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# Non-specific Patterns of Vector, Host, and Avian Malaria Parasite Associations in a Central African Rainforest

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## Abstract

Malaria parasites use vertebrate hosts for asexual multiplication and Culicidae mosquitoes for sexual and asexual development, yet the literature on avian malaria remains biased towards examining the asexual stages of the life cycle in birds. To fully understand parasite evolution and mechanism of malaria transmission, knowledge of all three components of the vector-host-parasite system is essential. Little is known about avian parasite-vector associations in African rainforests where numerous species of birds are infected with avian haemosporidians of the genera *Plasmodium* and *Haemoproteus*. Here we applied high resolution melt qPCR-based techniques and nested PCR to examine the occurrence and diversity of mitochondrial cytochrome *b* gene sequences of haemosporidian parasites in wild-caught mosquitoes sampled across 12 sites in Cameroon. In all, 3134 mosquitoes representing 27 species were screened. Mosquitoes belonging to four genera (*Aedes, Coquillettidia*, *Culex*, and *Mansonia*) were infected with twenty-two parasite lineages (18 *Plasmodium* spp. and 4 *Haemoproteus* spp.). Presence of *Plasmodium* sporozoites in salivary glands of *Coquillettidia aurites* further established these mosquitoes as likely vectors. Occurrence of parasite lineages differed significantly among genera, as well as their probability of being infected with malaria across species and sites. Approximately one-third of these lineages were previously detected in other avian host species from the region, indicating that vertebrate host sharing is a common feature and that avian *Plasmodium* spp. vector breadth does not always accompany vertebrate-host breadth. This study suggests extensive invertebrate host shifts in mosquito-parasite interactions and that avian *Plasmodium* species are most likely not tightly coevolved with vector species.

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**Keywords**

mosquitoes; *Plasmodium*; avian malaria; vector-parasite interactions; HRM; PCR

## Introduction

Few studies have explored the role of culicine mosquitoes in the transmission of avian *Plasmodium* (Huff 1965; LaPointe *et al*. 2005; Gager *et al*. 2008; Ejiri *et al*. 2008; Ishtiaq *et al*. 2008; Njabo *et al*. 2009; Kimura *et al*. 2010), and the literature on avian malaria parasites remains heavily biased towards examining bird-parasite associations (i.e. van Riper *et al*. 1986; Beadell *et al*. 2004; Hellgren *et al*. 2004; Valkiūnas 2005, Beadell *et al*. 2009). Since the advent of PCR-based molecular diagnoses to detect presence of avian malaria parasites in vectors, new mosquito species, representing several genera, have been implicated in transmission of avian malaria parasites (Valkiūnas 2005, Ejiri *et al*. 2008; Ishtiaq *et al*. 2008; Kim et al., 2009a; Njabo *et al*. 2009). Unfortunately, very little is known about their vectorial capacities, their degree of refractoriness or susceptibility to infection, their host feeding preferences and longevity, or their abundance and distribution. All of these factors influence natural vector and host-parasite interactions and transmission patterns and influence the prevalence of vector-borne diseases (Ishtiaq *et al*. 2006, 2008; Hellgren *et al*. 2008).

Our knowledge of avian malaria vectors in Sub-Saharan Africa is particularly poor. Avian *Plasmodium* spp. were only recently detected and shown to develop to the sporozoite stage in wild mosquitoes of the genus *Coquillettidia* collected from the lowland forests of Cameroon (Njabo *et al*. 2009). The possibility remains that new mosquito genera and species may still be identified as major and minor avian vectors in Central Africa. This study fills a gap in the current research on vectors of avian malaria in Africa by combining high throughput molecular techniques and microscopic examination of common mosquitoes species collected from the lowland forests of Cameroon.

Avian haemosporidian parasites (genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) are cosmopolitan vector-transmitted parasites common in birds in virtually all regions of the world (Waldenström *et al*. 2002; Fallon *et al*. 2003; Beadell *et al*. 2004; Scheuerlein & Ricklefs 2004; Szymanski & Lovette 2005; Valkiūnas 2005; Ishtiaq *et al*. 2006; Hellgren *et al*. 2007). Traditionally, only blood stages of these parasites have been investigated in vertebrate blood films using microscopic examinations and species have been described based on morphology (Peirce 1981). With the application of both traditional parasitology methods (Garnham, 1966; Valkiūnas, 2005) and molecular diagnostic techniques (Palinauskas *et al*. 2008), these parasites have been shown to exhibit varying degrees of host specificity and various modes of transmission (Bensch *et al*. 2000; Ricklefs & Fallon 2002; Waldenström *et al*. 2002; Palinauskas *et al*., 2009). As with all haemosporidian parasites, the life cycle of *Plasmodium* spp. involves the sexual process and sporogony that occur in a definitive host (vector) and the merogony and development of gametocytes that occur in vertebrate hosts (Valkiūnas, 2005).

Given that mosquito species vary in their overall vectorial capacities to support parasite development and transmission, the relationship between the parasite and the vertebrate host in both evolutionary and ecological contexts can be altered in many ways (Ishtiaq *et al*. 2008). Ecological ranges and dispersal of mosquitoes (e.g. Lum *et al*. 2007) may play a role in reproductive isolation and gene flow between populations of parasites. Widely dispersing vectors would likely move parasite lineages among host populations, thus increasing the effective population size of the parasite and reducing losses of genetic diversity (May &

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Nowak 1994). The dependency of mosquito abundance on environmental conditions could regulate temporal variation in a vertebrate host's exposure to parasites. Depending on the availability of vectors in dry or wet habitats, parasite prevalence in hosts could differ on a rather small geographical scale (Wood *et al*. 2007). Vectors with broad blood-feeding tendencies could facilitate host switching in generalist parasites (Githeko *et al*. 1994). On the other hand, parasites with specialized vector associations should have a restricted host range (Killick-Kendrick 1978). Comparison of avian host and parasite phylogenies indicates that closely related haemosporidian parasites may be found in distantly-related host species and distantly-related parasites can share a single host species (Ricklefs & Fallon 2002; Beadell *et al*. 2004; Gager *et al*. 2008; Garamszegi, 2009). Our understanding of the level of vector-parasite specificity and the extent to which closely-related mosquito species share the same or closely-related haematozoan parasites is, however, limited (see Gager *et al*. 2008).

Several ornithophilic blood-feeding mosquito species have been identified in the lowland forests of Cameroon, suggesting that they could be potential vectors of avian malaria (Njabo *et al*. 2009). Detecting the presence of avian *Plasmodium* spp. in these mosquitoes could help to understand avian parasite-vector relationships when combined with previous research examining distribution of parasites lineages in avian hosts in southern Cameroon (Bonneaud *et al*. 2009; Chasar *et al*. 2009; Valkiūnas *et al*. 2009; Loiseau *et al*. 2010). The last comprehensive mosquito survey in Cameroon conducted in 1952, recorded quite a high diversity of 65 species across 10 genera (Rageau and Adam 1952). In this study, we used High Resolution Melt (HRM) analyses, nested PCR, and DNA sequencing of the cytochrome *b* gene of avian parasites in trapped mosquitoes to examine associations of parasites between vectors and their potential avian hosts at the same sites where birds were previously sampled for malaria (Bonneaud *et al*. 2009; Chasar *et al*. 2009). Because HRM and PCR-based detection approaches do not read differences between developmental stages of parasites and thus alone do not provide definitive evidence of parasite transmission, we also dissected the salivary glands of *Cq. aurites* to screen for presence of sporozoites, the last stage of *Plasmodium* spp. development in vectors. The detection of sporozoites in the salivary glands of the vectors as well as shared parasite lineages in vertebrate and dipteran hosts, provide stronger support of transmission.

In this study we screened several mosquitoes species collected from the lowland forests of Cameroon to: 1) identify further potential mosquito vectors of avian *Plasmodium* spp., 2) document patterns in lineage diversity of avian *Plasmodium* across mosquito vectors, and 3) explore *Plasmodium* lineages in local vectors and avian hosts. For the purpose of this study, we used the name *Aedes mcintoshi* because our field samples most closely resembled this species. However, genitalia of males collected in vegetation in the vicinity of the traps had genitalia morphology intermediate between *Aedes mcintoshi* and *Aedes lineatopennis* (Ludlow).

## Materials and Methods

### Mosquito collection and identification

Mosquitoes were collected from several locations in the lowland forests of Cameroon from June to August 2007 and from to April to May 2008 (Figure 1). In 2007, the mosquitoes were collected using six Center for Disease Control (CDC) Miniature Light Traps (Sudia and Chamberlain 1962) baited with $CO_2$ (John W. Hock, Gainesville, FL) (Njabo et al. 2009). Because mosquitoes were more numerous at two sites, Ndibi and Nkouak, we conducted a more extensive resampling at these two sites a year later (2008) using a more comprehensive mosquito collection scheme that included the use of six miniature CDC traps baited with $CO_2$, four net traps (Jupp and McIntosh 1967), four modified bird-baited

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Ehrenberg lard cans (Downing and Crans 1977), and sweep net collections of resting mosquitoes in forest vegetation.

Nkouak is generally more forested than Ndibi and has a lower human population density (fewer than 1,000 people). Ndibi is mostly characterized by secondary forest in various stages of degradation and seasonally flooded swamp forest (September to December) with floating grass plant communities along either side of the Nyong River (Smith 1990). Additionally, Ndibi is less than 2 km from the city of Akonolinga (population 25,700) across the Nyong River. Based on ecological differences, the two areas should have different patterns of vector diversity.

Traps were set out each day for at least 12 h (06.00 pm–06.00a m). Following each trapping period, the collection bags were removed from traps and the mosquitoes were transported alive from the field and maintained on sucrose solution in the shade of the forest (approximately 21° C) before immobilization with chloroform and/or smoke later that morning. On the day of collection and immobilization, mosquitoes were sorted by sex and identified to species with the aid of a stereomicroscope (×90) and morphological keys (Edwards 1941; Service 1990). Numerous mosquito species cannot be identified by female characters alone and require examination of male genitalia. Males are seldom captured in vertebrate/$C0_2$-baited traps and thus efforts were made to find males resting in vegetation in the locations where trapping was performed to assist in the correct identification, assuming that the species of males captured were consistent with the species obtained for females in the same locations. Males were stored in tubes (with dry silica to keep desiccated) for later dry mounting and separation and slide mounting of genitalia for morphological examination. Genitalia were cleared and slide-mounted in Euparal according to instructions provided in the mounting kit purchased from Bioquip Products Inc (Rancho Dominguez, California). Unfed mosquitoes were pooled according to species and kept in 95% alcohol in the shade in a cooler box in the field and at −20° C in the laboratory until DNA isolation. Female mosquitoes with visible undigested blood meals were removed in order to avoid sequencing *Plasmodium* DNA from blood meals.

## DNA extraction and molecular identification of haemosporidians from mosquitoes

In the laboratory, the head and thorax of each mosquito in each pool was carefully severed from the abdomens for DNA extraction. Pools which varied in size from 3–20 mosquitoes were homogenized with the aid of heat-sealed pipette tips and total DNA was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's protocol. We added 30μL of 100mg/mL dithiothreitol to the digestion buffer to help dissolve the hard exoskeleton (Cooper 1994) and total DNA was eluted in the final step with 200μL elution buffer.

High Resolution Melt (HRM), a recent enhancement to traditional DNA melting analysis that can be used to determine sequence variations within PCR amplicons, was used for initial parasite screening in mosquito pools. HRM requires initial amplification of the target sequence by PCR in the presence of a DNA-binding dye that generates a strong fluorescent signal when bound to DNA. HRM analysis was achieved by transferring the conventional PCR procedure to the Rotor-Gene 6000 platform (Corbett Research, Sydney, Australia) using the following primers: PlasmHRM.F 5’-CAGCTYTAAAAATACCCTTYTATCCA-3’ and PlasHRM 1.2R 5’-CCWGCWGTTTRTTAGGAATTGT-3’. These primers were designed in regions of the mtDNA cytochrome *b* gene that are highly conserved among avian *Plasmodium* parasites and shown through repeated trials to be robust enough to detect infection in a broad range of background host DNA. Fluorescence-based assays based on HRM analysis were developed to detect *Plasmodium* spp. DNA in all the different culicine mosquitoes. PCR reactions

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contained 1.8 ng/μL genomic DNA (1.8–3.9 ng/μL concentration/reaction was found to be best for this reaction), 5 μL of SensiMix DNA kit (Quantace) per 10 μL reaction volume, 0.5μM of each primer, and 0.88 μM $MgCl_2$ (Invitrogen). Samples were run on a Rotor-Gene 6000 (HRM) using real-time PCR thermocycling parameters of 10 minutes at 95° C followed by 40 cycles of 95° C for 5 seconds and 60° C for 10 seconds. This was followed by a melt step of 65–75° C in 0.1° C increments pausing for two seconds per step. The increase in SYTO 9 fluorescence was monitored in real time during PCR and the subsequent decrease during the melt phase by acquiring each cycle/step to the green channel (470 nm excitation and 510 nm emission) of the Rotor-Gene. Parasite lineages were scored by examining normalized and difference melt plots using the Rotor-Gene Software. All reactions were carried out in duplicate.

We also subjected the same mosquito samples to a nested PCR using the protocol described in Bensch *et al*. (2000). For the nested PCR, positive or negative amplifications were evaluated as the presence or absence of bands on 1.5% agarose gels. Samples that showed positive amplification were subjected to dye terminator cycle sequencing reactions (30 cycles, 55° C annealing) and sequenced on ABI 3730 Genetic Analyzer (Applied Biosystems) automated sequencers using Big Dye vs. 3.1. For all these samples, the mtDNA cyt *b* gene was sequenced in two overlapping fragments. Sequences were assembled with Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI) and aligned and manually corrected by eye. Sequences were then confirmed by BLASTN to be most closely related to avian *Plasmodium* spp. cyt *b*. Potentially new and unique sequences were checked by additional sequencing of the fragments. The electropherograms were also checked for double nucleotide peaks to infer possible cases of mixed infections of two or more different parasite lineages. For every set of 20 samples, we used a negative control (cocktail with no DNA) to control for the presence of false positives. This protocol amplifies a 498–502-bp long fragment of the cytochrome *b* gene of the mitochondria of the parasites. Unresolved sequences showing double peaks in the electropherograms were removed from the analyses. Each novel *Plasmodium* lineage found multiple times in independent PCRs, either within the same pool or in several different pools, was considered verified and assigned a lineage specific name. Lineages differing by one or two nucleotides were re-sequenced for verification purposes and once verified were considered separate distinct lineages. The new sequences have been deposited in GenBank (Accession numbers HM179147-HM179164).

### Phylogenetic Analysis

We estimated parasite phylogenetic relationships using all samples for which we had at least 400 base pairs of cytochrome *b* sequence, although 498–502 bp were available for most samples. We inferred taxonomic identity by assessing the phylogenetic affinities of mosquito-isolated *Plasmodium* spp. lineages with published sequences from GenBank that were reliably identified to morphological species, following the phylogeny developed by Chasar *et al*. (2009). In our analysis, we included morphospecies of *Plasmodium (Novyella)* known to be most prevalent in sub-Saharan Africa, including our study sites (Valkiūnas *et al*. 2008), and rooted all our trees with *Haemoproteus* spp. sequences (GenBank accession nos. FJ404690 and FJ404697). The program modeltest version 3.06 (Posada & Crandall 1998) indicated that the most likely model of base pair substitution was general time reversible (GTR + G). We used Bayesian analysis conducted with MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001) to reconstruct a phylogeny using these parameters. The Markov Chain was sampled every 200 generations for 10 million generations. Bayesian posterior branch probabilities were obtained by taking the majority rule consensus of the sampled trees, excluding the first 12,500 trees as burnin. Replicate runs of the software, each with one cold and three heated chains, produced essentially identical results. Node supports in the resulting phylogeny was tested using 200 ML bootstrap replications.

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Finally, we compared our mosquito-isolated *Plasmodium* spp. lineages to the previously published African avian *Plasmodium* spp. cytochrome *b* sequences of Bonneaud *et al*. (2009), Chasar *et al*. (2009) and using the MalAvi database (Bensch et al. 2009). MalAvi streamlines comparative analysis because it contains only haemosporidian cytochrome *b* sequences and greatly facilitates the identification of shared avian hosts (Kimura *et al*. 2010).

## Microscopic examination of salivary glands of mosquitoes

Malarial infection was also determined by microscopic examination of mosquito salivary glands. Nine wild-caught female *Cq. aurites* collected from Ndibi in February 2009 were dissected and salivary glands were isolated on glass slides using traditional mosquito dissection methods (Valkiūnas 2005). The heads of the insects were cut off with a razor and salivary glands were gently pressed out with a slight pressure by a blunt needle on the thorax near the base of the fore legs. The glands were placed in a small drop of the normal saline, ruptured by a gentle pressure of a needle, and mixed with a minute drop of the saline to produce a thin film. The preparations were air-dried and fixed in absolute methanol in the field, and then stained with Giemsa in the laboratory, as described by Valkiūnas (2005). An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine slides, prepare illustrations, and to take measurements. Entire films were examined at low magnification (× 400) and recorded sporozoites were studied at high magnification (× 1,000). Representative preparations of sporozoites (accession numbers 47721, 47722 NS) were deposited in the collection of the Institute of Ecology, Nature Research Centre, Vilnius, Lithuania.

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## Statistical Analysis

Statistical analyses were conducted in Genstat (release 11, VSN International, Rothamsted Experimental Station, Harpenden, UK). In all analyses, we investigated the probability of infection (0 for absence and 1 for presence) fitted as the response term in a generalized linear model with logit link function in which the binomial denominator and dispersal parameter were set to 1. The probability of detecting an infection in a given sample is likely to increase as a decelerating function of the number of mosquitoes sampled. As a consequence, we fitted both the number of mosquitoes in each sample and the number squared as co-variates to control for potential linear and non-linear variation in the probability of detecting infections arising as a consequence of variation in the number of mosquitoes used in each sample. Furthermore, the analyses were also weighted by the number of mosquitoes per sample to account for decreasing variance with increasing sample size. Significant differences among levels of a factor were determined within the same model without the need for multiple comparisons testing, by setting each level in turn as a reference. All means were back-transformed from their logit transformation in the analysis using the formula [1/(1+exponential (-mean)].

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The prevalence of infection with each of the individual *Plasmodium* spp. was too low to permit analysis at the level of each individual lineage. Consequently, we pooled all infections involving a *Plasmodium* spp. lineage and categorized each mosquito sample as either being infected or not with *Plasmodium* spp. lineages. Further, we tested for differences in the occurrence of avian *Plasmodium* spp. among all mosquito genera. Since some genera of mosquitoes had too few samples to permit analysis, we focused the subsequent analyses on species of the four most common mosquito genera only (*Aedes, Coquillettidia*, *Culex*, and *Mansonia*).

We first examined whether mosquitoes of the four most common genera differed in the probability of being infected. To do so, we used the above models to examine whether the

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four most common genera of mosquitoes differed in their probability of infection with avian *Plasmodium* spp. lineages. Two analyses were conducted, one each for the infection results obtained using HRM and Nested PCR techniques. Mosquito genus was fitted as the main explanatory term in both analyses as a four-level factor. Overall, we obtained 1–21 mosquitoes per sample (*Aedes*: N=26 samples, mean mosquitoes/sample = 10.8; *Coquillettidia*: N=268 samples, mean mosquitoes/sample = 4.9; *Culex*: N=86 samples, mean mosquitoes/sample= 8.5; *Mansonia*: N=32 samples, mean mosquitoes/sample = 16.1).

Any differences among genera in detection probability could be driven by differences in the sampling locality. As a consequence, we conducted further analyses investigating the probability of infection across the four genera within a single locality (i.e., Ndibi). In the two models, corresponding to the two detection techniques, genera was fitted as the primary explanatory term as a four-level factor. In these analyses, sample sizes were as follows: *Aedes*: N= 25 samples, mean mosquitoes/sample = 11.1; *Coquillettidia*: N=95 samples, mean mosquitoes/sample = 4.7; *Culex*: N=70 samples, mean mosquitoes/sample = 9.5; *Mansonia*: N=32 samples, mean mosquitoes/sample = 16.1.

Additionally, we investigated whether or not levels of infection differed across localities. In this case, the data was such that we could only consider a single genus (i.e., *Coquillettidia*) across two localities (i.e., Ndibi and Nkouak). Thus, in this case, site was fitted in the two models as the primary explanatory term as a two-level factor. Sample sizes were as follows, Ndibi: N= 95 samples, mean mosquitoes/sample = 4.7; Nkouak: N=173 samples, mean mosquitoes/sample = 5.1.

Finally, the maximum likelihood estimate (MLE) of infection rates for each species of mosquito was calculated as described by Biggerstaff (2006). MLE was utilized to estimate the proportion of infected individuals in field-pooled samples.

## Results

Mosquito species belonging to the genera *Uranotaenia, Ficalbia, Mimomyia, Eretmapodites, Hodgesia, Culex, Aedes, Anopheles*, *Coquillettidia, Lutzia*, and *Mansonia* were collected in the study. Ten species from four genera were positive for *Plasmodium* spp. by both HRM and nested PCR. Maximum likelihood estimates (MLE) of parasite infections range from 2.97/1000 mosquitoes in *Culex guiarti* Blanchard to 106.91/1000 mosquitoes in *Coquillettidia pseudoconopas* Theobald (Table 1).

From the nine wild-caught female *Cq. aurites* collected from Ndibi and dissected, sporozoites were observed in salivary glands of four mosquitoes. Up to four sporozoites were observed in each positive salivary gland smear. The sporozoites were of elongated shape typical for malaria parasites, with nuclei located approximately at the center. The size of these sporozoites averaged 9.9 μm in length and 0.9 μm in width (n=5).

Of the 452 pools screened, 135 (30%) pools were positive for malaria parasites by HRM. Notable discrepancies between HRM and nested PCR results were found such that 23 (17%) HRM positive pools were not positive for nested PCR and only three (3%) nested PCR-positive pools were negative by HRM. From the PCR pools, we recovered 102 (91%) *Plasmodium* and 10 (9%) *Haemoproteus* representing 22 unique lineages (18 *Plasmodium* and four *Haemoproteus*). Of these, 10 *Plasmodium* spp. lineages and two *Haemoproteus* spp. lineages were found in the *Coquillettidia* species, 12 *Plasmodium* spp. lineages and three *Haemoproteus* spp. lineages in the *Culex* species, one *Plasmodium* sp. lineage in *Aedes mcintoshi* Huang, and one *Plasmodium* sp. lineage in *Mansonia uniformis* Theobald (Table 1).

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### Occurrence of Plasmodium spp. in mosquitoes of the four most common genera

Combining all 12 sites from which mosquitoes were collected, we observed significant differences in probability of some mosquito genera being infected with avian *Plasmodium* spp. (HRM and Nested PCR). All analyses controlled for significant effects of the number of mosquitoes (range in $\chi^2 = 21.98$–$65.90$, df = 1, $p < 0.001$) and number of mosquitoes squared (range in $\chi^2 = 35.56$–$93.63$, df = 1, $p < 0.001$). HRM assays detected significant differences in probability of genera infected with avian malaria ($\chi^2 = 63.20$, df = 3, $p < 0.001$), with *Aedes* and *Coquillettidia* having significantly higher infection rates than *Culex* and *Mansonia*. There were no significant differences between *Culex* and *Mansonia* or between *Aedes* and *Coquillettidia* (Figure 2a). Nested PCR assays yielded different results ($\chi^2 = 25.38$, df = 3, $p < 0.001$) where probabilities of infection of *Coquillettidia* were significantly higher than the other three genera with no significant differences between *Aedes*, *Culex*, and *Mansonia* (Figure 2b). Similar results were observed using both HRM ($\chi^2 = 59.77$, df = 3, $p < 0.001$) and nested PCR ($\chi^2 = 38.34$, df = 3, $p < 0.001$) assays with respect to *Plasmodium* spp. probabilities of infection between the four genera in Ndibi, where most of the mosquitoes were collected (results not shown). In comparisons between Ndibi and Nkouak, the probabilities of infections of *Coquillettidia* were greater in Ndibi than in Nkouak using both HRM and nested PCR assays. However, only in the HRM analysis was there a significant source of bias effect of the number of mosquitoes per sample ($\chi^2 = 11.10$, df = 1, $p < 0.001$). In no other case was there a significant linear effect of the number of mosquitoes per sample on the probability of infection (nested PCR: $\chi^2 = 0.15$, df = 1, $p = 0.70$) and in no case were quadratic effects significant (results not shown).

### Phylogenetic analysis and patterns of lineage diversity

The Bayesian analyses tree for *Plasmodium* and *Haemoproteus* spp. is given in Figure 3. For convenience and easy comparison with lineages published in other studies, the tree is divided into five groups with strong to moderate support (68–100% posterior probability, Groups A–E). Interestingly, each of the groups was closely affiliated to at least one lineage previously found in birds and corresponding to the morphospecies of parasites shown in Chasar *et al*. (2009). Lineage diversity was particularly high in Group A, which had four lineages isolated from *Culex* species and two from *Coquillettidia* spp. mosquitoes. Nested within Group A were two known morphospecies, *Plasmodium globularis* (Genbank J404710) and *Plasmodium multivacuolaris* (Genbank FJ404720) that were both isolated from *Andropadus latirostris* Strickland. Group B, a sister Group to A, represented rare lineages isolated from *M. uniformis* and *Culex guiarti* Edwards. Most isolations from mosquitoes fell within Group C and showed the most similarity to the PV11 lineage previously isolated from *Ploceus aurantius* Vieillot (Boneaud *et al*. 2009). Interestingly, this lineage was found in mosquitoes of three genera, *Aedes, Coquillettidia*, and *Culex*. Group D consisted of lineages isolated from *Coquillettidia* spp. and *Culex* spp. and was more closely affiliated with lineage PV23L of Chasar *et al*. (2009), isolated from *Cyanomitra olivacea* Smith, A, while Group E consisted of lineages affiliated with *Plasmodium lucens* (FJ404708), PV12 (GQ150196, FJ404701), pGRW9 (DQ060773), Hap46 (DQ839085), and *Plasmodium megaglobularis* (FJ404703) isolated from *C. olivacea*. Figure 4 shows the distribution of the parasite lineages among the four most common mosquito genera.

## Discussion

### Distribution and patterns of parasite lineage diversity across mosquito genera and avian hosts

Although there is widespread belief that most haemosporidian parasites are strict host specialists (Bensch *et al*. 2000; Ricklefs & Fallon 2002), several recent studies on species of *Plasmodium* and *Haemoproteus* showed considerable variation in host breadth in numerous

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lineages of the haematozoa (Fallon *et al*. 2003; Beadell *et al*. 2004, 2009; Križanauskienė *et al*. 2006; Svensson and Ricklefs 2009). Our data show that some *Plasmodium* spp. lineages are found in mosquito species of different genera. This distribution of parasite lineages suggests that multiple assemblages of common parasite lineages may potentially be transmitted by different mosquito species that infect multiple avian host species. This level of sharing of lineages, though moderate, might be due to the similar ecology of these mosquito species. For example, in our phylogeny, *Plasmodium* lineage PV11 was detected in mosquitoes of three divergent genera (*Aedes, Coquillettidia*, and *Culex*), suggesting that some malaria lineages have low vector specificity (Figures 3 and 4). Their frequent capture in the same traps suggests that they have similar temporal and spatial foraging habits and, therefore, may encounter the same suite of potential hosts.

A comparison of these parasite lineages with those previously detected in avian hosts from this region revealed that three *Plasmodium* lineages are shared among vertebrate and invertebrate hosts. These roughly correspond to the clades of closely related *Plasmodium* lineages that likely belong to the same morphospecies of parasites (Chasar *et al*. 2009). Of note, these lineages showed marked vertebrate host-species fidelity in the Chasar *et al*. (2009) study. Of the 22 observed haemosporidian lineages isolated from these mosquitoes, 15 *Plasmodium* spp. were newly described. Four lineages have been found in previous studies in more than one vertebrate host species in different bird families and on different continents (Beadell *et al*. 2009; Bonneaud *et al*. 2009; Chasar *et al*. 2009). Lineage PV11, one of the most common lineages found in three mosquito genera, has been isolated in numerous bird species throughout West Africa (Loiseau *et al*. 2010) and other parts of the world (Ishtiaq *et al*. 2008). Lineages PV3, PV12, and PlasCoq6 have also been found in different bird species (Bonneaud *et al*. 2009; Chasar *et al*. 2009) and these lineages were found in at least two mosquito species of different genera. These agree with results from Gager *et al*. (2008) and other experimental studies which demonstrated successful development of the same strains of many *Plasmodium* species in mosquitoes belonging to the same and different genera (Valkiūnas 2005). Parasites with broad host range are generally thought to have low fitness cost to their hosts, but may achieve higher abundance and face reduced extinction risk relative to specialists (Woolhouse *et al*. 2001; Beadell *et al* 2009). Overall, the high *Plasmodium* spp. lineage diversity in mosquitoes suggests that avian malaria is very diverse in the rainforests of Cameroon.

Up to 15 unique *Plasmodium* parasite lineages found here in mosquitoes have not been detected in avian hosts to date. This may reflect the limited sampling of avian hosts at our study sites or biased capture methods. Most studies on avian malaria parasites are markedly biased towards small, common passerine birds or small birds of other avian orders that are easily captured in mist nets in the understory. This misses the larger birds and the great diversity of bird species that live in the canopy. Our results suggest that more hosts need to be sampled, using other capture methods, to realize the high diversity of parasites in vector communities. Additionally, some of the recorded lineages may have come from reptiles and not from birds, as reptile *Plasmodium* spp. frequently cluster with avian species in cyt *b* trees (Martinsen *et al*. 2008). It should also be noted that PCR could amplify the DNA of sporozoites that are injected into the bloodstream by vectors, but that these sporozoites may not develop in birds (Valkiūnas et al. 2009). Examining the records of blood stages will be needed to determine if lineages detected in avian hosts are those of developing parasites. We thus emphasize the value of both PCR and microscopy in studies on the distribution and ecology of avian hemosporidian parasites in natural populations.

Importantly, sporozoites of *Plasmodium* spp. which coincide with morphology and size of sporozoites of *Plasmodium (Novyella) rouxi*, a widespread malaria parasite of passeriform

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birds (Garnham 1966), were observed in the salivary glands of *Cq. aurites*, thus providing further evidence of the role of these mosquitoes as vectors of avian *Plasmodium*.

An interesting finding of this study was the detection of *Haemoproteus* spp. lineages in some mosquito species. There is only one other report of the isolation of *Haemoproteus* spp. lineages in culicine mosquitoes using PCR-based methods (Ishtiaq *et al*. 2008). A possible explanation for the isolation of *Haemoproteus* spp. in mosquitoes might be the result of amplifying parasite residue in the digestive system (located between mouth and midgut) picked up during bloodmeals on infected vertebrates (see Kim *et al*. 2009b). This should be considered, in particular, because our mosquito samples were initially pooled in the field. However, because we collected unfed mosquitoes, this seems unlikely. After about 50 hours of feeding, the bloodmeal in the gut of mosquitoes is sufficiently digested and degraded that it makes any identification by PCR-based techniques problematic (Yohannes *et al*. 2008). Additionally, ectopic development of haemosporidian parasites in non-vector mosquitoes, as reported for *P. gallinaceum* developing in a non-vector fruit fly *Drosophila melanogaster*, should also be considered (Schneider and Shahabuddin 2000). As of yet, there is no evidence of *Haemoproteus* spp. sporogony and transmission by mosquito species.

## Differences in Plasmodium spp. occurrence among mosquito species

Few species of mosquitoes have been documented as competent for the transmission of avian *Plasmodium* species. Those that have, are most commonly of the genera *Culex* (LaPointe *et al*., 2005), *Aedes*, and *Culiseta*. A few additional species have been recorded that support complete development from the genera *Anopheles, Psorophora*, and *Mansonia* (Valkiūnas 2005), and, most recently, in *Coquillettidia* (Ishtiaq *et al*. 2008; Njabo *et al*. 2009). We were not expecting to find equal levels of infection rates of *Plasmodium* spp. in all the mosquito species we tested. Only ten of the 27 species sampled were positive for malaria parasites, and our results are consistent with the general finding that the probability of infection with avian *Plasmodium* spp. should vary among mosquito species (LaPointe *et al*., 2005; Gager *et al*., 2008). Our results are also consistent with the fact that the evolution of major clades of parasites correlates with vector shifts into different dipteran families, presumably by giving parasites access to new hosts (Martinsen *et al*. 2008).

The high infection rates of *Plasmodium* spp. in *Culex* and *Coquillettidia* (Figure 4) relative to other mosquito species suggested that species of these two genera are important vectors of avian malaria at our study sites. The apparent absence of *Plasmodium* spp. infection in other species (*Ae. microstictus, Ae. domesticus, Cx. Vansomereni*), in any of the anophelines, or in any species of the other genera (*Hodgesia*, *Mimomyia*, *Uranotaenia, Lutzia*, *Eretmopodites*) might be due to these species not being competent vectors of avian *Plasmodium*. All species except for *Eretmopodites* spp. were captured in the bird-baited traps and hence would have opportunities to ingest and be exposed to the parasites. Major developmental losses during gametogenesis, ookinete, and oocyst development may cause them to be completely refractory (Alavi *et al*. 2003). *Anopheles gambiae* and *Ae. aegypti* have been shown to transmit *P. gallinaceum* in controlled experiments (Garnham 1966;Alavi *et al*. 2003), but in the wild, *Anopheles* spp. are known to be vectors of *Plasmodium* spp. of mammals (Killick-Kendrick 1978). This possibility of transmission of avian *Plasmodium* spp. (albeit in controlled environments) suggests that the shift of *Plasmodium* spp. into new hosts may be associated with specialization on vectors (Martinsen *et al*. 2008). It is worth noting that most *Plasmodium* species use mosquitoes (Culicidae) as vectors, with few exceptions (e.g., a lizard malaria parasite *Plasmodium mexicanum* is transmitted by sandflies) (Psychodidae; Ayala and Lee 1970;Fialho and Schall 1995). *Plasmodium agamae*, also a parasite of reptiles, completes its development in biting midges (Petit *et al*. 1983). Such a broad range of vectors for lizard malaria parasites might testify to the ancient origin of reptile malaria

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parasites in comparison to the *Plasmodium* spp. of birds and mammals (see Valkiūnas 2005).

### The use of molecular methods in detecting avian haemosporidian parasites

The application of polymerase chain reaction (PCR) and sequencing to determine parasite presence and identity has opened up avenues for understanding vector-parasite (Hellgren *et al*. 2007; Ejiri *et al*. 2008; Ishtiaq *et al*. 2008; Kimura *et al*. 2010; Njabo *et al*. 2009) and host-parasite interactions (Bensch *et al*. 2000; Perkins and Schall 2002; Ricklefs and Fallon 2002; Waldenström *et al*. 2002; Fallon *et al*. 2003; Beadell *et al*. 2004; Bensch *et al*. 2004) in many haemosporidian parasite communities. HRM was more effective in detecting positive pools (30%) than nested PCR (25%), which further highlights some limitations of nested PCR-based approaches and the need for more sampling methods in wild-caught mosquito species to capture the full parasite range. Although we cannot fully rule out false positives for some of the HRM positives, sequence data for most of these (97%), as shown by nested PCR, reduces this tendency. While usually highly effective, the success of HRM analysis depends largely on the particular sequence under investigation (Montgomery *et al*. 2007). We should point out that the PCR-based approach is limited by its inability to specifically target salivary gland sporozoites and that we cannot conclude that isolations made from all mosquitoes in this study necessarily confirmed that they were vectors (see Kim *et al*. 2009b). However, this study does confirm previous observations by Njabo *et al*. (2009) that *Plasmodium* lineages are capable of achieving a sporozoite stage in *Cq. aurites*. Further studies are needed to confirm the presence of sporozoites in other wild-caught mosquito species and to determine their transmission capabilities. Additional vector competence studies would also be useful, including tests involving the experimental infection of vectors.

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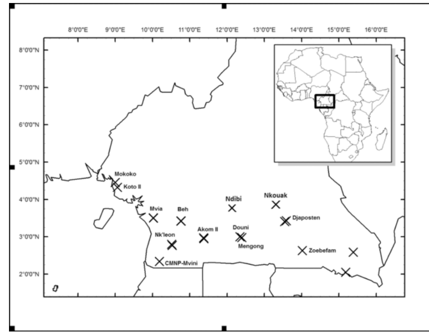


**Figure 1.**
Map showing sampling sites (crosses) where culicine mosquito species were collected in the lowland forest of Cameroon in West Africa.



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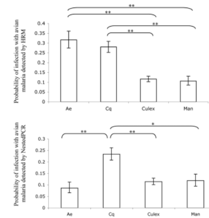


**Figure 2.**
The probability that species of the four most common mosquito genera were infected with avian malaria detected either by (a) HRM or (b) nested PCR was modeled using a generalized linear model. We show the back-transformed mean probabilities of infection (± SE). Single asterisk indicates significance at $p < 0.05$ and double asterisk indicates significance at $p < 0.001$. Discrepancy between (a) and (b) likely due to removal of mixed species infections



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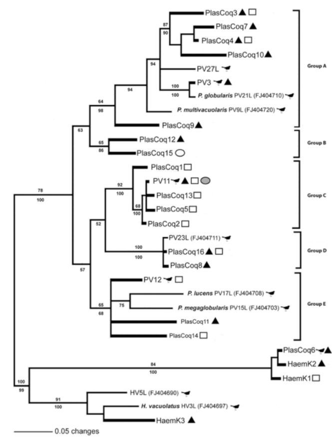


**Figure 3.**

Bayesian phylogenetic analysis of mosquito-isolated *Plasmodium* spp. cytochrome *b* lineages. Lineages isolated from mosquitoes are indicated with filled triangles representing *Culex* spp, open squares *Coquillettidia* spp., open circle *Mansonia uniformis*. grey circle *Aedes mcintoshi*, and open circle *Mansonia uniformis*. Lineages detected from this study are shown in bold lines and those previously isolated from birds are indicated with a bird icon. Major groups, A–E are indicated by vertical bars and correspond to closely related lineages that belong, or likely belong, to the same morphospecies of parasites. Numbers located on the top of the branches indicate bootstrap support (ML 200) and below are from Bayesian probability values. Scale bar indicates number of nucleotide substitutions per site.

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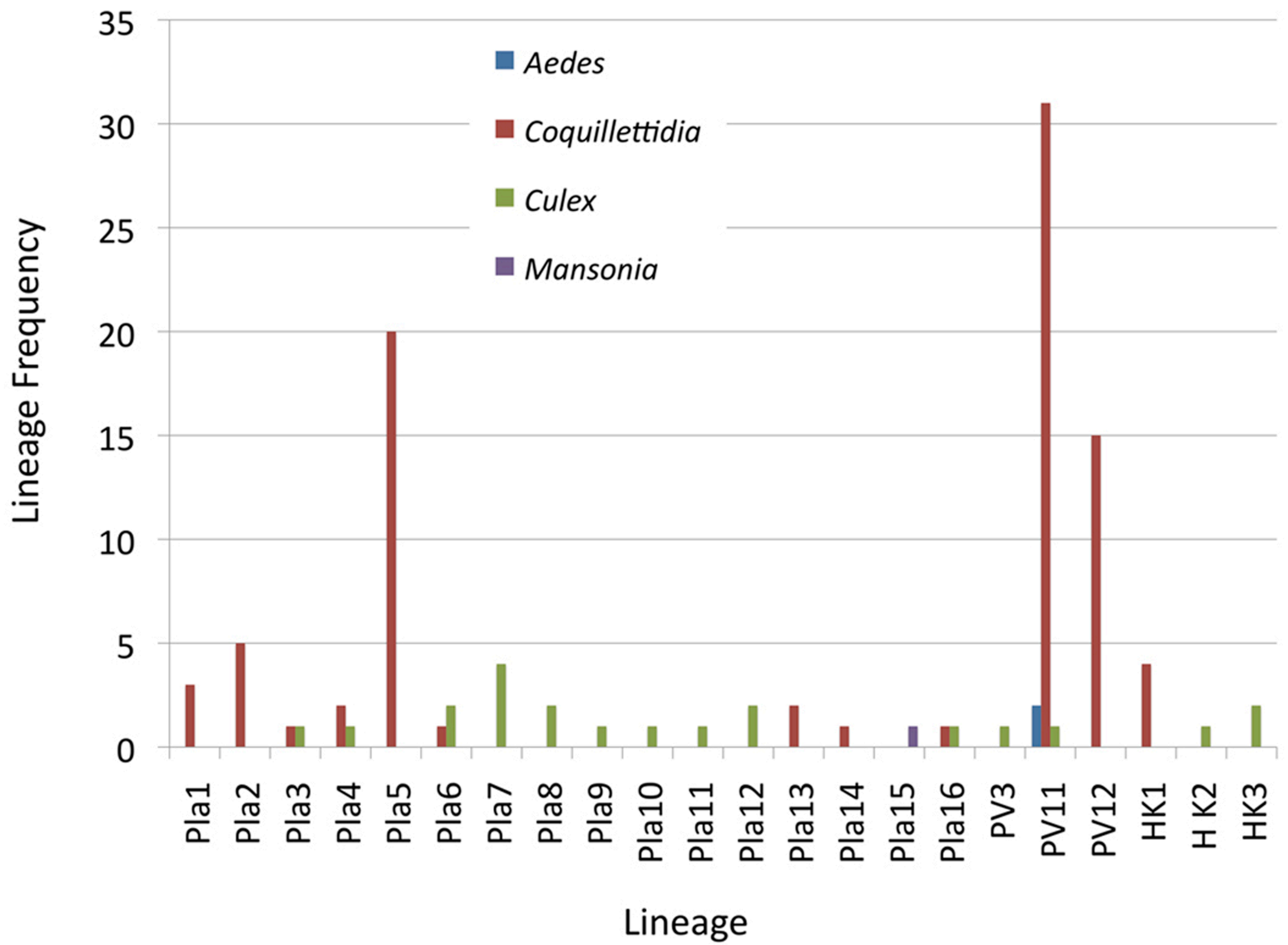


**Figure 4.**
Distribution of parasite lineages among the four most common mosquito genera.

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### Table 1

Avian *Plasmodium* parasite occurrence in female mosquito species collected in the lowland forests of Cameroon, 2007–2008

| Mosquito species | Sampling location | Number of individuals | Pool size | Proportion of positive pools (percentage) | MLE/1000 |
|---|---|---|---|---|---|
| *Aedes mcintoshi* Huang | Nd, Nk, Mp, Nkl, Mv, Ko, Mo | 277 | 25 | 4 (0.16) | 15.29 (36.46, 5.14) |
| *Aedes domesticus* Theobald and *Aedes microstictus* Edwards | Nd, Zo | 3 | 1 | 0 | 0.00 |
| *Anopheles coustani* Laveran | Nd, Mv, Mp, Be, Mv, Mo, Nk | 43 | 7 | 0 | 0.00 |
| *Anopheles funestus* Group | Nd | 4 | 2 | 0 | 0.00 |
| *Anopheles gambiae* complex | Nkl | 2 | 2 | 0 | 0.00 |
| *Anopheles hancocki* Edwards | Nd | 2 | 2 | 0 | 0.00 |
| *Anopheles nili* Theobald | Nd | 4 | 2 | 0 | 0.00 |
| *Coquillettidia aurites* Theobald | Nd, NK | 1118 | 230 | 96 (0.42) | 105.22 (126.78, 86.52) |
| *Coquillettidia metallica* Theobald | Nd,Nk | 21 | 6 | 2 (0.33) | 106.91 (332.99, 20.03) |
| *Coquillettidia pseudoconopas* Theobald | Nk, Mv | 184 | 32 | 11 (0.34) | 71.30 (122.70, 38.19) |
| *Lutzia tigripes* (de Grandpre and de Charmoy) | Kt, Nd, Nk | 4 | 2 | 0 | 0.00 |
| *Culex annulioris* var. *major* Edwards | Nd, Nk, Mv | 66 | 8 | 2 (0.25) | 30.40 (96.95, 5.88) |

| Mosquito species | Sampling location | Number of individuals | Pool size | Proportion of positive pools (percentage) | MLE/1000 |
|---|---|---|---|---|---|
| *Culex neavei* Theobald | Nd, Nk | 138 | 16 | 8 (0.50) | 75.67 (144.56, 36.30) |
| *Culex perfidiosus* | Nd | 138 | 16 | 7 (0.44) | 62.61 (123.59, 28.53) |
| Edwards *Culex poicilipes* Theobald | Nd, Nk | 25 | 3 | 2 (0.67) | 100.22 (400.22, 20.25) |
| *Culex guiarti* Blanchard | Nd, Nk Kt, Mv, Mp, Be, Nkl, Mo, Ak | 335 | 37 | 1(0.03) | 2.97 (14.35, 0.17) |
| *Culex vansomereni* Edwards | Nd | 28 | 4 | 0 | 0.00 |
| *Eretmapodites chrysogaster* Graham | Ko, Mo, Nd, Nk | 5 | 3 | 0 | 0.00 |
| *Hodgesia psectropus* Edwards | Nd | 61 | 5 | 0 | 0.00 |
| *Mansonia uniformis* Theobald | Nd | 515 | 32 | 2 (0.06) | 3.93 (12.87, 0.71) |
| *Mimomyia lacustris* Theobald and *hispida* Edwards | Nd | 13 | 1 | 0 | 0.00 |
| *Uranotaenia alboabdominalis* Theobald | Nd | 40 | 4 | 0 | 0.00 |
| *Uranotaenia coerulocephata* Theobald | Mo, Nd, Zo | 4 | 2 | 0 | 0.00 |
| *Uranotaenia bilineata* Theobald | Nd, Nk, Zo | 36 | 1 | 0 | 0.00 |
| *Uranotaenia balfouri* Theobald | Nd | 63 | 6 | 0 | 0.00 |
| *Uranotaenia mashonaensis Theobald* | Mo, Nd | 5 | 1 | 0 | 0.00 |



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Mv: Mvia, Mp: Mpombo, Be: Beh, Nkl: Nk'leon, Mv: Mvini, Ko: Koto II,Mk:Mokoko, Nd: Ndibi, Nk: Nkouak, Zo: Zoebefam; CMP: Campo Ma'an, Ak: Akom II. Pool size refers to the number of individuals combined by species/date/location into a single sample of DNA extraction. MLE represents Maximum Likelihood Estimation (per 1000 mosquitoes) (upper, lower 95% confidence limits) based on pool size