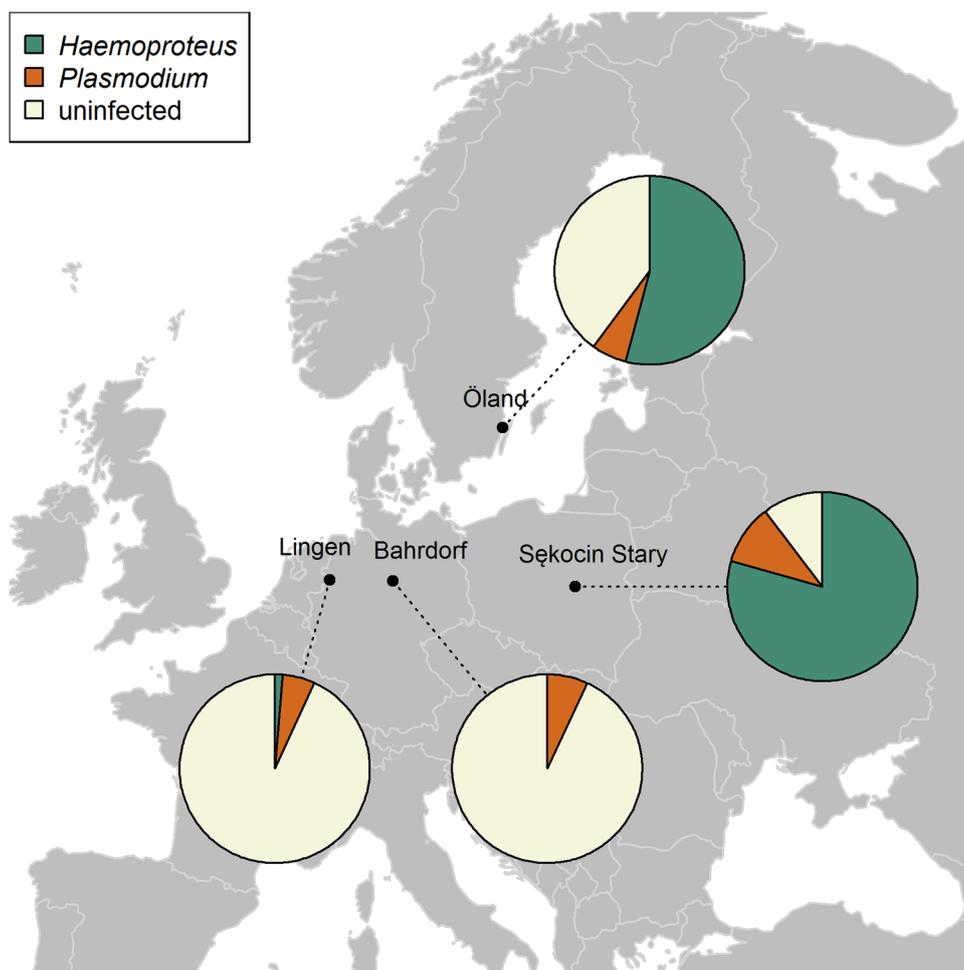

Fig. 1. Distribution and prevalence of *Plasmodium* and *Haemoproteus* parasites across four breeding populations of the pied flycatcher molecularly screened for the presence of parasites: Bahrdorf and Lingen in Germany (Wiersch *et al.* 2007), the island of Öland in Sweden (Kulma *et al.* 2013) and Sękocin Stary in Poland (present study). Pie charts show the parasite genus frequencies of each population. Sample sizes: $N_{Bahrdorf} = 72$; $N_{Lingen} = 147$; $N_{Öland} = 353$; $N_{Sekocin\ Stary} = 68$. Note that *Haemoproteus* infection rates recorded in Bahrdorf and Lingen populations may be underestimated as a consequence of the applied protocol (see 'Discussion' for details).

grounds either during or outside of the breeding season), which we extracted from the MalAvi database (version: Malavi8120153_04102016; Bensch *et al.* 2009). Bayesian reconstructions were carried out in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003), following a General Time Reversible model including invariable sites and variation among sites (GTR + I + G) suggested by the software jModelTest 2.1.10 (Darriba *et al.* 2012). The *Leucocytozoon* spp. lineage SISKIN2 was used as an outgroup and two separate runs of 10 million generations were conducted with a sampling frequency of every 100th generation. The burn-in fraction was set to 0·25.

Results

Infection rates of the study population of pied flycatchers varied from very frequent for *Haemoproteus* (79·4%), through moderate for *Trypanosoma* (39·7%) to rare for *Plasmodium* (8·8%). Parasites from the *Haemoproteus* and *Plasmodium* genera were represented by two and four cytochrome *b* lineages, respectively (Table 1). The most common lineage – PFC1, representing the morphospecies *H. pallidus* – was found in 96·3% of *Haemoproteus*-infected individuals. Other lineages occurred at a frequency of <5%, including two lineages detected only in a single individual each (Table 1B). Only two birds (2·9%) carried mixed haemosporidian infections: one individual with two *Haemoproteus* lineages and the other with *Haemoproteus* and *Plasmodium* lineages. In the case of *Trypanosoma* parasites, five types of parasite SSU rRNA sequences were detected: four assigned to a species level and one assigned to a genus level (Table 1C). The most common species – *T. culicavium*, occurred in 59·2% of *Trypanosoma*-infected individuals. Only one bird (1·5%) carried a mixed (double) infection.

Among all screened individuals, 35·3% carried simultaneous infections of *Haemoproteus* and *Trypanosoma* parasites. Because of differences between males and females in *Trypanosoma* infection rates (see below), the analyses of the association between the occurrences of these two parasite genera within an individual host were performed separately for each sex. The occurrence of *Haemoproteus* was not associated with the occurrence of *Trypanosoma* either in males or females (Fisher's exact test, males: $P = 0.565$, females: $P = 0.290$).

The probability of carrying a *Haemoproteus* infection was not associated with either the sex or age of the host (sex: males – 88·0%, females – 69·2%, age: yearlings – 83·3%, older birds – 70·6%; Table 2). In the case of *Trypanosoma*, the probability of infection varied in relation to the host's sex, with males being more frequently infected than females (sex: males – 56·0%, females – 30·8%, age: yearlings – 40·0%, older birds – 41·2%; Table 2).

The prevalence of haemosporidian infections varied among four breeding populations of the pied flycatcher, with the lowest incidence of infection in the two German populations, intermediate in Öland and the highest in the population from central Poland ($\chi^2 = 213.81$, df = 3, $P < 0.001$, Table 3). Also, the proportion of infections caused by each parasite genus differed among populations (Fisher's exact test, $P < 0.001$). In general, infections in the two German populations were dominated by *Plasmodium*, while in the two other populations, by *Haemoproteus* (Fig. 1, Table 3). Lineage richness was the highest in the Öland population with over twice as many lineages as in each of the three other populations (Table 3), while the Shannon index was over two times lower in Sękocin Stary than in the three other populations (Table 3). However, only the difference between the Sękocin Stary and Öland populations was statistically significant (Öland *vs* Sękocin Stary: z = 5·79, $P < 0.001$; Lingen *vs* Sękocin Stary: z = 1·92, $P = 0.08$; Lingen *vs* Öland: z = 0·82, $P = 0.42$; Lingen *vs* Bahrdorf: z = 0·66, $P = 0.53$; Öland *vs* Bahrdorf: z = 1·53, $P = 0.14$; Sękocin Stary *vs* Bahrdorf: z = 0·51, $P = 0.62$). Among 18 lineages detected in Lingen, Öland and Sękocin Stary, only one (pSGS1) was common for all populations, while 66·7% of the lineages occurred only in a single population (Fig. 2).

Lineages included in the phylogenetic analysis (18 lineages from Lingen, Öland, Sękocin Stary and five additional lineages detected in pied flycatchers on breeding grounds, Fig. 2) form two major clades of haemosporidian parasites: *Haemoproteus* and *Plasmodium*. The lineages found in all three breeding populations are dispersed in the clades and do not form monophyletic groups.

Table 1. The community of blood parasites [genera *Haemoproteus* (A), *Plasmodium* (B) and *Trypanosoma* (C)] in the pied flycatcher population from central Poland (Sękocin Stary) in 2011–2014 ($n = 68$)

Parasite taxon	Lineage	GenBank accession no.	$N_{occurring}$	Prevalence, %
(A)				
Haemoproteus pallidus	PFC1	JX026906	52	76·5
Haemoproteus majoris	PHSIB1	AF495565	2	2·9
(B)				
Plasmodium ashfordi	GRW02	AF254962	1	1·5
Plasmodium sp.	GRW09	FJ404701	3	4·4
Plasmodium sp.	PLOPRIO1	FJ404702	1	1·5
Plasmodium relictum	SGS1	AF495571	2	2·9
(C)				
Trypanosoma avium		AY099320	2	2·9
Trypanosoma bennetti		JF778738	5	7·4
Trypanosoma corvi		JN006854	4	5·9
Trypanosoma culicavium		HQ107967	16	23·5
Trypanosoma sp.		JN006850	1	1·5

The dataset includes only one record per individual for birds sampled more than once. The prevalence of each parasite taxon is presented as the percentage of individuals harbouring infection (either as a single or as a multiple infection) with a given taxon.

Table 2. The association between host's sex and age and the probability of infection with parasites from the *Haemoproteus* and *Trypanosoma* genera

	Predictor	Coefficient	S.E.	z	P
Haemoproteus	Sex	1.19	0.71	1.67	0.094
	Age	−0.75	0.63	−1.19	0.236
Trypanosoma	Sex	1.12	0.57	1.98	**0.048**
	Age	0.07	0.54	0.13	0.897

Data were analysed with generalized linear mixed models (GLMM) with sex and age as categorical explanatory variables and study year as a random variable (effect not shown). Non-significant interactions were removed from the model. Significant terms ($P < 0.05$) are shown in bold.

Table 3. Prevalence and diversity of haemosporidian parasites in four molecularly screened breeding populations of the pied flycatcher: Bahrdorf and Lingen in Germany (Wiersch *et al.* 2007), the island of Öland in Sweden (Kulma *et al.* 2013) and Sękocin Stary in Poland (present study)

Population	N	N_{inf}	Prevalence *Plasmodium*	Prevalence *Haemoproteus*	Species richness (*Plasmodium*, *Haemoproteus*)	Shannon index (asymptotic 95% CIs)
Bahrdorf	72	5	5 (6·9%)	0	4 (4, 0)	1.332 (0.50–1.33)
Lingen	147	10	8 (5·4%)	2 (1·4%)	6 (5, 1)	1.696 (0.94–1.75)
Öland	353[a]	216[b]	21 (5·9%)	191 (54·1%)	14 (10, 4)	1.473 (1.29–1.58)
Sękocin Stary	68	59[c]	7 (10·3%)	53 (77·9%)	6 (4, 2)	0.643 (0.31–0.92)

N and N_{inf} refer to the number of screened samples/individuals and the number of samples/individuals positive for the presence of a parasite.
[a]Includes multiple samples from the same individuals, the total number of screened individuals: 319.
[b]Includes four individuals with unidentified infection.
[c]Includes two individuals with mixed infections: hPFC1/pSGS1 and hPFC1/hPHSIB1.

Discussion

The incidence of infection with haemosporidians and trypanosomes in a population of the pied flycatcher located in central Poland is one of the highest reported so far for this species. Specifically, we show that the prevalence of infection with *Haemoproteus* and *Plasmodium* parasites reaches 86·8%, while infection with *Trypanosoma* – 39·7%. Previous studies in populations of this species screened either molecularly or using blood smears showed the prevalence range between 6.9 and 61·2% in the case of haemospiridians (Bennett *et al.* 1994; Dale *et al.* 1996; Siikamäki *et al.* 1997; Sanz *et al.* 2001*a*; Wiersch *et al.* 2007; Kulma *et al.* 2013) and between 1·1 and 40·6% in the case of trypanosomes (Bennett *et al.* 1994; Dale *et al.* 1996; Siikamäki *et al.* 1997; Sanz *et al.* 2001*a*). The detected haemosporidian prevalence is also much higher than the prevalence (estimated based on molecular screening) reported in two closely related species – the collared (*Ficedula albicollis*) and the semi-collared flycatchers (*Ficedula semitorquata*) (Szöllősi *et al.* 2008; Kulma *et al.* 2013; Peev *et al.* 2016).

The comparison of four pied flycatcher populations, which were screened molecularly for the presence of haemosporidian
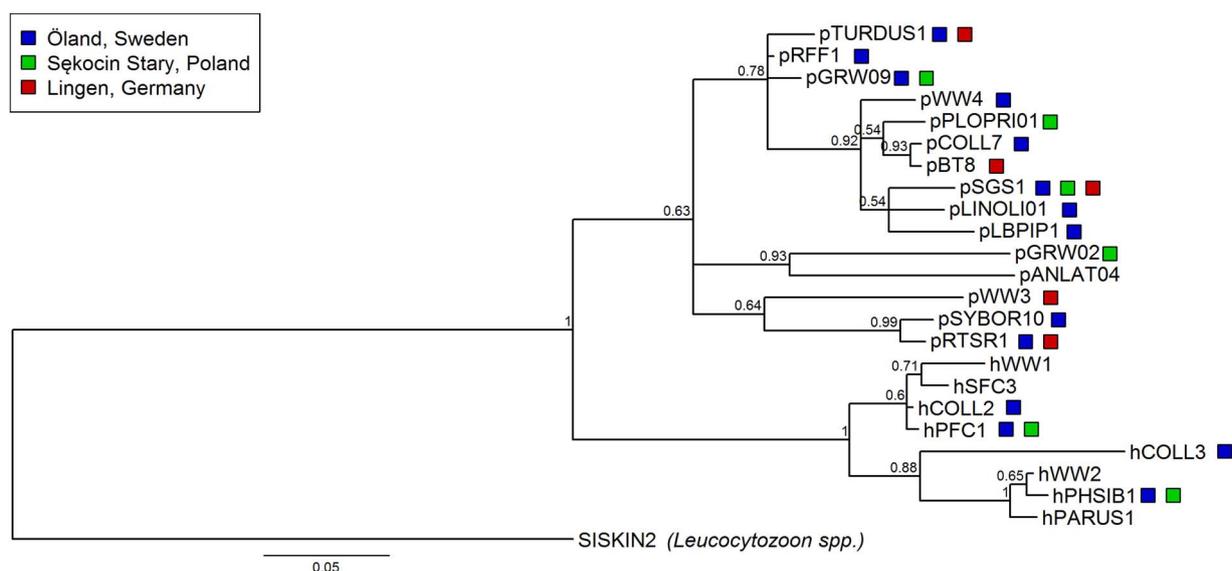

Fig. 2. Phylogenetic relationships of 23 parasite lineages detected in pied flycatchers (8 of *Haemoproteus* spp. and 15 of *Plasmodium* spp.) from the three analysed breeding populations [Lingen (Germany), Öland (Sweden) and Sękocin Stary (Poland); indicated with colour boxes] and previously found in this species (birds caught on breeding grounds either during or outside of the breeding season) extracted from the MalAvi database. In the case of the Lingen population one lineage was excluded from the analysis because, based on available information, it was not possible to match it with the lineage in the MalAvi database. The phylogenetic tree was constructed from partial cytochrome *b* sequences (479 bp) and rooted with *Leucocytozoon* spp. lineage SISKIN2. Nodal support values near branches indicate Bayesian posterior probabilities.

infections, showed some geographic differences both in the infection rate and the diversity of the haemosporidian community. Based on the overall prevalence and frequency of infection with *Plasmodium* and *Haemoproteus* parasites, four populations can be clustered into two groups: one containing both German populations (Wiersch *et al.* 2007), and the other containing the populations from Öland, Sweden (Kulma *et al.* 2013) and Sękocin Stary, Poland (present study). Populations from Bahrdorf and Lingen were characterized by a low prevalence (6·9%) and the dominance of *Plasmodium* infections, while populations from Öland and central Poland by a high prevalence (61.2–86·8%) and the dominance of *Haemoproteus* lineages. It has to be noted, however, that *Haemoproteus* infection rates recorded in two German populations, may be underestimated as a consequence of the applied protocol. In contrast to Kulma *et al.* (2013) and our study, which assessed infection status based solely on Waldenström *et al.* (2004) protocol, Wiersch *et al.* (2007) additionally carried out an initial screening and chose for the subsequent analysis only positive samples. Since the primer set used in this step, targeting 18 SSU rRNA gene fragment, presumably detects mostly *Plasmodium* specimens (Hulier *et al.* 1996), it may, in general, fail at amplification of the majority of *Haemoproteus* parasites. Because this specific primer set was only very occasionally used in avian haemosporidian studies (Ribeiro *et al.* 2005; Wiersch *et al.* 2007), it is very difficult to reliably assess the success rate of *Haemoproteus* detection with its use. Although Öland and Sękocin Stary populations are similar in some aspects, they differ in some indices of parasite diversity. For example, the Öland population is characterized by higher species richness (the total number of detected lineages) and ecological diversity (as described by the Shannon index). In the case of the *Haemoproteus* community a noticeable difference between these two populations is a fairly common occurrence of two lineages on Öland (PFC1 – 42·0%, PHSIB1 – 34·4%) and an almost complete dominance of one lineage in Sękocin Stary (PFC1 – 96·3%). The higher lineage richness reported for Öland may be at least partly associated with the much larger sample size, since the number of detected lineages increases with the number of screened individuals (Neto *et al.* 2015). In general, the geographic variation of parasite infection rates and diversity indicates that pied flycatchers from different breeding populations may be exposed to different parasite-mediated selection pressures.

Observed differences in the composition of haemosporidian communities as well as in the incidence of infection in geographically distant populations of the same host species might result from various ecological, climatic and population factors, which affect haemosporidian distribution directly or indirectly by shaping the abundance and diversity of vectors (e.g. Lachish *et al.* 2011; Krama *et al.* 2015). It is currently not possible to assess to what extent vectors drive the observed patterns because the distribution of vectors, both on wintering and breeding grounds, is poorly understood. That is especially true for *Haemoproteus* vectors.

The current study is one of the few molecularly characterizing the incidence and richness of trypanosomes in populations of a specific avian host, as opposed to avian guilds (e.g. Soares *et al.* 2016; Valkiūnas *et al.* 2016). The infection rate with trypanosomes in the population from central Poland is over three times higher than the estimates in most pied flycatcher populations screened up to date. Specifically, the incidence of infection varied between 1·1% in south-eastern Sweden, 12–16·2% in central Finland, 15% in south-eastern Norway, 16% in central Spain and 40·6% in northern Finland (Bennett *et al.* 1994; Dale *et al.* 1996; Siikamäki *et al.* 1997; Sanz *et al.* 2001*a*). Because the aforementioned prevalences were derived from blood smear screening, they may be underestimated in comparison with PCR-based

diagnostics, which have been shown to be generally more sensitive in detecting *Trypanosoma* (Sehgal *et al.* 2001; Nagata, 2006, but see Valkiūnas *et al.* 2016). Moreover, actual prevalences, regardless of the technique used (smear- or PCR-based diagnostics using blood), may be underestimated because in infected birds *Trypanosoma* parasites are abundant in the bone marrow, while they usually occur at very low densities or may be absent in the peripheral blood (Apanius, 1991).

Trypanosome infections in the study population were dominated by *T. culicavium* – a recently described novel species of avian trypanosomes, which is transmitted by mosquitoes (subgenera *Culex* and *Barraudius*) (Votýpka *et al.* 2012). Votýpka *et al.* (2012), based on experimentally identified transmission mode (ingestion of infected vectors) and detection of the parasite in two individuals of the collared flycatcher, suggested that insectivorous songbirds are natural vertebrate hosts of this species. By demonstrating the detection of this trypanosome in another insectivorous bird species, our study confirms this statement. Information about the diet of adult pied flycatchers is very limited, but available data indicate that they ingest Diptera, although at a much lower frequency than other prey (mainly Hymenoptera and Coleoptera) (Bibby and Green, 1980; Silverin and Andersson, 1984). So far, *T. culicavium* has only been detected in European mosquitoes (Votýpka *et al.* 2012; Svobodová *et al.* 2015*a*). Among the three other detected trypanosome species, *T. corvi* [vector: hippoboscid flies (Mungomba *et al.* 1989; Votýpka *et al.* 2004) has been shown to be transmitted in Europe (Zídková *et al.* 2012)], *T. avium* [vector: blackflies (Bennett, 1961; Votýpka and Svobodová, 2004)] – both in Europe and Africa (Svobodová *et al.* 2015*b*; Valkiūnas *et al.* 2016), while *T. bennetti* (vectors currently unknown) – in Europe (Zídková *et al.* 2012). However, since information about the vectors and occurrence of trypanosome species in birds is currently very limited, the areas of transmission of these parasites are not definite.

The incidence of infection with *Haemoproteus* and *Trypanosoma* in the study population was unrelated. In birds, the correlated occurrence of these parasites has been found in some [a positive association in white-winged crossbills (*Loxia leucoptera*)] (Deviche *et al.* 2010), but not in other studies (Oakgrove *et al.* 2014; Svobodová *et al.* 2015*a*). In general, a non-independent incidence of infection may occur if, e.g. infection with one parasite causes immunosuppression allowing the other parasite to successfully invade and develop in the host (positive association) or if infection with one parasite prevents the development of the other parasite *via* cross-immunity (negative association) (Pedersen and Fenton, 2007).

We also looked at each parasite genus separately to search for any relationship between infection incidence and two host traits: age and sex. Despite the fact that susceptibility to parasitic infection is known to increase with age as new infections accumulate during life (Deviche *et al.* 2001; Podmokła *et al.* 2014), we did not find such associations. In the case of the host sex, we found that males were more prone to be infected with *Trypanosoma* than females, while there was no difference between sexes in the probability of infection with *Haemoproteus*. Sex differences in parasite prevalence are generally being attributed to an unequal exposure of the sexes to vectors (van Oers *et al.* 2010; Egizi *et al.* 2014) and differences between males and females in the immune-endocrine system (Schuurs and Verheul, 1990).

In conclusion, by using the molecular approach we showed that the pied flycatcher population from central Poland exhibits one of the highest infection rates with haemoparasites across the breeding range of this species. The comparison of molecularly screened breeding populations revealed some geographic differences in haemosporidian communities, which indicates that pied flycatchers from different populations may experience

diverse parasite-mediated selection pressures. However, to be able to confidently make such a conclusion, more thorough study is needed, including of populations with balanced and synchronized sampling, conditions which were not met by the current study. The latter condition means that samples should be collected in the same years as well as at the same point in a breeding period, since both factors are known to be associated with changes in the prevalence and/or composition of the parasite community (annual variation: Bensch and Åkesson, 2003; Bensch *et al.* 2007; Knowles *et al.* 2011, but see Pagenkopp *et al.* 2008; variation within a season: Szöllősi *et al.* 2016, Dubiec *et al.* 2017).

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