Research

Ecography 38: 842–850, 2015
doi: 10.1111/ecog.01158

Subject Editor: Catherine Graham. Editor-in-Chief: Miguel Araújo. Accepted 23 October 2014

Global phylogeography of the avian malaria pathogen *Plasmodium relictum* based on MSP1 allelic diversity

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Knowing the genetic variation that occurs in pathogen populations and how it is distributed across geographical areas is essential to understand parasite epidemiology, local patterns of virulence, and evolution of host-resistance. In addition, it is important to identify populations of pathogens that are evolutionarily independent and thus 'free' to adapt to hosts and environments. Here, we investigated genetic variation in the globally distributed, highly invasive avian malaria parasite *Plasmodium relictum*, which has several distinctive mitochondrial haplotyps (cyt b lineages, SGS1, GRW11 and GRW4). The phylogeography of *P. relictum* was accessed using the highly variable nuclear gene merozoite surface protein 1 (MSP1), a gene linked to the invasion biology of the parasite. We show that the lineage GRW4 is evolutionarily independent of GRW11 and SGS1 whereas GRW11 and SGS1 share MSP1 alleles and thus suggesting the presence of two distinct species (GRW4 versus SGS1 and GRW11). Further, there were significant differences in the global distribution of MSP1 alleles with differences between GRW4 alleles in the New and the Old World. For SGS1, a lineage formerly believed to have both tropical and temperate transmission, there were clear differences in MSP1 alleles transmitted in tropical Africa compared to the temperate regions of Europe and Asia. Further, we highlight the occurrence of multiple MSP1 alleles in GRW4 isolates from the Hawaiian Islands, where the parasite has contributed to declines and extinctions of endemic forest birds since it was introduced. This study stresses the importance of multiple independent loci for understanding patterns of transmission and evolutionary independence across avian malaria parasites.

Parasites causing avian malaria (e.g. *Plasmodium* spp. and related haemosporidian parasites) have active transmission on all continents except Antarctica. To date, fewer than 60 *Plasmodium* species that infect birds have been described morphologically (Valkiunas 2005, Hellgren et al. 2007, Bensch et al. 2009), but over 500 (MalAvi database 15 May 2014, <http://mbio-serv2.mbioekol.lu.se/Malavi/>) lineages have been found by barcoding the parasites' mtDNA cytochrome b gene (cyt b) (Bensch et al. 2000, Hellgren et al. 2004, 2013a, Waldenström et al. 2004). Avian *Plasmodium* spp. and related haemosporidian parasites exhibit considerable variation in host specificity (Beadell et al. 2004, Reullier et al. 2006, Pérez-Tris et al. 2007, Hellgren et al. 2009, Loiseau et al. 2012), geographic range (Beadell et al. 2006, Hellgren et al. 2007, Wood et al. 2007, Bensch et al. 2009, Marzal et al. 2011), seasonal occurrence and relapses (Perez-Tris and Bensch 2005, Wood et al. 2007, Cosgrove et al. 2008, Hellgren et al. 2013b), and effects on host individuals (Marzal et al. 2005, Bensch et al. 2006, Hellgren et al. 2007, Palinauskas et al. 2008, 2011, Lachish et al. 2011). One of the most widespread species, *Plasmodium relictum,* is included in the IUCN list of 100 of the World's worst invasive species (Lowe et al. 2000) because of its devastating impacts on isolated island ecosystems (Van Riper et al. 1986), including the decline and extinction of many of Hawaii's endemic honeycreepers (Warner 1968, Samuel et al. 2011).

Plasmodium relictum is a morphologically defined species that includes several cyt b lineages. This species has an extremely broad transmission area, spanning all the continents but Antarctica (Bishop and Bennett 1992). Three of its cyt b lineages are particularly widespread (SGS1, GRW11, and GRW4) and have to date been found in individuals of 64 species in 21 families (SGS1), 28 species in 13 families (GRW11) and 59 species in 18 families (GRW4) (MalAvi database 15 May 2014, Bensch et al. 2009), respectively. As well as being extreme host generalists, these three lineages occur in both temperate and tropical regions (Fig. 1). One of the lineages, SGS1, has confirmed transmission in both tropical Africa and northern temperate areas of Europe (Hellgren et al. 2007, Marzal et al. 2011). No information exists on geographic or host specific genetic structuring among populations of these lineages because past technical difficulties have made it difficult to develop nuclear markers for haemosporidian parasites of birds. However, the introduction of high throughput sequencing has overcome some of these problems (Bensch et al. 2014).

One highly variable gene in the genome of the human malaria parasite *Plasmodium falciparum,* is the merozoite surface protein 1 (msp1, Noranate et al. 2009). In studies of primate *Plasmodium*, regions of this gene have been used to infer population structure and phylogeography (Noranate et al. 2009, Kang et al. 2012, Pacheco et al. 2012, Tanabe et al. 2013). The MSP1 gene encodes a 190 kDa protein that during erythrocytic schizogony (merogony) is anchored to the parasite's cell membrane (Gerold et al. 1996). Antibodies to the peptide are frequent in human populations with high malaria prevalence and can be associated with resistance to the parasite (Hui and Hashimoto 2007, Ngoundou-Landji et al. 2010). Because MSP1 is one of the major proteins that coats the parasite during its extracellular state and is associated with the initial attachment to host red blood cells, it is likely to interact with the immune system and parasite evasion mechanism of the host. Accordingly, MSP1 is expected to exhibit considerable geographic variation from coevolutionary interactions with the immune systems of host populations.

Here, we used a highly variable region of the *P. relictum* MSP1, block 14 (MSP1 b14) (Hellgren et al. 2013a), and a global sample of the lineages GRW4, GRW11, and SGS1 to gain insight into their phylogeography and to investigate 1) the extent of MSP1 variation within populations defined by the cyt b haplotypes, 2) the rate of evolution of MSP1b14 relative to cyt b, 3) gene flow or incomplete lineage sorting between these populations as evidenced by sharing of MSP1 alleles between mitochondrial lineages, and 4) global structuring of the MSP1 alleles.

Methods

Global distribution of *Plasmodium relictum* cyt b lineages

Transmission areas for the lineages SGS1, GRW11 and GRW4 were evaluated using data obtained from the MalAvi database together with data from Hellgren et al. (2007). Transmission was confirmed if the lineage had been found in either a resident bird species or a juvenile before migration. Presence of the lineages in Japan is based on their identification in blood-fed mosquitoes. Although infections can be

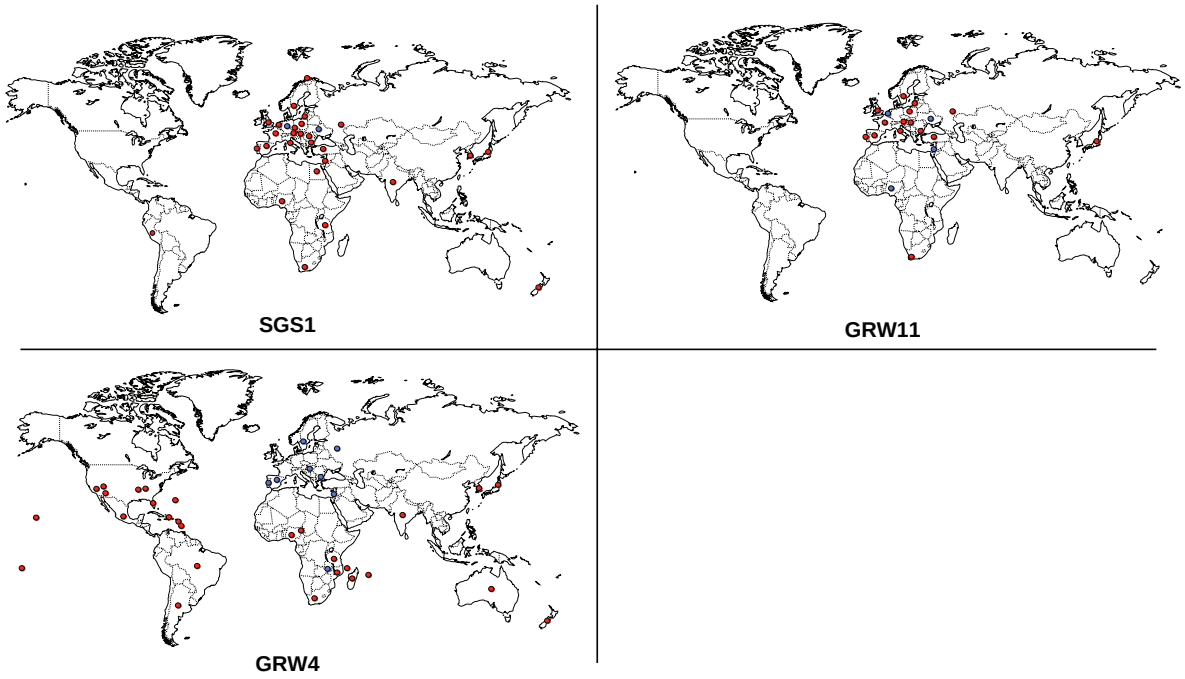

Figure 1. Global distribution of *Plasmodium relictum* cyt b lineages (SGS1, GRW11 and GRW4) obtained from the database MalAvi (15 May 2014). Red dots represent confirmed transmission events represented by the lineage being found in a resident bird species or in a juvenile individual before migration. Cases of transmission in Japan are based on the identification of the lineages in blood-fed mosquitoes. Blue dots represent cases where the lineages have been found in adult migratory birds with tropical wintering areas.

aborted at different stages in the mosquito and thus not indicate active transmission per se, the blood meals were shown to have been taken from resident bird species, indicating local transmission. Locations were plotted based on country or state (United States) (Fig. 1).

Molecular screening of variation in the MSP1 gene

Altogether 108 isolates with known infections of one of the *P. relictum* lineages SGS1, GRW11, or GRW4, originating from 21 different locations, were sequenced and evaluated for allel variation at the MSP1_b14 locus (Table 1). All samples had been collected in other studies and were known by prior screening to be infected with *P. relictum*. A nested protocol for MSP1_b14 was developed using outside primers MSP1_3F (5′-TAGAGCATTAAGTGAAACGGAAGA-3′) and MSP1_3R (5′-AGGAAGAAGTTTTTCATCCTGT GA-3′), according to Hellgren et al. (2013a) and a new set of internal primers MSP1_3FN 5′-GGTTCAGTTTCTT CTGATGTACCC-3′ and MSP1_3RN 5′-TGGTGAAT CTAATGATGCAAATGG-3′, which were developed by aligning the MSP1 lineages of GRW4 (GenBank; KC969176) with SGS1 (GenBank; KC969175). The first PCR reactions (MSP1_3F and MSP1_3R) were run according to Hellgren et al. 2013a. The second PCR reactions (MSP1_3FN/ MSP1_3RN) were run in 25 microliter reactions using 2 microliter of the first PCR product as a template with an annealing temperature of 54°C for 40 cycles. Both PCRs were run using Taq-Platinum (Invitrogen, Carlsbad, CA, USA). Positive amplification was confirmed as positive bands on a 2% agarose gel. The samples were sequenced using the primer MSP1_3FN. Sequences were aligned against the SGS1_MSP1 gene (KC969175) using the software Geneious ver. 6.1.6. Genetic differences between different MSP1 alleles and cyt b lineages was calculated using a Jukes–Cantor model as implemented in MEGA 5.2

Phylogenetic reconstruction

Phylogenetic reconstruction of the MSP1 alleles was done with MrBayes (Ronquist and Huelsenbeck 2003) as implemented in Geneious ver. 6.1.6. The homolog sequence of *P. gallinaceum* (AJ809338.1) was used as an out-group. A Bayesian phylogeny was constructed using 1 000 000 iterations and sampling every 200th tree under a GTR + invariable site model allowing for 6 rates of substitution. After discarding 25% of the sampled trees as a burn-in period, the remaining trees were used to construct a majority consensus tree. For visualisation of genetic mixing between the different *P. relictum* cyt b lineages, a Bayesian phylogeny was constructed using the same settings as above, including the 10 most closely related lineages to SGS1, GRW11, and GRW4 in MalAvi (Bensch et al. 2009).

Global structuring

Separation of populations was tested using an analysis of similarities (ANOSIM) as implemented in the vegan package in R (R Core Team). The ANOSIM detects differences between two or more sampling units by comparing between group dissimilarity with the mean of within group dissimilarity. All tests were done using a 'Bray–Curtis' dissimilarity matrix iterated over 10 000 times. First, we used individual information on both cyt b and MSP1 to test overall geographical dissimilarity of *P. relictum* types. Second, we tested separately for SGS1/GRW11 and GRW4 whether MSP1 alleles varied between sampling locations (i.e. North America, South America, Europe, Africa, Hawaii, and Japan). Due to the few samples obtained, New Zealand was not included. For sample sizes see Table 1.

Results

Global distribution of *Plasmodium relictum* cyt b lineages

Although transmission has been confirmed across a large range of climatic zones from tropical areas to the most northern parts of Scandinavia when considering all the cyt b lineages of *P. relictum* (Fig. 1), a more differentiated pattern emerges when global distribution is viewed for individual cyt b lineages (Fig. 1). GRW11 have so far not been recorded in the New World. SGS1 is transmitted among several different species at different locations from tropical Africa to northern Scandinavia, as well as New Zealand and recently SGS1 was also found in several resident bird species in Peru (Marzal et al. 2014). In contrast to SGS1, GRW11 appears to be restricted to temperate sites, with the exception of the recovery of GRW11 from a single resident species (the Cape bulbul *Pycnonotus capensis*) in South Africa (Fig. 1, Supplementary material Appendix 1).

GRW4 is distinct among the three lineages in having a more global distribution. However, unlike SGS1 and GRW11, GRW4 has only a single case of confirmed transmission in the temperate regions of the Old World (one infected bluetit *Cyanistes caeruleus* in Spain (Ferrer et al. 2012)), with the exception of Japan, where oocysts and sporozoites of GRW4 were detected in mosquito vectors. GRW4 is, however, frequently observed in adults of migratory bird species with tropical wintering ranges (Bensch et al. 2006, Hellgren et al. 2007) (Fig. 1).

MSP1 sequencing

The nested protocol amplified a 279 bp long fragment of the MSP1 gene (excluding primers). After pruning the sequences, we obtained a 269 bp fragment that was of good quality across all samples to be used in the analysis. Alignment against the SGS1 MSP1 gene (KC969175) confirmed specific amplification of the MSP1 block 14 (MSP1_b14). We found nine MSP1_b14 alleles among all samples: five in the GRW4 samples and four in the SGS1 samples, three of which were shared with GRW11 (Fig. 2, GenBank accession numbers KJ850275-KJ850283). The Jukes–Cantor distance (J–C) of the MSP1_b14 alleles associated with either SGS1 or GRW4 ranged between 0.074 and 0.112 (mean 0.095) while the J–C distance between

Table 1. MSP1 allele distribution for the *Plasmodium relictum* lineages GRW4, SGS1 and GRW11, across different locations and host species.

Cyt b lineage	MSP1 allel	Location	Species
SGS1	Pr 1 (5)	Kenya (2)	House sparrow *Passer domesticus* (2)
		Nigeria, Jos (2)	Black scrub robin *Cercotrichas podobe* (1)
			Roufustailed scrub robin *Cercotrichas galactotes* (1)
		Nigeria, Malafatori (1)	Sudan golden sparrow *Passer luteus* (1)
	Pr 2 (20)	Italy (4)	House sparrow *Passer domesticus* (4)
		Turkey (3)	House sparrow *Passer domesticus* (3)
		Bulgaria (2)	House sparrow *Passer domesticus* (1)
			Great reed warbler *Acrocephalus arundinaceus* (1)
		Spain (4)	House sparrow *Passer domesticus* (4)
		Lithuania (3)	Crossbill *Loxia curvirostra* (2)
			Tree sparrow *Passer montanus* (1)
		Japan (3)	*Culex pipiens pallens* (vector) (3)
		South Africa	Cape bulbul *Pycnonotys capensis* (1)
	Pr 3 (11)	Japan (11)	*Culex pipiens pallens* with blood meal of jungle crow *Corvus macrorhynchos* (1)
			Culex pipiens pallens (vector) (10)
	Pr 7 (1)	Japan (1)	*Culex pipiens pallens* (vector) (1)
GRW11	Pr 2 (10)	Bulgaria (2)	House sparrow *Passer domesticus* (1)
			Great reed warbler *Acrocephalus arundinaceus* (1)
		Italy (3)	House sparrow *Passer domesticus* (3)
		Spain (1)	House sparrow *Passer domesticus* (1)
		Lithuania (2)	House sparrow *Passer domesticus* (1)
			Lesser whitethroat *Sylvia curruca* (1)
		Japan (1)	*Culex pipiens pallens* (vector) (1)
		South Africa	Cape bulbul *Pycnonotys capensis* (1)
	Pr 3 (1)	Japan (1)	*Culex pipiens pallens* with blood meal of Asian house martin *Delichon dasypus* (1)
	Pr 7 (1)	Japan (1)	*Culex pipiens pallens* (vector) (1)
GRW4	Pr 4 (2)	Japan (1)	*Culex pipienspallens* (vector) (1)
		South Africa (1)	Cape white-eye *Zosterops v. Capensis* (1)
	Pr 5 (6)	Bulgaria (1)	Great reed warbler *Acrocephalus arundinaceus*, adult (1)
		Zambia (3)	Great reed warbler *Acrocephalus arundinaceus* (3)
		Japan (2)	*Culex pipiens pallens* with blood meal of Oriental reed warbler *Acrocephalus orientalis* (1)
			Culex pipiens pallens (vector) (1)
	Pr 6 (3)	Nigeria, Malafatori (2)	Lesser swamp warbler *Acrocephalus gracilirostris* (1)
			African reed warbler *Acrocephalus baeticatus* (1)
		Zambia (1)	Great reed warbler *Acrocephalus arundinaceus* (1)
	Pr 8 (13)	USA, Tampa (1)	House sparrow *Passer domesticus* (1)
		Bermuda (6)	House sparrow *Passer domesticus* (6)
		Argentina (3)	House sparrow *Passer domesticus* (3)
		Hawaii, Kauai (2)	Kauai amakihi *Hemignathus kauaiensis* (2)
		New Zealand	House sparrow *Passer domesticus* (1)
	Pr 9 (36)	USA, Tampa (5)	House sparrow *Passer domesticus* (5)
		USA, St. Louis (4)	House sparrow *Passer domesticus* (4)
		Bermuda (1)	House sparrow *Passer domesticus* (1)
		Brazil (1)	House sparrow *Passer domesticus* (1)
		Hawaii, Molokai (8)	Apapane *Himatione sanguinea* (6)
			Hawaii amakihi *Hemignathus virens* (2)
		Hawaii, Hawaii (6)	Hawaii amakihi *Hemignathus virens* (4)
			Apapane *Himatione sanguinea* (2)
		Hawaii, Maui (5)	Apapane *Himatione sanguinea* (3)
			Hawaii amakihi *Hemignathus virens* (1)
			Hawaiian goose, nene, *Branta sandvicensis* (1)
		Hawaii, Kauai (6)	Apapane *Himatione sanguinea* (1)
			Elepaio *Chasiempis sandwichensis* (5)

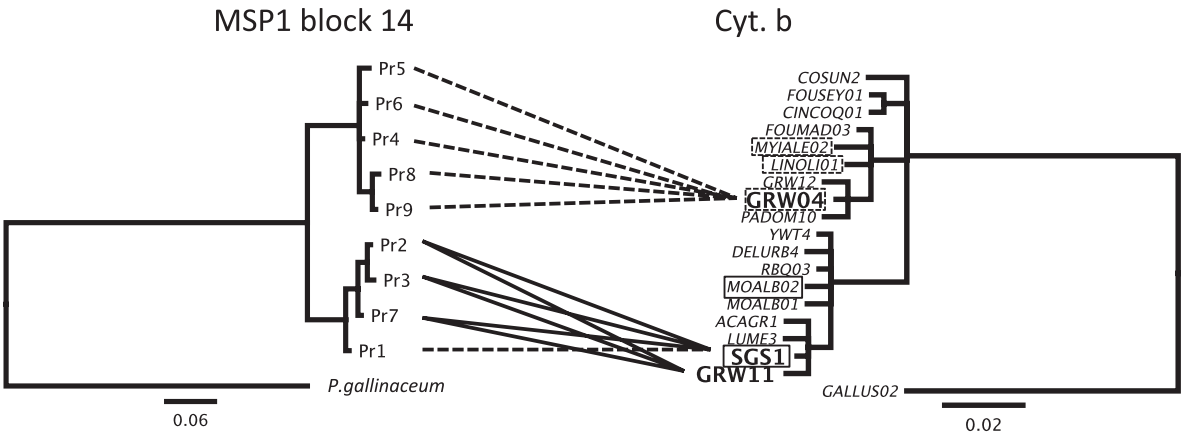

Figure 2. Phylogenies of the MSP1 alleles obtained from *P. relictum* (lineage SGS1, GRW11 and GRW4) and their link with the cyt b lineages. The cyt b phylogeny includes the closest lineages to each of SGS1, GRW11 and GRW4. Broken lines represent novel MSP1 alleles to a cyt b lineage whereas solid lines represent MSP1 alleles shared between different cyt b lineages. Same boxes (solid or broken) around cyt b lineage names corresponds to sharing the same nuclear gene DHFR-TS allel (Beadell et al. 2006).

the cyt b haplotypes was 0.023. Thus, the divergence rate is about 3.2–4.8 times faster at the MSP1_b14 compared to the cyt b gene, assuming that the MSP1 alleles co-diversified with the cyt b lineages. To confirm that the most common shared allele (Pr 2) between SGS1 and GRW11 was indeed identical over a longer section of the MSP1 gene, we sequenced an additional 2572 bp of an isolate of GRW11 obtained from house sparrow *Passer domesticus* in Lithuania using the primers MSP1_3F/3R, MSP1_1F2/1_R2 and MSP1_F2/R2 as described in (Hellgren et al. 2013a). The longer sequence confirmed that the MSP1_b14 allele Pr 2 was identical between SGS1 (KC969175) and the sequenced GRW11 lineage.

Phylogeny and geographical distribution of MSP1 alleles

The MSP1_b14 alleles formed two distinct, well-supported phylogenetic clusters, one originating from GRW4 isolates and one originating from SGS1 and GRW11 isolates (Fig. 2 and 3). The allele Pr 1, which is sister to the remaining SGS1/GRW11 alleles, was found only in SGS1 isolates orig-

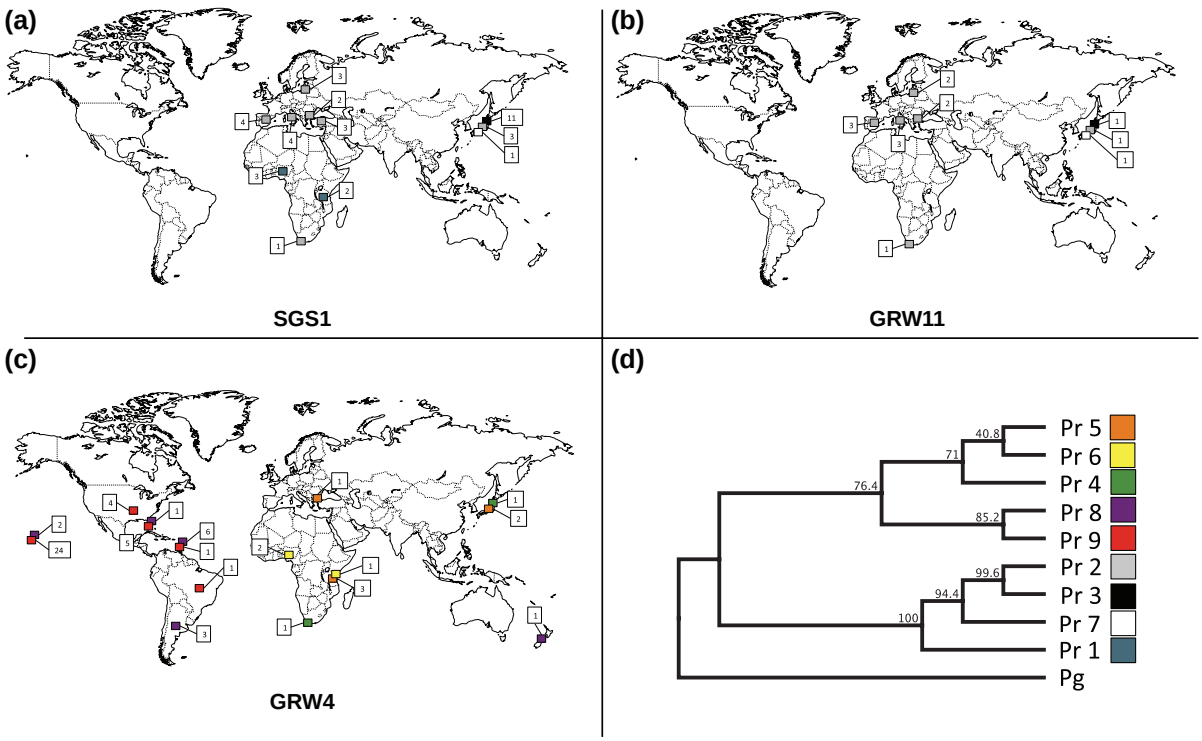

Figure 3. Geographical distribution of MSP1 alleles across the cyt b lineages: (a) SGS1; (b) GRW11; and (c) GRW4. The colour of each symbol in (a–c) corresponds to the different MSP1 alleles represented by different colours in (d). Numbers in boxes represent the number of cases of each allele.

inating from the tropical part of Africa; the three additional alleles (Pr 2, 3 and 7) were shared among GRW11 and SGS1 isolates (Fig. 2). The sorting of MSP1 alleles between Europe and Africa was statistically unlikely to have happened by chance (Fisher's exact test, SGS1 MSP1 alleles in Europe vs MSP1 alleles in Africa; $p = 0.0003$, for sample sizes see Table 1). The only MSP1 allele of SGS1/GRW11 (Pr 2) recovered from Europe was also found in Japan in both GRW11 and SGS1 isolates. However, the most common MSP1 allele of SGS1 in Japan (Pr 3) was not observed outside of that country. Allele Pr 7 was also exclusive to Japan and found in low frequency in both SGS1 and GRW11 isolates (Fig. 3).

The GRW4 cluster contained two closely related MSP1 alleles (Pr 8 and 9), both recovered from New World and Pacific (including Hawaii and New Zealand (NZ; only Pr 8)) isolates, and one group of alleles (Pr 4, 5, and 6) found in Old World isolates. Within the Old World cluster, two of the alleles (Pr 4 and 5) were found both in Africa and in Japan while allele Pr6 was only found in Africa. The two New World alleles (Pr 8 and 9) were both found to be transmitted in North and South America as well as in Bermuda and on Hawaii. Among four Hawaiian Islands that were sampled (Kauai, Maui, Molokai, Hawaii), Pr 9 was found to be the most frequent, whereas Pr 8 occurred in only two of the 26 isolates, both originating from the island of Kauai (Fig. 4).

Naturally infected hosts can be infected by more than one parasite. When amplifying parasite DNA from such samples it is hard to know which of parasites have been amplified. However, in our case the MSP1 alleles all fell into well-defined clusters broadly corresponding to the cyt b haplotypes obtained from the same sample. The two most closely related lineages (SGS1 and GRW11) were the lineages that shared MSP1 alleles. That the sharing of alleles between SGS1 and GRW11 should be cases of double infections, where alleles belonging to one of the lineages are amplified, is not likely as the patterns were consistent across all of the European samples (20 cases for SGS1 and 10 for GRW11).

Allel frequencies were significantly dissimilar among different geographic areas studied (North America, South America, Europe, Africa, Japan and Hawaii), both when combining cyt b and MSP1 alleles (ANOSIM; $R = 0.50$, $p < 0.001$) and when testing the MSP1 alleles across the individual cyt b lineages (ANOSIM; SGS1/GRW11: $R = 0.59$, $p < 0.001$, GRW4: $R = 0.54$, $p < 0.001$).

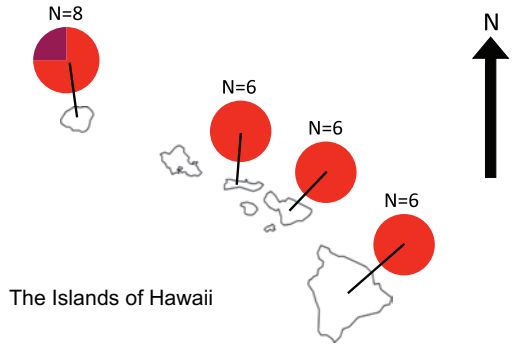

Figure 4. Frequency distribution of the MSP1 alleles, Pr 8 (purple) and Pr 9 (red), on the islands of Hawaii.

Host distribution of MSP1 alleles

The most common host of *P. relictum* in this study was the house sparrow *Passer domesticus*, which harboured four of the nine different MSP1 alleles. There was a lack of evidence towards strict host specificity by any of the MSP1 alleles, as all MSP1 alleles that were found more than once also had multiple host origins. Further, no statistical support was found when testing the distribution of MSP1 alleles in house sparrows compared to 'other host species' (ANOSIM: $R = 0.03$, $p > 0.05$). It should be noted, however, that Pr 5 and Pr 6 from African isolates of GRW4 were all found infecting host species belonging to the Old World warbler genus *Acrocephalus*, whereas the MSP1 alleles originating from the New World were all found in house sparrows or species endemic to the islands of Hawaii (Table 1). To fully understand the linkage between host species and MSP1 allele types will require samples of *P. relictum* (GRW4) from the New World from hosts other than house sparrows, as well as investigating *P. relictum* in house sparrows originating from Africa.

Discussion

Our discovery of multiple independent loci of avian *Plasmodium* parasites provides insight into species limits, levels of differentiation, and gene-flow among parasite populations. Allelic variation at a gene locus such as MSP1, which is known to be linked to immunity (Ngoundou-Landji et al. 2010) as well as being a protein involved in the invasion of the red blood cells (Kadekoppala and Holder 2010, Wright and Rayner 2014), allows us to study how this variation might be linked to differences in host–parasite interactions. Such studies are crucial to understanding the evolution of local patterns of virulence and host resistance. Further, rapidly evolving genetic markers, such as MSP1, will provide finer population resolution, making it possible to follow the introduction of malaria to new host populations and resulting epidemic disease outbreaks.

The discovery of considerable geographically structured genetic variation in MSP1 across different cyt b lineages of the *P. relictum* morphospecies suggests that different cyt b lineages of *P. relictum* should not be considered as one panmictic population. Instead, these parasites appear to behave as different populations that may present differences in their impact on individual hosts and host communities.

Gene flow between different cyt b lineages

Morphologically-defined species of avian malaria often include multiple cyt b lineages with different geographical distributions (Beadell et al. 2006, Marzal et al. 2011) and life history traits (Perez-Tris and Bensch 2005). In *Haemoproteus*, which is a sister genus to *Plasmodium*, several closely related cyt b lineages represent independently evolving entities with distinct and independent associations of mitochondrial and nuclear alleles (Bensch et al. 2004). The level of gene mixing between different cyt b lineages has not been investigated to the same extent among species of avian *Plasmodium*. However, when comparing the distribution of nuclear MSP1

alleles across the cyt b lineages isolated in this study, a somewhat mixed result emerges. The GRW4 and SGS1/GRW11 clades (Fig. 2) presented no evidence of mixing; each of the clades were associated with different, monophyletic sets of MSP1 alleles. This result complements a previous study that focused on another, more slowly evolving nuclear gene (DHFR-TS (Beadell et al. 2006), in which GRW4 shared DHFR-TS alleles with the closely related lineages (LINILI01 and MYIALE02), and SGS1 shared lineages with a different closely related lineage (MOAL02) (Fig. 2). Again no sharing of nuclear alleles was observed between SGS1 and GRW4. Therefore we strongly suggest that SGS1 and GRW4 should be considered as separate species and call for more morphological and life-cycle studies to find specific characters for the two groups of lineages in order to assign them to different morphospecies.

For SGS1/GRW11 the outcome was different. This group shared MSP1 alleles across the two different cyt b lineage isolates (Fig. 2) and only the Pr 1 allele was unique to SGS1. The isolates of *P. relictum* obtained from Europe (SGS1/GRW11) shared an identical MSP1 allele (Pr 2); in Japan they shared three alleles (Pr 2, Pr 3, Pr 7). As the SGS1parasites in Europe and Japan share several nuclear alleles with GRW11, either they are the same evolutionary entity ('species') or the MSP1 alleles represent ancestral variation that has not yet sorted out between the descendant lineages of SGS1 and GRW11. Because the MSP1 gene evolves three times as fast as the cyt b gene, incomplete lineage sorting seems unlikely. The geographic distributions of SGS1 (excluding the likely tropical-transmitted allele Pr 1) and the GRW11 lineages are essentially coincident (Fig. 1 and 3), which again suggests that they represent parasites belonging to the same gene pool. The African allele of SGS1 (Pr 1) was not found in any isolate of GRW11 and is basal to the other MSP1 alleles in the phylogeny (Fig. 3d). These central African populations of SGS1 might not be transmitted elsewhere, but more samples are needed to determine whether other MSP1 alleles are absent from tropical regions of Africa.

Geographical distributions

MSP1 alleles of SGS1/GRW11

SGS1 was previously suggested to be one of the few *Plasmodium* lineages with active transmission in both tropical Africa and the far north temperate regions of Europe (Hellgren et al. 2007). However, data presented here suggest that there may be two different SGS1 lineages in the European-African migratory flyway, one with the MSP1_Pr1 allele that is transmitted in tropical Africa and one with the MSP1_Pr2 allele that is transmitted in Europe. The MSP1_Pr2 allele was also found in a Cape bulbul *Pycnonotys capensis*, a non-migratory bird species endemic to parts of South Africa that are climatically similar to the Mediterranean region of Europe. This pattern suggests that climate zones act as transmission barriers to the SGS1 lineages with different MSP1 alleles. Such a 'barrier' might arise because of limitations on competent vector communities between the regions or differences in extrinsic parasite development affected by abiotic factors such as temperature (Vanderberg and Yoeli 1966, Billker et al. 1997). In fact, differences in development success of *P. relictum* in relation to temperature was shown under experimental conditions for its vector on Hawaii, *Culex quinquefasciatus* (LaPointe et al. 2010).

One intriguing observation regarding the geographical distribution of SGS1/GRW11_MSP1 alleles was the higher number of MSP1 alleles found in Japan compared to the monomorphic MSP1 patterns in Europe. This pattern might stem from a sampling artefact (which is unlikely due to the higher number of isolates and host species among the European samples), or it might indicate a recent rapid population expansion of one SGS1/GRW11 lineage in Europe or a long period of differentiation in Japan.

GRW4 and the origin of malaria on Hawaii

One intriguing finding from the study was the occurrence of both New World alleles (Pr 8 and Pr 9) of lineage GRW4 in the Hawaiian Islands. GRW4 appears to be the sole lineage of *P. relictum* that was introduced to Hawaii (Beadell et al. 2006, Jarvi et al. 2013). One study has used another nuclear gene (TRAP) to investigate the genetic diversity of the GRW4 lineage on Hawaii (Farias et al. 2012). In this study, the authors found that five different alleles occurred on the islands of Hawaii. However, to date there exist no data on how these alleles are distributed with respect to MSP1 alleles or how the diversity of the TRAP gene is distributed across other GRW4 populations outside the island of Hawaii. The vector *Culex quinquefasciatus* was first introduced to Hawaii in 1826. Before its introduction, no appropriate vectors were present, thus precluding the establishment of *Plasmodium* spp. in the endemic avifauna of Hawaii before 1826 (Warner 1968). The most parsimonious explanation for the origin of the parasite has been the introduction of infected birds of Old World origin early in the 20th century, when local bird clubs introduced dozens of passerine species to replace declining native birds (Warner 1968, Van Riper et al. 1986, Beadell et al. 2006). However, our data suggests an alternative hypothesis; that the parasites reached the islands from the New World, possibly following the introduction of one or more species of non-native passerines from North America (Moulton et al. 2001). In particular, at least four species of North American origin were released in large numbers on one or more islands prior to 1938, when *P. relictum* was first documented on Hawaii Island (Baldwin 1941). These include house finch *Carpodacus mexicanus* (1859), western meadowlark *Sturnella neglecta* (1928), northern cardinal *Cardinalis cardinalis* (1929–1931), and northern mockingbird *Mimus polyglottis* (1928–1933) (Pyle and Pyle 2009). In order to confirm this hypothesis, more samples are needed from areas and species from other potential introduction areas.

We found two MSP1 alleles (Fig. 4) in Hawaiian isolates of GRW4. The most common allele (Pr 9) occurred on all four islands in 24 of 26 samples that were screened, while allele Pr 8 was recovered from only two amakihi *Hemignathus kauaiensis* from the island of Kauai. The presence of two different MSP1 alleles on this island suggests either that both were introduced together or that there were multiple introductions to the Islands. Interestingly, we documented the presence of Pr 8 in house sparrows from New Zealand, raising the possibility that this less common allele reached Hawaii as a separate introduction when house sparrows of

New Zealand origin were released on Oahu in 1871 (Pyle and Pyle 2009).

Regardless, these new data highlight the importance of investigating the degree to which these different allels affect their hosts. If the two alleles differ in pathogenicity or if weak cross-immunity (Bruce and Day 2002) occurs in the hosts between the alleles, causing differences in the immune response elicited from their hosts, the spread of Pr8 to other islands in the archipelago may lead to new epizootics that could affect some lowland native bird populations presently evolving tolerance to *P. relictum* (Atkinson et al. 2013).

Throughout this study we found geographical MSP1 variation both between and within the different cyt b lineages. This might represent either an outcome of historical isolation between lineages causing divergence, or cases of local adaptations driven by different local selection regimes. In human malaria (i.e. *P. falciparum*), the diversity and allele distribution at genes involved in host invasion, such as msp1, have from time to time been found to be driven by selection from host immune genes, resulting in parasite alleles differing in frequency between populations (Remarque et al. 2008, Barry et al. 2009, Noranate et al. 2009). In order to elucidate whether this is also the case in avian malaria species, there is a need for more extensive sampling in different local populations. This would confirm whether the variation observed in this study is an effect of geographic isolation with small or no effects on how they interact with the hosts, or a result of different selection regimes between host populations. Thus, increasing sampling within continents might reveal finer geographical structure than observed in this study, which might be linked to local selection regimes.

Future research directions

The results and methods of this study illustrate the use of multiple, independent loci to examine 'species' limits, differentiation, and gene flow within malaria parasite populations, as well as the evolution of local patterns of virulence, questions that will remain in focus for years to come in the field of wildlife malaria. The results presented here raise important questions. For example, 1) would lineages of GRW4 from the Old World be as virulent as the New World lineages when introduced into novel host populations; 2) is the high mortality observed in native Hawaiian birds and transplanted birds in zoological gardens (Bueno et al. 2010) associated with specific high-virulence strains of *P. relictum*, or are all equally pathogenic; and 3) is the minor MSP1 allele on Hawaii a more recent introduction and if so what are the risks that this strain will cause new outbreaks if it spreads to new host populations on other islands? Experimental studies and development of additional nuclear markers may help to shed light on these questions.

Acknowledgements – This work was supported in part by the Swedish Research Council (Svenska Vetenskapsrådet) and the Crafoord Foundation (OH), the U.S. Geological Survey Wildlife and Terrestrial Resources Program (CA). Any use of trade, product, or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. Government. Sample collection by LBM was supported by NSF-IOS 0920475.

References

Atkinson, C. T. et al. 2013. Experimental evidence for evolved tolerance to avian malaria in a wild population of low elevation Hawaii amakihi (*Hemignathus virens*). – Ecohealth 10: 366–375.

Baldwin, P. H. 1941. Checklist of the birds of the Hawaii National Park, Kilauca-Mauna Loa Section, with remarks on their present status and a field key for their identification. – Hawaii National Park Natural History Bulletin.

Barry, A. E. et al. 2009. Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum*. – PLoS One 4: e8497.

Beadell, J. S. et al. 2004. Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. – Mol. Ecol. 13: 3829–3844.

Beadell, J. S. et al. 2006. Global phylogeographic limits of Hawaii's avian malaria. – Proc. R. Soc. B 273: 2935–2944.

Bensch, S. et al. 2000. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. – Proc. R. Soc. B 267: 1583–1589.

Bensch, S. et al. 2004. Linkage between nuclear mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? – Evolution 58: 1617–1621.

Bensch, S. et al. 2006. Temporal dynamics and diversity of avian malaria parasites in a single host species. – J. Anim. Ecol. 76: 112–122.

Bensch, S. et al. 2009 MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. – Mol. Ecol. Res. 9: 1353–1358.

Bensch, S. et al. 2014. Genomic resources notes accepted 1 June 2013–31 July 2013. – Mol. Ecol. Res. 14: 218–218.

Billker, O. et al. 1997. The roles of temperature, pH and mosquito factors as triggers of male and female gametogenesis of *Plasmodium berghei* in vitro. – Parasitology 111: 539–545

Bishop, M. A. and Bennett, G. F. 1992. Host–parasite catalogue of the avian haematozoa: supplement 1, and bibliography of the avian blood-inhabiting haematozoa: supplement 2. – Occ. Pap. Biol. Memorial Univ. Newfoundland 15: 1–244

Bruce, M. C. and Day, K. P. 2002. Cross-species regulation of malaria parasitaemia in the human host. – Curr. Op. Microbiol. 5: 431–437.

Bueno, M. G. et al. 2010 Identification of *Plasmodium relictum* causing mortality in penguins (*Spheniscus magellanicus*) from São Paulo Zoo, Brazil. – Vet. Parasitol. 173: 123–127.

Cosgrove, C. L. et al. 2008. Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. – J. Anim. Ecol. 77: 540–548.

Farias, M. E. M. et al. 2012. Analysis of the trap gene provides evidence for the role of elevation and vector abundance in the genetic diversity of *Plasmodium relictum* in Hawaii. – Malaria J. 11: 305.

Ferrer, E. S. et al. 2012. Molecular characterization of avian malaria parasites in three Mediterranean blue tit (*Cyanistes caeruleus*) populations. – Parasitol. Res. 111: 2137–2142.

Gerold, P. et al. 1996. Structural analysis of the glycosylphosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. – Mol. Biochem. Parasitol. 75: 131–143.

Hellgren, O. et al. 2004. A new PCR assay for simultaneous studies of Leucocytozoon, *Plasmodium*, and *Haemoproteus* from avian blood. – J. Parasitol. 90: 797–802.

Hellgren, O. et al. 2007. Detecting shifts of transmission areas in avian blood parasites: a phylogenetic approach. – Mol. Ecol. 16: 1281–1290.

Hellgren, O. et al. 2009. A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. – Ecology 90: 2840–2849.
Hellgren, O. et al. 2013a. Identification and characterization of the merozoite surface protein 1 (msp1) gene in a host-generalist avian malaria parasite, *Plasmodium relictum* (lineages SGS1 and GRW4) with the use of blood transcriptome. – Malaria J. 12: 381.
Hellgren, O. et al. 2013b. Circannual variation in blood parasitism in a sub-Saharan migrant passerine bird, the garden warbler. – J. Evol. Biol. 26: 1047–1059.
Hui, G. and Hashimoto, C. 2007. *Plasmodium falciparum* anti-MSP1–19 antibodies induced by MSP1-42 and MSP1-19 based vaccines differed in specificity and parasite growth inhibition in terms of recognition of conserved versus variant epitopes. – Vaccine 25: 948–956.
Jarvi, S. I. et al. 2013. Next-generation sequencing reveals cryptic mtDNA diversity of *Plasmodium relictum* in the Hawaiian Islands. – Parasitology 140: 1741–1750.
Kadekoppala, M. and Holder, A. A. 2010. Merozoite surface proteins of the malaria parasite: the MSP1 complex and the MSP7 family. – Int. J. Parasitol. 40: 1155–1161.
Kang, J.-M. et al. 2012. Genetic polymorphism and natural selection in the C-terminal 42 kDa region of merozoite surface protein-1 among *Plasmodium vivax* Korean isolates. – Malaria J. 11: 206.
Lachish, S. et al. 2011. Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure. – J. Anim. Ecol. 80: 1196–1206.
LaPointe, D. A. et al. 2010. Thermal constraints to the sporogonic development and altitudinal distribution of avian malaria *Plasmodium relictum* in Hawai'i. – J. Parasitol. 96: 318–324.
Loiseau, C. et al. 2012. Host and habitat specialization of avian malaria in Africa. – Mol. Ecol. 21: 431–441.
Lowe, S. et al. 2000. 100 of the world's worst invasive alien species: a selection from the global invasive species database. – <www.issg.org>.
Marzal, A. et al. 2005. Malarial parasites decrease reproductive success: an experimental study in a passerine bird. – Oecologia 142: 541–545.
Marzal, A. et al. 2011. Diversity, loss, and gain of malaria parasites in a globally invasive bird. – PLoS One 6: e21905.
Marzal, A. et al. 2014. Invasive avian malaria as an emerging parasit disease in native birds of Peru. – Biol. Invasion doi: 10.1007/s10530-014-0718
Moulton, M. P. et al. 2001. Patteins of success among introduced birds in the Hawaiian Islands. – Stud. Avian Biol. 22: 31–46.
Ngoundou-Landji, J. et al. 2010. Fine specificity of anti-MSP119 antibodies and multiplicity of *Plasmodium falciparum* merozoite surface protein 1 types in individuals in Nigeria with sub-microscopic infection. – Malaria J. 9: 287.
Noranate, N. et al. 2009. Population diversity and antibody selective pressure to *Plasmodium falciparum* MSP1 block2 locus in an African malaria-endemic setting. – BMC Microbiol. 9: 219.
Pacheco, M. A. et al. 2012. The origin of malarial parasites in orangutans. – PLoS One 7: e34990.
Palinauskas, V. et al. 2008. *Plasmodium relictum* (lineage P-SGS1): effects on experimentally infected passerine birds. – Exp. Parasitol. 120: 372–380.
Palinauskas, V. et al. 2011. *Plasmodium relictum* (lineage SGS1) and *Plasmodium ashfordi* (lineage GRW2): the effects of the co-infection on experimentally infected passerine birds. – Exp. Parasitol. 127: 527–533.
Perez-Tris, J. and Bensch, S. 2005. Dispersal increases local transmission of avian malarial parasites. – Ecol. Lett. 8: 838–845.
Pérez-Tris, J. et al. 2007. Within-host speciation of malaria parasites. – PLoS One 2: e235.
Pyle, R. L. and Pyle, P. 2009. The birds of the Hawaiian Islands: occurrence, history, distribution, and status. – B. P. Bishop Museum, Honolulu, HI, USA, ver. 1, <http://hbs.bishopmuseum.org/birds/rip-monograph/>.
Remarque, E. J. et al. 2008. Apical membrane antigen 1: a malaria vaccine candidate in review. – Trends Parasitol. 24: 74–84.
Reullier, J. et al. 2006. Diversity, distribution and exchange of blood parasites meeting at an avian moving contact zone. – Mol. Ecol. 15: 753–763.
Ronquist, F. and Huelsenbeck, J. P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. – Bioinformatics 19: 1572–1574.
Samuel, M. D. et al. 2011. The dynamics, transmission, and population impacts of avian malaria in native Hawaiian birds: a modeling approach. – Ecol. Appl. 8: 2960–2973.
Tanabe, K. et al. 2013. Within-population genetic diversity of *Plasmodium falciparum* vaccine candidate antigens reveals geographic distance from a central sub-Saharan African origin. – Vaccine 31: 1334–1339.
Valkiunas, G. 2005. Avian malaria parasites and other haemosporidia. – CRC.
Van Riper, C. et al. 1986. The epizootiology and ecological significance of malaria in Hawaiian land birds. – Ecol. Monogr. 56: 327–344.
Vanderberg J. P. and Yoeli, M. 1966. Effects of temperature on sporogonic development of *Plasmodium berghei*. – J. Parasitol. 52: 559.
Waldenström, J. et al. 2004. A new nested polymerase chain reaction method very efficient in detecting *Plasmodium* and *Haemoproteus* infections from avian blood. – J. Parasitol. 90: 191–194.
Warner, R. E. 1968. The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. – Condor 70: 101–120.
Wood, M. J. et al. 2007. Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. – Mol. Ecol. 16: 3263–3273.
Wright, G. J. and Rayner, J. C. 2014. *Plasmodium falciparum* erythrocyte invasion: combining function with immune evasion. – PLoS Pathog. 10: e1003943.

Supplementary material (Appendix ECOG-01158 at <www.ecography.org/readers/appendix>). Appendix 1.