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Detecting shifts of transmission areas in avian blood parasites — a phylogenetic approach

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Abstract

We investigated the degree of geographical shifts of transmission areas of vector-borne avian blood parasites (*Plasmodium*, *Haemoproteus* and *Leucocytozoon*) over ecological and evolutionary timescales. Of 259 different parasite lineages obtained from 5886 screened birds sampled in Europe and Africa, only two lineages were confirmed to have current transmission in resident bird species in both geographical areas. We used a phylogenetic approach to show that parasites belonging to the genera *Haemoproteus* and *Leucocytozoon* rarely change transmission area and that these parasites are restricted to one resident bird fauna over a long evolutionary time span and are not freely spread between the continents with the help of migratory birds. Lineages of the genus *Plasmodium* seem more freely spread between the continents. We suggest that such a reduced transmission barrier of *Plasmodium* parasites is caused by their higher tendency to infect migratory bird species, which might facilitate shifting of transmission area. Although vector-borne parasites of these genera apparently can shift between a tropical and a temperate transmission area and these areas are linked with an immense amount of annual bird migration, our data suggest that novel introductions of these parasites into resident bird faunas are rather rare evolutionary events.

Keywords: bird-migration, *Haemoproteus*, *Leucocytozoon*, PCR, *Plasmodium*, transmission area

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Introduction

To what extent can parasites or microorganisms use the migratory routes of their hosts to shift or extend their transmission areas? Annually, billions of birds migrate from their breeding areas in temperate regions of the Northern Hemisphere to their wintering areas in the tropics (Alerstam 1990). Along with these migrants parasites and microorganisms may hitch-hike, thereby potentially being spread to new areas and hosts along the migratory flyways (Smith *et al*. 1996; Mackenzie *et al*. 2004; Ishiguro *et al*. 2005; Ricklefs *et al*. 2005; Olsen *et al*. 2006). When parasites are introduced into new host species or faunas they can have devastating impact on population sizes and viability of the naïve host populations, as illustrated by the introduction of a globally distributed avian *Plasmodium* parasite (Beadell *et al*. 2006) to the Hawaiian Islands, which apparently has caused the extinction of several native bird species (Van Riper III *et al*. 1986). Although this introduction was presumably caused by human interference, the fate of the Hawaiian birds illustrates the potentially severe negative impact that novel parasite introductions may have on natural populations. Therefore, it is important to ask the question how common are such parasite introductions in ecologically common host faunas that are interconnected by migratory birds?

In this study, we investigate how common shifts have been in host-faunas and changes of transmission areas over ecological and evolutionary timescales in three genera of

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avian blood parasites occurring in the Palaearctic-African bird migration system. Blood parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are all transmitted by dipteran vectors and have cosmopolitan distributions (Valkiunas 2005). Traditionally these parasites have been investigated on blood films using microscopic examination and species have been identified using morphological cues. Of the around 200 morphological identified parasite species (morpho-species), about half have been found to occur in two or more of the five biogeographical zones (Holarctic, Ethiopian, Oriental, Australian or Neotropic) (data from Valkiunas 2005). However, recent molecular data show that evolutionary independent entities can be identified below the level of morpho-species (Bensch *et al*. 2004) and that these can have their own independent ecological properties (Perez-Tris & Bensch 2005; Reullier *et al*. 2006). Analyses carried out at the level of morpho-species might therefore be less useful, as a morpho-species may consist of different cryptic species, many with unique associations to hosts and vectors.

The recent discovery of cryptic diversity in hamosporidian parasites challenges the common idea that migratory birds may facilitate the spread of blood parasites between different areas and faunas of resident bird communities. Although parasites belonging to the same morpho-species are known to be transmitted over broad geographical areas, the various genetic lineages that form such morpho-species could be much more localized. Here we use sequence-based information from a large collection of malaria-like parasites from Europe and Africa to investigate the movement of parasites between different transmission areas. The aim is to investigate the occurrence of parasite introduction over ecological and evolutionary timescales.

In this system, two extreme scenarios are possible:

1 Shifts in ecological time; both geographical areas are functionally one transmission area where the parasites can use migratory birds to move freely between the two bird communities.
2 Shifts in evolutionary time; introduction events between the different bird faunas are rare and can be detected mainly over evolutionary time.

We investigated the validity of these two scenarios in our system. In an ecological time frame, we investigate to what extent resident and migrant birds share the same parasite and whether it is common that the same molecular parasite lineages are found among resident bird species at both ends of a migratory route. If so, parasites may be freely transmitted with the help of the migratory hosts. In an evolutionary time frame, we examine the interchange of parasite faunas between Africa and Europe using a phylogenetic approach. If the exchange of parasites between regions is rarer than the within region rate of lineage diversification, there should be a phylogenetic signal of transmission area. This suggestion has been made earlier by Waldenström *et al*. (2002) who found indications that African and European transmitted lineages were separated into different phylogenetical clades. We test this hypothesis using a phylogenetic approach on a large data set. A strong phylogenetic signal of transmission area would mean that molecular parasite lineages are restricted to a specific transmission area over a time span long enough to generate diversification events, that is, shifts in transmission areas mainly occurs on an evolutionary timescale.

Method

Sampling of parasite lineages

We screened 5886 blood samples from birds caught in Europe or sub-Saharan Africa using polymerase chain reaction (PCR) methods for detection of parasite mtDNA. These samples yielded 2477 cases of haemosporidian infections from 139 different bird species. The birds were trapped at various localities in 16 different countries (13 in Europe and 3 in sub-Saharan Africa) between 1993 and 2005 (for detailed geographical description of sampling sites see appendix). The sampling areas in Africa and Europe are interconnected with the Western European-Western Africa flyways (in the case of the sampling areas, Nigeria, Cameroon and Gabon), which is one of the main migratory bird routes for west European bird species (Moreau 1972; Alerstam 1990). Some of the most common migratory species used in this study, have been ringed at or around the sampling locations and later retrapped in the same geographical regions where the sampling sites at the opposite area of the migratory route were located (Pettersson *et al*. 1986; Zink 1987, 1995; Ottenby bird observatory, unpublished). All European birds were identified to species according to Svensson (1992), and resident African bird species according to Borrow & Demey (2001). We divided the investigated species into three ecological classes: (i) European resident species, for birds that stay year around within the continent; (ii) African resident species, for birds that are resident in sub-Saharan Africa, and (iii) migrant species, for all birds that make seasonal movements between breeding areas in Europe and nonbreeding areas in sub-Saharan Africa. The occurrence of different lineages within these sets of birds were used to locate the probable transmission area of each parasite lineage to take place either in Africa, in Europe or in both continents (see below). Of the screened birds, 427 individuals (of which 184 were infected) belonged to African resident species, 1143 (626 infected) belonged to European resident species, and the remaining 3916 individuals (1663 infected) comprised of migrant bird species. Of the migrants 543 individuals (180 infected)

were sampled in nonbreeding areas in Africa and 3373 individuals in Europe (1483 infected). Furthermore, we compared our sequences with a data set from Cameroon and Gabon, consisting of 527 individuals from 93 African resident bird species and sequenced for the same cytochrome *b* (cyt *b*) region (Beadell 2006; Beadell, unpublished). From this data set we could determine that five lineages previously only found in migratory bird species have transmission in resident African species (see also appendix).

Parasites were detected from blood samples using recently developed molecular methods (Bensch *et al*. 2000; Hellgren *et al*. 2004; Waldenström *et al*. 2004). In short, the DNA from the avian blood samples were extracted by phenol–chloroform or ammonium acetate protocols, and screened for the presence of parasite mtDNA using primer pairs designed to amplify *Plasmodium*, *Haemoproteus* and *Leucocytozoon* parasites using one of three similar methods that all target the same region of the cyt *b* gene (Bensch *et al*. 2000; Hellgren *et al*. 2004; Waldenström *et al*. 2004). We used negative controls, one for each eighth sample, to detect false PCR amplifications; in the very few cases, we got presence of false-positives the whole PCR-batch was re-run from the beginning until no false-positives were detected. The three methods we used differ slightly; one consists of a standard PCR while the other two methods are based on nested PCR protocols, and hence they differ in their sensitivity of detecting parasite DNA. However, as no prevalence data are analysed in this study the choice of PCR method does not affect the analyses. All samples were screened for *Plasmodium* and *Haemoproteus*, while only a portion of the sample (1746 individuals) were screened also for *Leucocytozoon*. Amplified PCR products were sequenced using the Amplicycle sequence kit on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems), following the manufacturer's recommendations. The different methods all obtained a fragment of the same 479-bp region of cyt *b* gene on the mitochondria.

Phylogenetic reconstruction

All obtained sequences together with formerly identified lineages (Bensch *et al*. 2000; Hellgren *et al*. 2004; Hellgren 2005) were used to construct a neighbour-joining tree with a Kimura 2-parameter distance matrix under a gamma distribution of 0.758 obtained from MODELTEST version 3.7 (Posada & Crandall 1998) using the software MEGA 3.1 (Kumar *et al*. 2004). Lineages were unambiguously assigned to parasite genera by their position in the neighbour-joining tree (Fig. 1). Bayesian phylogenetic reconstructions were performed separately for each genus using the software MRBAYES version 3.1 (Ronquist & Huelsenbeck 2003) and a model of evolutionary change suggested by the software MRMODELTEST version 2.2 (Nylander 2004). The phylogenies of *Haemoproteus* and *Plasmodium* were rooted using a *Leucocytozoon* sequence (L-SYBOR7′ YDQ897238) and the phylogeny of *Leucocytozoon* was rooted using a sequence of *Haemoproteus* (H-RB1′ YDQ060768). Each bayesian reconstruction were sampled every 500 steps over 20 million generations, using one cold and four hot Markov Coupled Monte Carlo–chains. The temperature for the chains were set to 0.1 after low observed rate of change between the chains using the default setting of 0.2 during test runs. The runs were allowed six different rates of substitutions where a portion of the site is invariable and the rest are drawn from a gamma distribution (similar to the GTR + I + G model) as suggested by the software MRMODELTEST version 2.2 (Nylander 2004). Of the 40 000 obtained trees, 10 000 (25%) were discarded as burn-in period after visualizing the runs with the software TRACER (Rambaut, A. & Drummond, A., available at http://evolve.zoo.ox.ac.uk) and AWTY (Wilgenbusch *et al*. 2004). The remaining 30 000 trees were used to construct majority-rule trees and were visualized using MEGA 3.1 (Kumar *et al*. 2004).

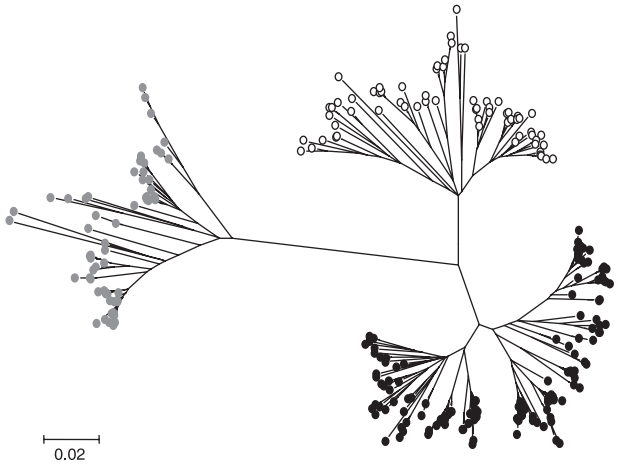

Fig. 1 Neighbour-joining tree of all found lineages based on 479 bp of the cytochrome *b* gene. Clusters belonging to either of the genera are separated with bootstrap values between 100 and 87% (*Leucocytozoon* vs. *Plasmodium* and *Haemoproteus* 100%, *Haemoproteus* vs. *Plasmodium* and *Leucocytozoon* 96%, *Plasmodium* vs. *Haemoproteus* and *Leucocytozoon* 87%). Black circles denote *Haemoproteus* lineages, white circles *Plasmodium* lineages and grey circles *Leucocytozoon*.

Phylogenetic signal of transmission area

On the constructed phylogenies of each parasite genus, we assigned if a lineage was transmitted in the resident bird fauna of Africa, Europe or both on the basis of occurrence in resident bird species within the data set (Fig. 2a–c). For the nodes in the trees, we noted how many lineages with known transmission area originated from that node and whether all the lineages were transmitted in the same area

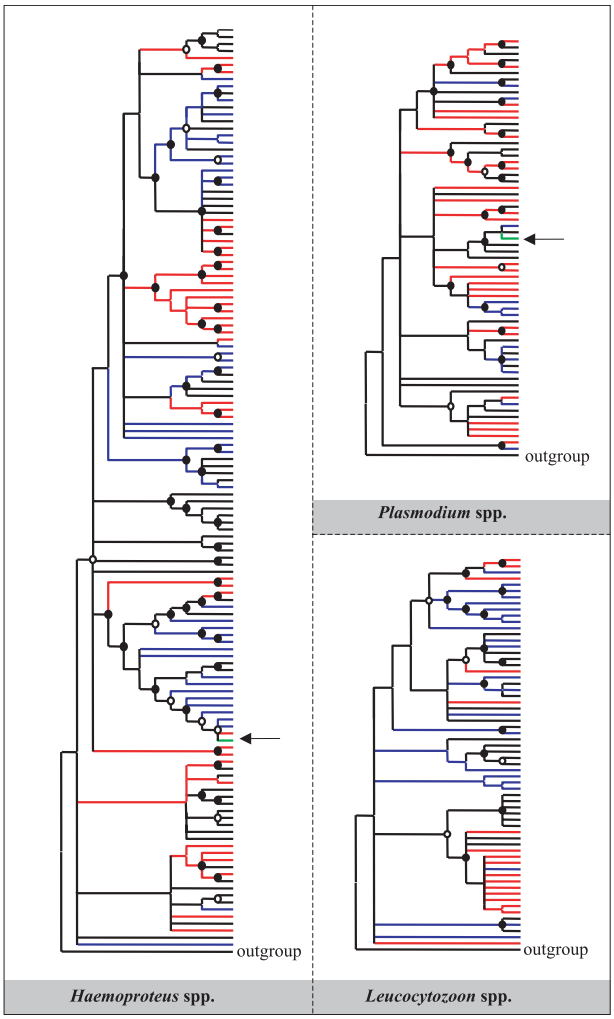

Fig. 2 Majority rule trees constructed from bayesian phylogenetic inference for each of the genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*. In each of the tree, lineages are marked with red if they have been found in resident African host species, blue if they have been found in resident European host species, green and marked with an arrow if they had been found in resident host species both in Africa and Europe and black if the lineage have not been found in resident species either in Africa or Europe. We present consensus trees constructed from 30 000 trees sampled every 500 generation over 20 million generations. Filled circles denote nodes supported with a posterior probability > 90. Open circles denote nodes supported with a posterior probability between 80 and 89.

or not. We used nodes regardless of node support, because if lineages had been assigned incorrectly in the phylogeny this would then only introduce noise in the data and reduce our chances to observe a phylogenetic effect of transmission area. To reduce problems with pseudo-replication, but still being able to use informative nodes, we based the tests only on those nodes that had the ability to change the character state. In other words, going from the leaves in the phylogeny towards the deeper nodes, we only included nodes when a change in character state was possible (i.e. discarding the nodes deeper in the phylogeny than those that for the time containing lineages from both transmission areas). These data were tested against the expected probability given the distribution of the data set. From the node data, we then conducted logistic regressions. With the use of the logistic regression, we calculated the probability that closely related lineages shared transmission area and compared this probability with the expected probability calculated from randomly sample lineages in the data set. A significantly higher probability would mean that lineages have been confined to the same area over diversification events. A similar approach has previously been used to determine the phylogenetic signal of host fidelity (Ricklefs & Fallon 2002; Beadell *et al*. 2004). The obtained slopes and intercepts from the logistic regression were used to calculate the probability curve for the outcome that all lineages originating from one node in the tree were found on the same transmission area. The observed probability of all lineages having the same transmission area ($P_{(obs)}$) was calculated using the logistic model;

$$\ln[P_{(obs)}/1 - P_{(obs)}] = a + b * n;$$
$$\{\text{i.e. } P_{(obs)} = \exp(a + bn)/[1 + \exp(a + b * n)]\},$$

where a is the intercept and b is the slope obtained from the logistic regression. By using the cluster sizes in the analysis instead of phylogenetic depth we could compensate for skewed ratios in our phylogenies (the ratio of African/European lineages, e.g. having a ratio of 95 African/5 European lineages would give a very high probability of closely related lineages being transmitted at the same area just by chance) and that the expected probability that all lineages in a cluster are transmitted at the same area is not equal at all depths, or cluster sizes in the phylogeny. The expected probability of all lineages originating from a node to be transmitted at the same area is not equal over the phylogeny if transmission areas were to be plotted randomly at the tips of the phylogeny. This is because the probability is forced to zero when the number of leaves in a cluster is higher than the number of lineages in the largest group of transmission area. For example if five lineages are originating from a node in a phylogeny that contains four lineages transmitted in Africa and four lineages transmitted in Europe, that node must contain lineages from the two different areas, thus the probability must be zero. To compensate for this, we calculated the negative 95% confidence interval (–95% C.I.) from the obtained probability [$P_{(obs)}$] and tested this against the expected probability [P_n(tot)] given the distribution of our sample (Fig. 3a–c). The expected probability was calculated for the given distribution of lineages with different transmission areas in the different phylogenies using the hypergeometric probability function;

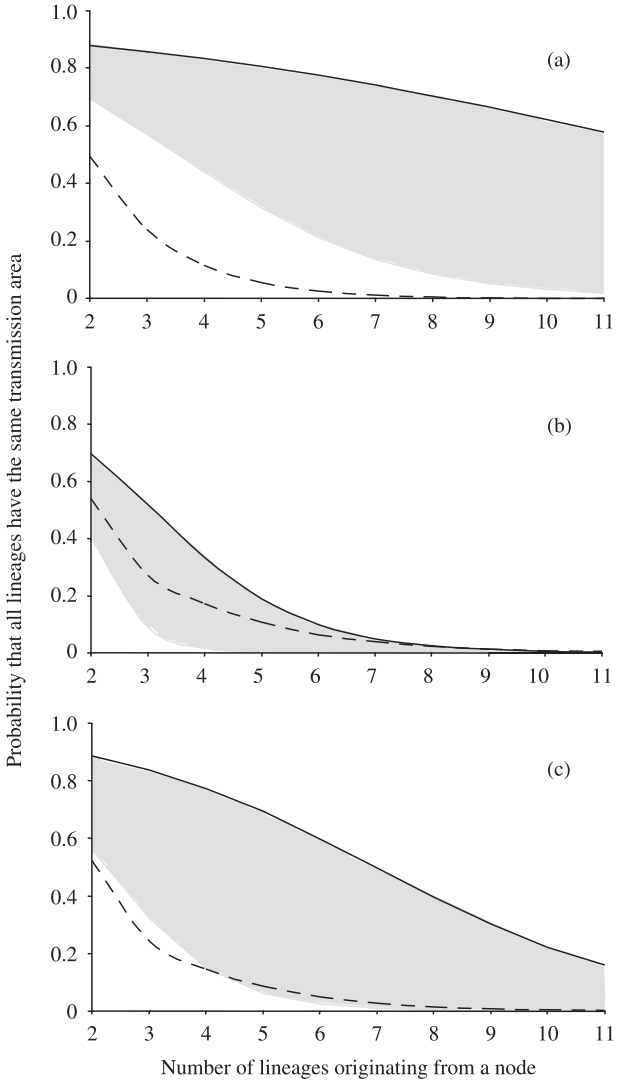

Fig. 3 Curves representing the probability whether all lineages originating in a certain node in the phylogenies are found in resident birds in the same area. (a) *Haemoproteus* lineages (Intercept = 1.97, S.E. = 0.59, Slope coefficient = –0.18, S.E. = 0.18). (b) *Plasmodium* lineages (Intercept = 0.84, S.E. = 0.63, Slope coefficient = –0.763, S.E. = 0.60). (c) *Leucocytozoon* lineages (Intercept = 2.1, S.E. = 0.93, Slope coefficient = –0.41, S.E. = 0.29). Observed probability curves (black solid lines) and the lower 95% negative C.I. (shaded area) are based on coefficients obtained from logistic regressions (see above). Broken lines indicate the expected probabilities given the actual sample distributions. A phylogenetic signal of transmission area is supported if the lower 95% confidence intervals of the observed probability curves are excluding the expected probability curve.

$$P_n(\text{tot}) = P_n(A) + P_n(A0);$$

$$P_n(A) = \frac{\begin{bmatrix} D \\ k \end{bmatrix}\begin{bmatrix} (N\text{-}D) \\ (n\text{-}k) \end{bmatrix}}{\begin{bmatrix} N \\ n \end{bmatrix}} \quad P_n(A0) = \frac{\begin{bmatrix} D \\ 0 \end{bmatrix}\begin{bmatrix} (N\text{-}D) \\ (n\text{-}0) \end{bmatrix}}{\begin{bmatrix} N \\ n \end{bmatrix}}$$

where $P_n(\text{tot})$ is the probability of drawing either only African transmitted or only European transmitted lineages with n tries. $P_n(A)$ is the probability of only drawing lineages transmitted on area A in a cluster of the size n and $P_n(A0)$ is the probability of drawing no lineages of transmission area A (i.e. drawing all from the opposite area) given the cluster size n [n = cluster size in $P_{(\text{obs})}$]. The number of total lineages in the phylogeny is represented with N, D is the total number of lineages transmitted at area A, n is the cluster size, k is the number of lineages from area A [in $P_n(A)$, $k = n$, as all lineages should be the same].

To get another independent test of phylogenetic signal, we calculated the probability that the number of sister-lineage clades with similar transmission areas could have occurred by chance in the obtained phylogenies, given the distribution of lineages with different transmission areas. This was carried out by randomly constructing the same amount of sister-lineage clades as found in the different phylogenies, using the obtained distribution of African and European transmitted lineages. The simulation ran over 10 000 generations and the probability was calculated as the average times the same or more cases of area homogeneic clusters occurred compared to the real data set.

To estimate how long lineages have been confined to the same transmission area we calculated the maximum genetic distance between lineages in well-supported clades (posterior probability > 90) that were geographically homogenous. This was carried out for all three genera separately. The genetic distances were calculated using Jukes-Cantor distances, with equal substitution rate. We noted the minimum, maximum and median distances for geographical-homogenous clusters in each genus.

The ability of a parasite to infect both a resident and a migratory bird species could affect the dispersal potential of the parasite. Hence, we tested if parasites from the three genera showed different properties in whether they were only found in resident species or also in migratory bird species. This was carried out by comparing the proportion of lineages only found in resident bird species vs. lineages found both in resident and migratory bird species between the different genera using a chi-square test.

All statistical tests were carried out using SPSS version 12.0.1.

Results

Observed transmission areas

Of the total of 259 lineages of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* only two were observed in resident bird species in both Africa and Europe. The first case is the *Haemoproteus* lineage H-SYAT1, which is frequently found in resident (and migratory) populations of blackcaps, *Sylvia atricapilla* in Europe (Perez-Tris & Bensch 2005) that was also found in the African hill-babbler *Sylvia abyssinica*,

a related species resident in Tanzania. The second case is the widespread *Plasmodium* lineage P-SGS1, which has been found in five resident species in Europe (robin, *Erithacus rubecula;* great tit, *Parus major;* house sparrow, *Passer domesticus;* blackcap, *Sylvia atricapilla;* and Sardinian warbler, *Sylvia melanocephala*) as well as in three resident species in Africa (Sudan golden sparrow, *Passer luteus;* black scrub-robin, *Cercotrichas podobe;* and cinnamon-breasted rock bunting, *Emberiza tahapisi,* all occurring in Nigeria). The lineage SGS1 was also found in 13 species of intercontinental migrants (see appendix).

A total of 131 *Haemoproteus* lineages were obtained from 1582 individuals of 66 host species. Of these, 37 lineages were found in African resident species, 40 in European resident species, one in both African and European resident species (H-SYAT1), 47 in only intercontinental migrant species and six lineages have only been found in areas outside Africa and Europe (e.g. Asia) and are not used in further analysis. In *Haemoproteus*, 33 lineages (25%) were found in more than one bird species.

The 65 *Plasmodium* lineages were obtained from 618 individuals of 59 host species. Of these, 11 lineages were found in European resident species, 25 in African resident species, one lineage in both African and European resident species (P-SGS1), and 27 only in intercontinental migrant species. One lineage was found only in a resident species in Asia. In *Plasmodium*, 30 lineages (46%) were found in more than one bird species.

A total of 63 *Leucocytozoon* lineages were obtained from 334 infected individuals of 41 host species. Of these, 17 lineages were found in African resident species, 22 in European resident species, and 24 were found only in intercontinental migrant species. In *Leucocytozoon*, 17 lineages (27%) were found in more than one bird species. See appendix for number of individuals screened in each bird species.

Phylogenetic reconstruction and evolutionary signal of transmission area

Using a logistic regression approach, we detected a significant phylogenetic signal of area-restricted transmission among *Haemoproteus* and *Leucocytozoon,* whereas no such signal was detected for *Plasmodium* lineages (Fig. 3a–c). For *Haemoproteus* the observed probability that all lineages in a cluster were transmitted within the same area was significantly higher than expected throughout the depth of the investigated phylogeny [i.e. for all n, $P_n(\text{tot}) < P_{\text{obs}}$ (–95% C.I.), Fig. 3(a)]. Lineages of *Leucocytozoon* showed a significant phylogenetic signal at the 'tips' of the phylogeny but the signal was lost as clades became larger, 'deeper' in the phylogeny [i.e. for $n \leq 3.94$, $P_n(\text{tot}) < P_{\text{obs}}$ (–95% C.I.), Fig. 3(c)]. For *Plasmodium* the observed negative 95% C.I. probability was not separated from the expected [i.e. for all n, $P_n(\text{tot}) > P_{\text{obs}}$(–95% C.I.), Fig. 3(b)]. Hence, *Plasmodium* showed no significant signal in the phylogeny of being geographically restricted.

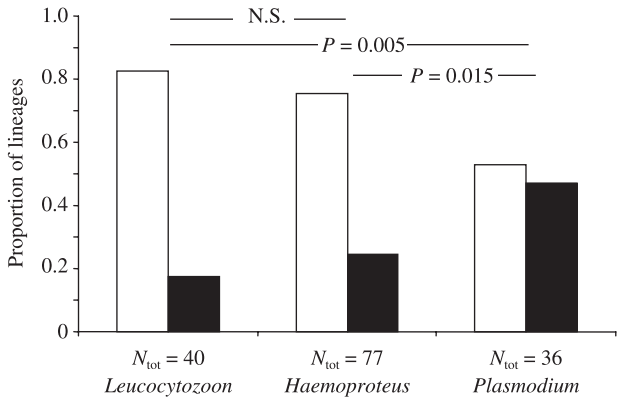

Fig. 4 Proportions of lineages found in resident bird species only vs. lineages found in both resident and migratory bird species, for *Haemoproteus*, *Leucocytozoon* and *Plasmodium*. White, proportion of lineages only found in resident bird species. Black, proportion of lineage found in both resident and migratory bird species. *Plasmodium* showed a higher proportion of lineages that were found both in resident and migratory species than *Haemoproteus* and *Leucocytozoon* (χ^2_2; $P = 0.01$).

The simulation of sister lineage clades gave a low probability that the number of observed area-homogenous clusters had occurred by chance in *Haemoproteus* (15 out of 18; $P < 0.0001$) and *Leucocytozoon* (six out of six; $P = 0.02$), whereas for *Plasmodium* the number of obtained area-homogenous clusters could have occurred by chance (6 out of 10; $P = 0.65$). This further strengthens the occurrence of a phylogenetic signal of transmission area for *Haemoproteus* spp. and *Leucocytozoon* spp.

The maximum genetic distance in geographically homogenous-clades ranged between 0.2% and 4.1% (median 0.6%) in *Haemoproteus*, between 0.2% and 5.8% (median 2.2%) for *Leucocytozoon* and between 0.2% and 4.5% (median 0.5%) for *Plasmodium*.

The migratory birds

The proportion of lineages only found in resident bird species vs. lineages found in both resident and migratory bird species was significantly different between the genera when testing the three genera together ($\chi^2_2 = 9.1$, $P = 0.01$). The different genera were then tested separately against each other. *Plasmodium* had a significantly higher proportion of lineages found in both resident and migrant birds than either *Haemoproteus* ($\chi^2_1 = 5.74$, $P = 0.017$) or *Leucocytozoon* ($\chi^2_1 = 7.7$, $P = 0.005$). Between *Haemoproteus* and *Leucocytozoon* there was no significant difference ($\chi^2_1 = 0.38$, $P = 0.48$) (Fig. 4 and Table 1 for exact number of lineages). As the exact same individual birds were tested

Table 1 Number of lineages found in the different combination of resident and migratory bird species. For example, the first row marked with an X in the Resident Africa column, show that 28 (or 22.4%) *Haemoproteus* lineages have been found in resident African bird species. The second row is thus representing the number of lineages that have been found both in resident African birds species and in intercontinental migratory bird species caught in Africa.

	Resident		Migrant			
Genus	Africa	Europe	Africa	Europe	Juvenile migrant Europe	Number of lineages
Haemoproteus	X					28 (22.4%)
	X		X			1 (0.8%)
	X			X		2 (1.6%)
	X		X	X		5 (4%)
	X		X	X	X	1 (0.8%)
		X				30 (24%)
		X		X		3 (2.4%)
		X		X	X	2 (1.6%)
		X	X	X		2 (1.6%)
		X	X	X	X	3 (2.4%)
	X	X				1 (0.8%)
			X			10 (8%)
				X		26 (20.8%)
				X	X	2 (1.6%)
					X	2 (1.6%)
			X	X		4 (3.2%)
			X	X	X	3 (2.4%)
	X	X	X	X	X	0 (0%)
Other geographical areas						6
Plasmodium	X					13 (20.3%)
	X		X			2 (3.1%)
	X			X		6 (9.4%)
	X		X	X		4 (6.3%)
	X		X	X	X	0 (0%)
		X				6 (9.4%)
		X		X		1 (1.6%)
		X		X	X	2 (3.1%)
		X	X	X		1 (1.6%)
		X	X	X	X	1 (1.6%)
	X	X				0 (0%)
			X			5 (7.8%)
				X		19 (29.7%)
				X	X	2 (3.1%)
					X	0 (0%)
			X	X		1 (1.6%)
			X	X	X	0 (0%)
	X	X	X	X	X	1 (1.6%)
Other geographical areas						1
Leucocytozoon	X					15 (23.8%)
	X		X			2 (3.2%)
	X			X		0 (0%)
	X		X	X		0 (0%)
	X		X	X	X	0 (0%)
		X				17 (27.0%)
		X		X		4 (6.3%)
		X		X	X	0 (0%)
		X	X	X		0 (0%)
		X	X	X	X	1 (1.6%)
	X	X				0 (0%)
			X			6 (9.5%)
				X		13 (21%)
				X	X	1 (1.6%)
					X	0 (0%)
			X	X		3 (4.8%)
			X	X	X	1 (1.6%)
	X	X	X	X	X	0 (0%)

both for *Plasmodium* and *Haemoproteus* there should be no sampling differences that could bias this analysis. Hence, lineages belonging to the genera *Plasmodium* showed a higher degree of infecting both resident and migratory bird species compared to the genera *Haemoproteus* and likely also *Leucocytozoon*.

Discussion

Restrictions of dispersal in evolutionary time

Our data suggest that novel introductions of parasites into resident bird faunas are rare events and that dispersing from one biographical zone to another is a slow evolutionary process that leaves phylogenetic markings in the genera *Haemoproteus* and *Leucocytozoon*, but have occurred more frequently in the genus *Plasmodium*. This affiliation to transmission areas is retained despite the vast numbers of birds that perform annual migrations between Africa and Europe, and the rather high frequency of the migrating birds that are infected with Haematozoan blood infections (Rintamäki *et al*. 1998; Rintamäki *et al*. 1999; Waldenström *et al*. 2002; Scheuerlein & Ricklefs 2004).

Parasites of *Haemoproteus* and *Leucocytozoon* show significant affiliation to a single resident bird fauna (either in the European or African biogeographical zone) over a time span long enough to generate parasite diversification events. To calculate the time span over which parasites have been area restricted, we found that all such clades confined to a single area had diverged by more than 0.2% with maximum values between 4.1% and 5.8% for the different genera. Hence, with knowledge of the divergence rates we can roughly estimate how long the lineages have been affiliated within the same transmission area. Unfortunately, there is no generally accepted molecular clock for Haematozoan parasites, thus precluding more exact estimations of divergence time. However, Ricklefs & Fallon (2002) suggested that the divergence rate of *Haemoproteus* and *Plasmodium* cyt *b* genes was approximately three times slower than that of their bird hosts. The divergence rate for birds' cyt *b* is about 2% per million years (Lovette 2004), which would mean a divergence rate of 0.67% per million years for the cyt *b* gene of Haemosporidian parasites. An alternative estimate can be achieved by using the separation of *Plasmodium falciparum* found in humans and *Plasmodium reichenowi* in the chimpanzee, which have diverged by 3.3% at the cyt *b* gene since their host lineages split apart 4–4.75 million years ago (Escalante *et al*. 1998). This estimate would give a divergence rate of 0.83–0.69% per million years for haemosporidian parasites. Hence, these two methods suggest similar estimates of divergence rates that are slower than their avian hosts. Based on these estimates of the divergence rate we find that all geographically homogenous clusters would have been homogenous for about 0.25–0.31 million years. In all of the three genera, the oldest homogenous clusters would have been confined to the same fauna for more than 5 million years. Note that parasite divergence time based on host divergence time may be underestimated if the parasite has shifted host after the diversification event took place. However, even if we have made a 25-fold underestimation of the divergence rate we still are in a time frame where all supported homologous clusters have been confined to the same area for at least 10 000 years.

Natural shifts of host fauna

Several adaptations are required for vector-borne parasites to shift host fauna under natural conditions. First, the parasite must be able to complete its development in a vector that comes in contact with migratory bird species. The parasite must then be able to infect and become adjusted to the new host species (in this case the migratory bird) and to complete its development in this new environment. After being transported within the migratory bird, the parasite must find and be able to be transmitted by a vector species in its new area. The abiotic factors differ profoundly between sub-Saharan Africa and temperate regions of Europe, including factors such as temperature, humidity and day length. Finally, the parasite must then do yet another host-shift from the migratory bird to a resident bird species at the new transmission area to complete the geographical shift. In the present study, we have only identified two cases (one in *Haemoproteus* and one in *Plasmodium*) where the required series of adaptations allowing transmission in resident bird faunas of both Europe and Africa have occurred within a time span short enough to preclude divergence in cyt *b* of the parasites. However, more cases have undoubtedly occurred during evolutionary time as can be traced in the phylogenies of all three genera, represented by well-supported clades of parasites that include related lineages transmitted both in Africa and Europe (Fig. 2a–c).

Distribution vs. transmission area

Our large-scale study of European and African birds clearly suggests that the previously held view that many Haemosporidian parasite species had large (global) distributions (in terms of where distribution and transmission area were thought to coincide) is incorrect. Largely, this conclusion is based on new information gathered through the possibility of identifying cryptic entities with the use of molecular methods and that even very small molecular differentiations seems to be linked with novel ecological properties of the parasites, such as dispersal patterns, transmission areas and host preference (Perez-Tris & Bensch 2005; Reullier *et al*. 2006). Our data

imply that lineages of *Haemoproteus* and *Leucocytozoon* have restricted transmission areas, although the distribution area might be larger than the transmission area. The former view was based on morphological identification alone. Lineages belonging to morphological distinct species of *Haemoproteus* have been found to acquire a molecular divergence of up to 2.7% without having obtained any noticeable morphological differentiation (Hellgren, Krizanauskiene, Valkiūnas and Bensch, unpublished). Applying the same estimates of divergence time as above, these lineages would have diverged 4 million years ago, but they are still morphologically indistinguishable. This gives the parasites time to shift transmission area, differentiate, become reproductively isolated and adapted to the new transmission area. As a result of the apparently much slower morphological differentiation, examinations of blood smears would group lineages with many very restricted transmission areas, giving the impression of one large transmission area.

The role of migratory birds as bridges between transmission areas

The finding that *Plasmodium* parasites more often infected both resident and migratory bird species than *Haemoproteus* and *Leucocytozoon*, together with previous studies that have also shown that *Plasmodium* exhibit lower degree of host fidelity compared to *Haemoproteus* (Ricklefs & Fallon 2002; Beadell *et al*. 2004) could potentially be one reason why we found no phylogenetic signal of transmission area in *Plasmodium*. Thus, lower host specificity might increase the chance for a parasite to spread between resident and migratory bird species thereby facilitating the transfer of parasites between resident bird faunas by means of migratory movements of birds.

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