OIKOS

Research

Diversification by host switching and dispersal shaped the diversity and distribution of avian malaria parasites in Amazonia

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Oikos
127: 1233–1242, 2018
doi: 10.1111/oik.05115

Subject Editor: Silke Bauer
Editor-in-Chief: Dries Bonte
Accepted 16 February 2018

Understanding how pathogens and parasites diversify through time and space is fundamental to predicting emerging infectious diseases. Here, we use biogeographic, coevolutionary and phylogenetic analyses to describe the origin, diversity, and distribution of avian malaria parasites in the most diverse avifauna on Earth. We first performed phylogenetic analyses using the mitochondrial cytochrome *b* (cyt *b*) gene to determine relationships among parasite lineages. Then, we estimated divergence times and reconstructed ancestral areas to uncover how landscape evolution has shaped the diversification of *Parahaemoproteus* and *Plasmodium* in Amazonia. Finally, we assessed the coevolutionary patterns of diversification in this host–parasite system to determine how coevolution may have influenced the contemporary diversity of avian malaria parasites and their distribution among Amazonian birds.

Biogeographic analysis of 324 haemosporidian parasite lineages recovered from 4178 individual birds provided strong evidence that these parasites readily disperse across major Amazonian rivers and this has occurred with increasing frequency over the last five million years. We also recovered many duplication events within areas of endemism in Amazonia. Cophylogenetic analyses of these blood parasites and their avian hosts support a diversification history dominated by host switching. The ability of avian malaria parasites to disperse geographically and shift among avian hosts has played a major role in their radiation and has shaped the current distribution and diversity of these parasites across Amazonia.

Keywords: macroevolution, parasite diversity, parasite dispersal

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Synthesis

Pathogens must overcome ecological and evolutionary barriers to infect their hosts. A broad survey of malaria infections of Amazonian birds shows that parasite diversity and distributions across Amazonia are outcomes of macroevolutionary patterns of host switching, host phylogenetic relationships, and recent dispersal across ecological barriers. Diversification of avian malaria parasites in the most diverse region on Earth primarily follows host relatedness, and does not support the Riverine Barrier Hypothesis as the primary mechanism underlying diversification of this group of organisms in the Amazon Basin.

Introduction

Dispersal followed by isolation and allopatric speciation is likely a key driver of diversification in parasite lineages (Poulin 2007, Hoberg and Brooks 2008, Ricklefs 2010) and has played a significant role in the macroevolutionary relationships between parasites and their hosts (Johnson et al. 2003, Jenkins et al. 2012). For example, a study on the diversity and distribution of avian protozoan ('malaria') parasites has shown that, despite their distributions being constrained across six Amazonian areas of endemism, these pathogens were not dispersal-limited among avian communities within or between areas of endemism (Fecchio et al. 2017a). Recent studies in Amazonia have shown that multiple malaria lineages coexist in the same bird communities with varying levels of host prevalence and specialization, with host exploitation ranging from infecting one to several distantly-related avian host species (Svensson-Coelho et al. 2013, Moens and Pérez-Tris 2016, Fecchio et al. 2017a, b). Host switching has been implicated as the main pattern in the evolutionary history and diversification of this group of parasites in both the New World (Ricklefs et al. 2004, 2014, Galen and Witt 2014, Santiago-Alarcon et al. 2014, Ellis et al. 2015, Fecchio et al. 2017a, c) and the Old World (Bensch et al. 2000, Hellgren et al. 2007, Lauron et al. 2015). Therefore, knowing how and when parasites and pathogens disperse among areas or shift to a new host species is essential for predicting the occurrence of emerging infectious diseases and to understanding how microevolutionary processes such as dispersal have shaped macroevolution (host switching) and thus the contemporary diversity of these organisms.

Evolutionary processes are thought to determine contemporary patterns of parasite diversity due to their potential influence on colonization and extinction of these organisms (Poulin 1997, 2007, Johnson et. al. 2003, Clayton et al. 2016). At macroevolutionary timescales, parasite communities are assembled on a given set of hosts as a result of five possible host–parasite coevolutionary events: cospeciation, sorting (extinction and 'missing the boat' i.e. failing to colonize all descendants of a host speciation event), duplication (intra-host speciation), cohesion (also called 'inertia' or 'failure to speciate'), and host switching (Clayton et al. 2016). Cospeciation is thought to be the most important mechanism structuring host–parasite assemblages for parasites having a close association with their host species and limited ability to disperse between hosts (e.g. some lineages of feather lice) (Clayton et al. 2016), although studies of some louse lineages indicate that ongoing dispersal and 'inertia' likely affect the macroevolutionary history of these host parasite systems (Weckstein 2004). However, for such vector-transmitted parasites as avian malaria parasites, which may be less dispersal limited, codivergence and thus cospeciation are also thought to be less important in their host–parasite macroevolutionary history (Ricklefs et al. 2004, 2014, Santiago-Alarcon et al. 2014, Lauron et al. 2015). Accordingly, investigation of evolutionary processes other than cospeciation, especially in a spatiotemporal framework, might help us to understand the diversification of avian malaria parasites.

Amazonia, the largest and most speciose tropical forest on Earth, has long been famous for its astonishing levels of vertebrate diversity and endemism (Wallace 1852). Many hypotheses have been postulated for the evolutionary processes that have promoted diversification within Amazonia, of which two predominate. First, the Pleistocene refuge hypothesis (Haffer 1969), postulates that climatic change periodically caused the retraction and fragmentation of this rainforest, producing isolated forest refugia surrounded by savanna and seasonally dry forest, thus facilitating allopatric speciation in Amazonia. Second, the riverine barrier hypothesis (Wallace 1852), proposes that large Amazonian rivers acted as barriers to dispersal and promoted speciation. Recent studies have shown that the large rivers of the Amazon Basin are important barriers to gene flow and evidently have been responsible for allopatric speciation in avian taxa (Ribas et al. 2012, Thom and Aleixo 2015, Ferreira et al. 2017). However, Smith et al. (2014) questioned the association between river drainage systems and the diversification of avian taxa. They argued that, rather than landscape changes acting as principle drivers of diversification in the basin, the ability of avian taxa to disperse and persist in the landscape, were the principle drivers of speciation within 27 widespread bird lineages across the Neotropics. This view highlights the importance of using lineages with different dispersal capacities, and taxa other than vertebrates, to uncover how biogeography has generally influenced diversification within Amazonia.

Parasites from the genera *Haemoproteus* (including the subgenera *Haemoproteus* and *Parahaemoproteus*) and *Plasmodium* comprise a diverse group of pathogens that are distributed worldwide and infect virtually all avian clades (Valkiūnas 2005, Clark et al. 2014). These intracellular parasites reproduce asexually in their vertebrate avian hosts and undergo complete sexual reproduction in hematophagous

female mosquitos (Diptera: Culicidae), biting midges (Diptera: Ceratopogonidae), and hippoboscid flies (Diptera: Hippoboscidae) (Valkiūnas 2005, Santiago-Alarcon et al. 2012), which act as vectors of *Plasmodium*, *Parahaemoproteus*, and *Haemoproteus*, respectively (Valkiūnas 2005, Santiago-Alarcon et al. 2012). Over the past two decades, the development of molecular tools for detecting and identifying avian malaria parasites (Bensch et al. 2000, 2009) has led to a greater understanding of host-specificity and host–parasite evolutionary relationships (Bensch et al. 2000, Waldenström et al. 2002, Ricklefs et al. 2004, Križanauskienė et al. 2006, Beadell et al. 2009, Moens and Pérez-Tris 2016).

Here we use the mitochondrial cytochrome *b* (cyt *b*) gene to delineate haemosporidian lineages and explore the evolutionary history of these parasites and their avian hosts throughout the Amazon Basin. Integrating phylogenetic and distributional relationships of parasite lineages, we estimated divergence times and reconstructed ancestral areas to unravel the role of rivers, as well as areas of endemism, in shaping diversification of Amazonian *Parahaemoproteus* and *Plasmodium*. We also assessed the coevolutionary patterns of diversification in this host–parasite system to reconstruct the patterns of macroevolutionary history among hosts and parasites and to determine how they may have influenced the contemporary distribution and diversity of malarial parasites in Amazonian birds.

Material and methods

Data collection

We analyzed 4178 samples from Amazonia, of which 2661 were published by Fecchio et al. (2017a) and 345 by Moens and Pérez-Tris (2016). The published samples include 25 locations in Brazil and one in Ecuador. To cover all areas of endemism in Amazonia, we analyzed 1172 additional samples from Brazil and Peru. In Peru, blood samples were collected from birds netted in the Centro de Investigación y Conservación de Río Los Amigos (CICRA). After blood collection in Peru, birds were ringed and released. In Brazil, liver samples were taken during specimen preparation at Floresta Nacional (FLONA) Caxiuanã, in the state of Pará, in accordance with corresponding permits. All blood and liver samples were stored in 95% ethanol until DNA extraction. All tissue samples and birds were collected under appropriate permits in Brazil and Peru. Our final sampling encompassed eight Amazonian areas of endemism from 28 locations across Brazil, Ecuador and Peru (Fig. 1, Supplementary material Appendix1).

Molecular screening of haemosporidian parasites

DNA was extracted using either phenol-chloroform or the Qiagen DNeasy 96 Blood and Tissue kit, following the Qiagen tissue protocol for both blood and liver stored in 95% ethanol. The protocols of Bell et al. (2015) were used to screen the 1172 samples for haemosporidian DNA with real-time PCR and then to amplify a 477 bp region of the cytochrome *b* gene from positive samples using nested PCR. All real-time PCRs were carried out using iTaq universal SYBR Green Supermix on a

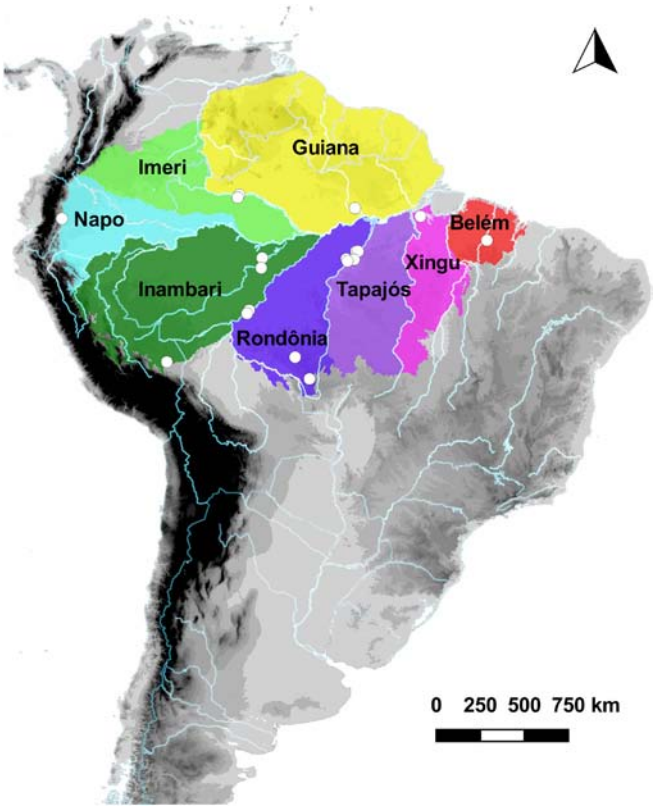

Figure 1. Sampling locations across eight areas of endemism in Amazonia. Coordinates for each site, number of individuals captured, and bird and parasite richness are available as Supplementary material Appendix 1.

real-time thermocycler, whereas nested PCRs were run using OneTaq Quick-Load 2X Master Mix with standard buffer. All samples identified as positive by real-time PCR underwent nested PCR amplifications of the *Haemoproteus/Plasmodium* mitochondrial cytochrome *b* gene. All PCR products were run on 1.25% agarose gels, stained with ethidium bromide, visualized under UV light, and photographed.

Positive PCR products were purified using ExoSAP-IT and sequenced using BigDye terminator v3.1 cycle sequencing kit. Cycle sequencing reaction products were purified using ethanol precipitation and were then re-suspended in 10 µl of dH_2O, and run on an DNA sequencer. The primers FIFI and R2 (Ishtiaq et al. 2007) were used for sequencing purified *Haemoproteus*/*Plasmodium* positive nested PCR products.

Forward and reverse sequences were visualized and assembled using Sequencher ver. 5.0.1. Chromatograms that showed the presence of multiple infections were scored as coinfections. Coinfections were separated using the program PHASE 2.1.1 (Stephens et al. 2001, Stephens and Donnelly 2003) following the protocol of Harrigan et al. (2014). Coinfections that could not be resolved were removed from further analyses.

Assembled sequences were aligned using BioEdit ver. 7.2.0 (Hall 1999) and collapsed to unique haplotypes using the FaBox haplotype collapser and converter tool (Villesen 2007). Sequence identities were verified with a local BLAST against the MalAvi database (Bensch et al. 2009) using BioEdit

ver. 7.2.0 (Hall 1999). New lineages were named after the host of origin following standard protocol (Bensch et al. 2009), using a six letter code produced by using the first three letters of both the host genus and specific epithet followed by a number to denote multiple lineages from a single host species. For example, lineage PHEGEN01 is the first lineage obtained from *Pheugopedius genibarbis*. DNA sequences of new lineages were deposited in GenBank and the MalAvi database (Supplementary material Appendix 6 and 7 for lineage names and associated accession numbers for all lineages used in this study).

Phylogenetic reconstruction

Assembled sequences of unique haplotypes were used to infer molecular phylogenies. We used the GTR+I+G model of nucleotide substitution as determined by jModelTest (Guindon and Gascuel 2003, Darriba et al. 2012). For clarity of visualization, lineages were split into two separate alignments, one for the subgenus *Parahaemoproteus* and one for the genus *Plasmodium*. For both alignments, *Leucocytozoon fringillarum* (accession no. FJ168564) was used as the outgroup. The subgenus *Haemoproteus* was excluded from the analyses because only 11 individuals were infected by this subgenus and the lineages were not represented in all areas of endemism.

We obtained a time-calibrated tree for each parasite lineage alignment using the Bayesian relaxed clock model (Drummond et al. 2006) in BEAST ver. 1.8.4 (Drummond et al. 2012) implemented on the CIPRES Science Gateway (Miller et al. 2010). The analysis was conducted for 64 lineages of *Parahaemoproteus* and 260 lineages of *Plasmodium*. We generated two independent runs for each alignment with parameters as follows: an uncorrelated lognormal relaxed clock, Yule process, 100 million generations of MCMC (Markov chain Monte Carlo), parameters sampled every 5000 generations, and 10% of generations discarded as burn-in. To obtain absolute ages for cladogenetic events through malaria trees, we used the mutation rate of 0.006 per lineage per million years estimated by Ricklefs and Outlaw (2010). Secondly, we conducted a second set of time tree analyses using a recently published substitution rate for avian malaria parasites estimated by Pacheco et al. (2018) based on whole mitochondrial genome sequences, as a uniform prior ranging from 0.00334 to 0.00487 substitutions per lineage per million years. Convergence and performance of runs were inspected using Tracer 1.6 (<http://beast.bio.ed.ac.uk/Tracer>), to ensure that ESS (effective sample size) values exceeded 200. The maximum clade credibility (MCC) tree for each malaria lineage was generated using TreeAnnotator. Time trees were visualized in FigTree ver. 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

To determine whether area of endemism had a significant phylogenetic signal in the evolutionary history of *Parahaemoproteus* and *Plasmodium* within Amazonia, we conducted a randomization test (Maddison and Slatkin 1991) for each haemosporidian clade in R ver. 3.3.2 (<www.r-project.org>), as described by Bush et al. (2016). A significant result would indicate that the area of endemism distribution within the tree topology is more conserved than expected by chance, showing a significant biogeographic constraint within the phylogeny.

Ancestral area reconstruction

We reconstructed ancestral areas on internal nodes of the avian malaria parasite MCC trees using the package BioGeoBEARS (BioGeography with Bayesian Evolutionary Analysis in R Scripts; Matzke 2014) implemented in R ver. 3.3.2. We tested six distinct biogeographic models available in BioGeoBEARS: DEC, DIVALIKE and BAYAREALIKE, each of them with a parameter *j* that corresponds to founder event speciation and allows for long-distance dispersal events. We considered eight geographical areas in Amazonia (Fig. 1): Belém, Guiana, Imeri, Inambari, Napo, Rondônia, Tapajós and Xingu, following Smith et al. (2014). Lineage distributions were coded as presence or absence in each of the eight areas. We set the maximum number of areas to six and pruned the outgroup from the tree prior to analysis. Analyses were implemented without constraints. Likelihood values of each of the six different BioGeoBEARS models were compared using Akaike information criterion (AIC).

Coevolutionary analysis

To reconstruct the coevolutionary history of *Parahaemoproteus* and *Plasmodium* and their avian hosts within Amazonia, we conducted cophylogenetic analyses using CoRe-PA. CoRe-PA (Merkle et al. 2010) is an event cost analysis, which determines the most probable coevolutionary history based on specific event costs. This analysis identifies the events that provide the best explanation of the cophylogenetic patterns observed within a tanglegram of host and parasite phylogenetic trees. The events include codivergence (cospeciation), sorting (extinction), duplication (within-host speciation), and host switching (Merkle et al. 2010). As for the ancestral reconstruction, *Parahaemoproteus* and *Plasmodium* were analyzed separately.

Tanglegrams were constructed using the above described parasite trees with the host trees reconstructed in BEAST (Drummond et al. 2012) using avian cytochrome oxidase I (COI) sequences available from Genbank and aligned in BioEdit ver. 7.2.0 (Hall 1999). For host species without available Genbank sequences, we used sequences from the closest related species available for phylogenetic reconstruction.

Four separate analyses, each with a different cost matrix for the four events, were conducted. Event cost values used within cost matrices were taken directly from the previous studies of Ricklefs and Fallon (2002), Ricklefs et al. (2004) and Santiago-Alarcon et al. (2014). For each of the four analyses, 100 randomizations were conducted to determine whether the number of each event differed significantly from random associations between host and parasite trees.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.c1d1f8h> (Fecchio et al. 2018) and the MalAvi database (DOI: 10.1111/j.1755-0998.2009.02692.x).

Results

Biogeography of haemosporidian parasites within Amazonia

Divergence time estimates revealed that, in both haemosporidian genera, diversification of Amazonian malaria parasites took place in the late Tertiary to Quaternary, beginning in the middle Miocene (~15 Mya; Fig. 2–3). Under the six biogeographical models evaluated using BioGeoBEARS, the best fit model for both *Parahaemoproteus* and *Plasmodium* was DEC+J (Supplementary material Appendix 2, 3). The ancestral area reconstruction among the eight areas of endemism using the DEC+J model showed a noticeable impact of some areas of endemism on parasite relatedness for *Parahaemoproteus*, with several clades formed entirely of lineages recovered from a single area of endemism. Furthermore, endemic lineages for some areas formed distinct clades in the phylogeny (e.g. Rondônia lineages) and endemic lineages of different geographical regions diversified over distinct times (Fig. 2). The Madison–Slatkin test indicated that areas of endemism have significant phylogenetic signal for *Parahaemoproteus* ($p < 0.001$). The Rondônia area of endemism was especially well represented in our data set, which allowed a more definite conclusion regarding the impact of area of endemism on parasite relatedness. A number of *Parahaemoproteus* lineages from Rondônia clustered together in the phylogenetic tree.

For *Plasmodium* lineages, the biogeographic signal was weaker, with many larger clades composed of lineages recovered from several areas of endemism (Fig. 3). Although

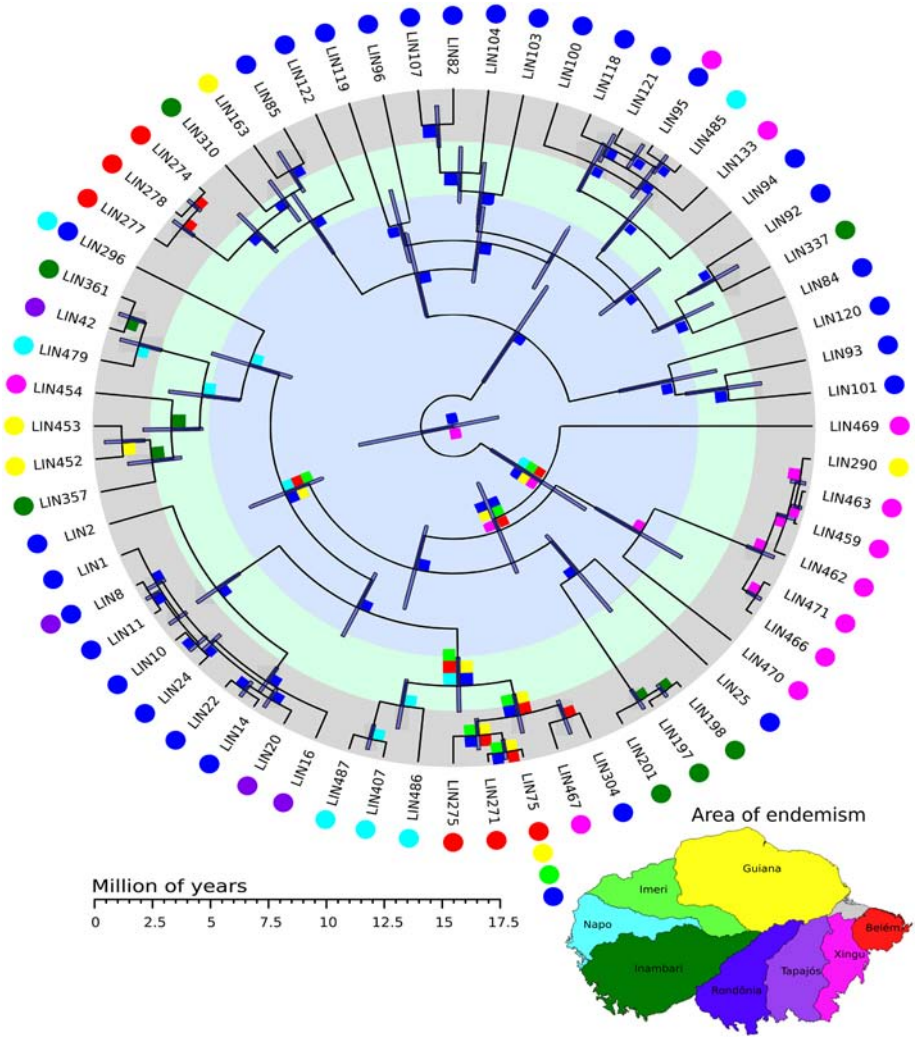

Figure 2. Phylogeny reconstructed using Bayesian inference of 64 *Parahaemoproteus* lineages and Amazonian ancestral area reconstructions. Blue bars labeling each node are the 95% credibility interval of divergence time estimates for each lineage. The Miocene (23–5.3 Mya), Pliocene (5.3–2.6 Mya), and Quaternary (2.6 Mya–present) periods are shaded in light blue, light green and light grey, respectively. Terminal taxa are colored according to the area of endemism in which they were found. Colored squares labeling ancestral nodes indicate the most likely ancestral area estimated by analysis in BioGeoBEARS on DEC+J model. The color scheme for terminal taxon labels and ancestral areas matches the eight areas of endemism colored in the inset map and on Fig. 1. Lineage names and associated accession numbers used in phylogeny reconstruction along with the host species infected by each lineage are available in Supplementary material Appendix 6.

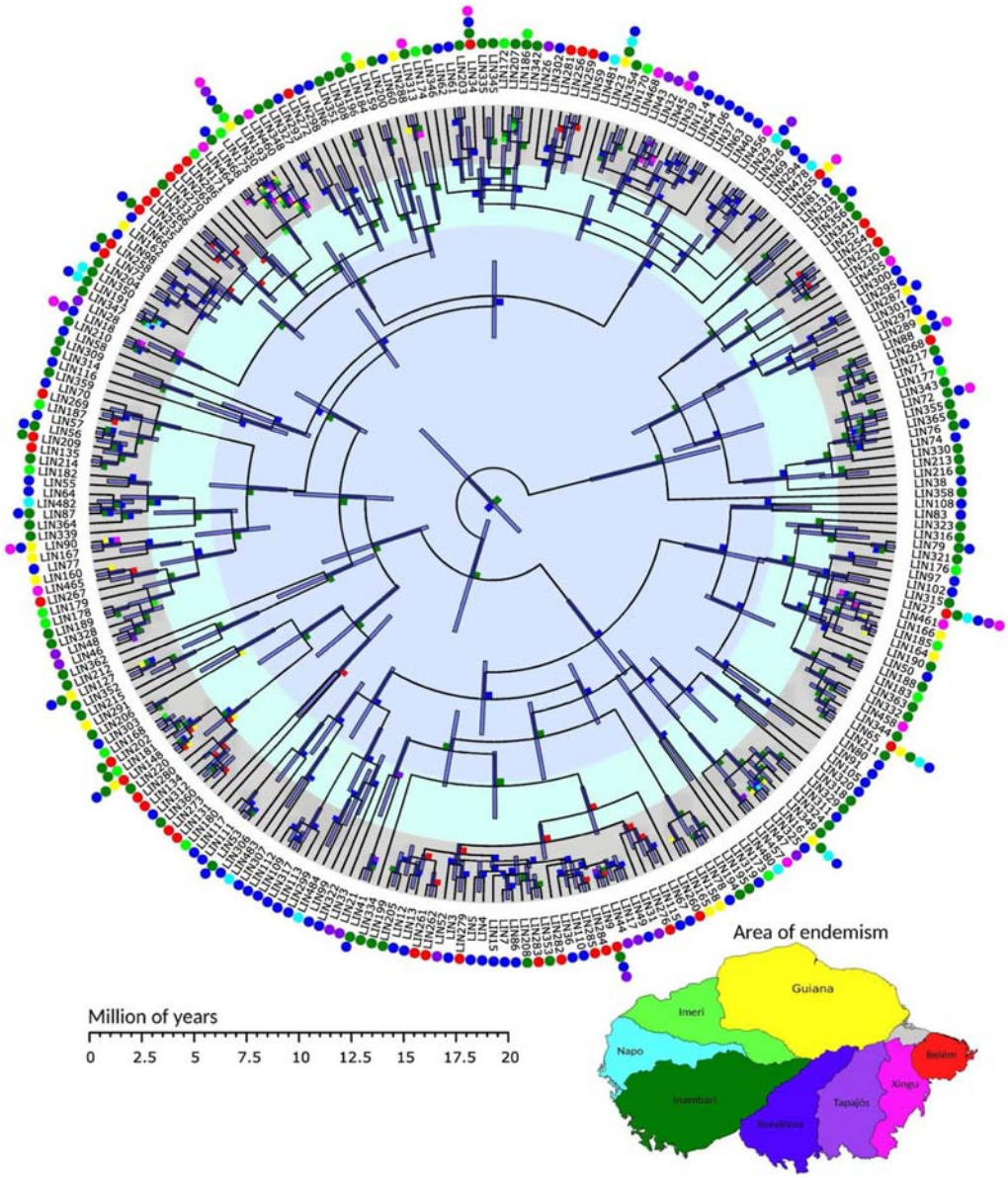

Figure 3. Phylogeny reconstructed using Bayesian inference of 260 *Plasmodium* lineages and Amazonian ancestral area reconstructions. Blue bars labeling each node are the 95% credibility interval of divergence time estimates for each lineage. The Miocene (23–5.3 Mya), Pliocene (5.3–2.6 Mya), and Quaternary (2.6 Mya–present) periods are shaded in light blue, light green and light grey, respectively. Terminal taxa are colored according to the area of endemism in which they were found. Colored squares labeling ancestral nodes indicate the most likely ancestral area estimated by analysis in BioGeoBEARS on DEC+J model. The color scheme for terminal taxon labels and ancestral areas matches the eight areas of endemism colored in the inset map and on Fig. 1. Lineage names and associated accession numbers used in phylogeny reconstruction along with the host species infected by each lineage are available in Supplementary material Appendix 7.

the Madison–Slatkin test did find that areas of endemism had significant phylogenetic signal for *Plasmodium* ($p < 0.001$), mapping *Plasmodium* lineages to their geographical locations did not reveal a strong biogeographical pattern (Fig. 3).

Duplication events occurred more frequently than dispersal for both *Parahaemoproteus* and *Plasmodium*. Most dispersal events occurred in the last five million years, after the contemporary pattern of river drainage became established, meaning that those lineages were capable of crossing the largest Amazonian rivers. Extinction events were not evident in either *Plasmodium* or in *Parahaemoproteus*, and vicariance events were rare in both groups of parasites (Fig. 2–3).

Historical association between haemosporidian parasites and their avian hosts

Tanglegrams of *Parahaemoproteus* and *Plasmodium* and their avian hosts were used to construct coevolutionary cost-event

Table 1. Cost-event cophylogenetic analysis of the association between Amazonian *Parahaemoproteus* lineages and their avian hosts. Instances where the number of events significantly differed from 100 randomized trees are in bold

Event costs[a]				Total costs	Codivergence	Duplication	Sorting	Switching
0	2	1	3	131	**22[b]**	**5[c]-6[c]**	**13[b]-14[b]**	**35[c]-36[c]**
0	0	1	2	82	**13[b]-19[b]**	**8[c]-11[b]**	**4[b]-9[b]**	**37[c]-39[c]**
1	1	1	0	1	0	0	**0[c]**	62
1	0	1	1	51	**0[c]-8[b]**	12	0	**43[c]-51[b]**

[a]event costs of codivergence, duplication, sorting and host switching, respectively
[b]the number of events significantly exceeds that of 100 randomized trees ($p < 0.05$)
[c]the number of events is significantly less than that of 100 randomized trees ($p < 0.05$)

analyses. Based on 100 randomizations of host–parasite associations, total event costs between 51 and 131 were statistically well supported for *Parahaemoproteus* (Table 1), whereas for *Plasmodium*, event costs between 189 and 648 were statistically supported (Table 2). Both *Parahaemoproteus* and *Plasmodium* support a coevolutionary history dominated by host switching with occasional codivergence and duplication. However, sorting (extinction) had far less influence on the coevolutionary pattern, especially in *Parahaemoproteus*. When host switching was made very costly (event costs 0213 and 0012), it was still identified as the most frequent pattern within the coevolutionary history of haemosporidians and their avian hosts (Table 1–2).

Discussion

Two major patterns emerge from this study. First, biogeographic analyses of haemosporidian lineages provided strong evidence that these protozoan parasites are capable of dispersing across major Amazonian rivers and that this occurred with increasing frequency during the last five million years. Second, host switching was the main coevolutionary pattern in the diversification of these parasites within Amazonia.

Table 2. Cost-event cophylogenetic analysis of the association between Amazonian *Plasmodium* lineages and their avian hosts. Instances where the number of events significantly differed from 100 randomized trees are in bold

Event costs[a]				Total costs	Codivergence	Duplication	Sorting	Switching
0	2	1	3	648	**42[b]-47[b]**	**33[c]-49[b]**	**31[b]-41[b]**	**175[c]-181[c]**
0	0	1	2	367	11-**25[b]**	**64[c]-69[b]**	**9[b]-27[b]**	**171[c]-179[c]**
1	1	1	0	11	0	11	0	248
1	0	1	1	189	**0[c]**-2	70	0	187-**189[b]**

[a]event costs of codivergence, duplication, sorting and host switching, respectively
[b]the number of events significantly exceeds that of 100 randomized trees ($p < 0.05$)
[c]the number of events is significantly less than that of 100 randomized trees ($p < 0.05$)

The role of biogeography in the diversification of avian malaria parasites

We found significant phylogenetic signal for biogeographic effects in both *Parahaemoproteus* and *Plasmodium*, with some clades composed only of lineages from a single area of endemism. This pattern was most evident in *Parahaemoproteus*, where many Rondônia lineages fell out in a large Rondônia-specific clade. The patterns revealed by phylogenetic analysis show a moderate effect of area of endemism on phylogeny of this haemosporidian subgenus. Because areas of endemism constrain host distributions (Cracraft 1985, Cracraft and Prum 1988) and *Parahaemoproteus* has higher host specificity than *Plasmodium* (Beadell et al. 2004, 2009, Ishtiaq et al. 2010), one might expect to find a strong biogeographic pattern for this subgenus within Amazonia. Host family associations are also strongly constrained within the *Parahaemoproteus* phylogeny (Fecchio et al. 2017a). We did not expect *Plasmodium*, with its lower host specificity, general lack of host cospeciation (Ricklefs and Fallon 2002, de Vienne et al. 2013, Lauron et al. 2015), and absence of an effect of host family in its evolutionary history in Amazonia (Fecchio et al. 2017a), to show strong biogeographic effects. This finding suggests that the success of *Plasmodium* dispersal and colonization, with subsequent diversification, more likely has depended on local ecological conditions than on historical factors.

Ancestral area reconstructions for both genera failed to recover the pattern of vicariance between distinct areas of endemism predicted to shape lineage diversification by both the Refuge (Haffer 1969) and Riverine (Wallace 1852) hypotheses. In fact, duplication, followed by dispersal, was the predominant process generating diversification in avian malaria parasites throughout Amazonia, which accords with recent studies showing weak effects of barriers on the evolution of the Amazonian avifauna (Smith et al. 2014, Oliveira et al. 2017). Movement between areas of endemism can occur either when parasites move with infected hosts, by host switching, by a combination of these two processes, or perhaps even via movement of dipteran vectors.

Our estimates of the timing of cladogenic events differ from previous studies (Bensch et al. 2013, Silva et al. 2015). The greatest conflict was with Silva et al. (2015), who estimated an older time of origin and radiation of mammalian *Plasmodium* compared to Ricklefs and Outlaw (2010). To assess the effect of using different published clock rates, we ran an alternative BEAST dating scheme using the most recent molecular clock substitution rate published by Pacheco et al. (2018) and compared that to our analysis using the substitution rate published by Ricklefs and Outlaw (2010). As expected, the topologies for both *Plasmodium* and *Parahaemoproteus* did not change (Supplementary material Appendix 4 and 5 for trees). However, when using the Pacheco et al. (2018) rates, the first cladogenic events were older for both genera. Nevertheless, the diversification of most lineages within each parasite genus occurred in the late Miocene, with increasing

frequency of lineage splitting in the Pliocene and Quaternary. This timing overlaps with the formation of the largest Amazonian rivers (Hoorn et al. 2010). Thus, using this recently published clock rate, in concert with biogeographic reconstructions, our results continue to support the importance of dispersal after the formation of the Amazonian river drainage in shaping biogeographic diversification patterns of avian haemosporidians. Future studies using genomic rather than mitochondrial data could more precisely estimate the ages of cladogenesis for haemosporidian parasites.

Evolutionary relationships and diversification of haemosporidian parasites in Amazonia

Host switching is an important evolutionary pattern among avian haemosporidians, with higher host taxa tending to share closely related haemosporidian lineages (Bensch et al. 2000, Ricklefs and Fallon 2002, Waldenström et al. 2002, Ricklefs et al. 2004, 2014, Križanauskienė et al. 2006, Ellis et al. 2015). Dispersal followed by isolation, specialization, and evolution within a new host lineage can lead the formation of new haemosporidian lineages (Hoberg and Brooks 2008, Loiseau et al. 2012, Santiago-Alarcon et al. 2014, Ricklefs et al. 2014). This process would shift the parasite lineage across hosts and increase local parasite diversity (Ricklefs et al. 2014).

In Amazonia, host switching was the most frequent event in the coevolutionary history of *Parahaemoproteus* and *Plasmodium* with their hosts. Only when host switching was made very costly did the other evolutionary events (codivergence, duplication, sorting) increase in prevalence. The second most common event for *Parahaemoproteus* was codivergence, whereas for *Plasmodium* it was duplication (Table 1–2). Ricklefs et al. (2004) found duplication (within-host speciation) to be a frequent event for avian haemosporidians when event costs were low, at times even exceeding host switching frequency. However, this pattern was not observed for *Parahaemoproteus* and *Plasmodium* in Amazonia. Although duplication was the second most common event, its frequency was far below that of host switching. The lower frequency of duplication might reflect the greater host diversity sampled in our study. Duplication in the present analysis was not affected by differences in the duplication event costs, and increased only when host switching was costly. The dominance of host switching at higher taxonomic levels in Amazonia matches what is known for the evolutionary history of avian haemosporidians (Ricklefs et al. 2014) and is consistent with similar analyses conducted solely on *Haemoproteus* lineages (Galen and Witt 2014, Santiago-Alarcon et al. 2014). Galen and Witt (2014) found that *Haemoproteus* lineages recovered from Andean house wrens *Troglodytes aedon* in Peru diversified by host switching between distantly related avian species within this region. In *Plasmodium*, the generally poor matching of host and parasite phylogenies is attributed to a high frequency of host switching compared to other evolutionary events (Ricklefs and Fallon 2002, de Vienne et al. 2013, Lauron et al. 2015).

Many haemosporidian lineages have been found to infect distantly related hosts (Ricklefs et al. 2014). Even extremely phylogenetically distant hosts can become infected, as in the successful infection of mice with avian *Plasmodium lophurae* (McGhee 1951) and the susceptibility of erythrocytes from several mammalian species to avian *Plasmodium* parasites (McGhee 1957). Because most vector species often come in contact and can transmit a diverse array of haemosporidian lineages (Gager et al. 2008, Santiago-Alarcon et al. 2012, Medeiros et al. 2013, Valkiūnas et al. 2013), ongoing dispersal is relatively common and could facilitate host switching, while at the same time reducing the relative frequency of cospeciation between avian hosts and their haemosporidian parasites.

Although geographic barriers are thought to reduce the importance of dispersal, and thus host switching, and to promote cospeciation (Desdevises et al. 2002), we found that this is not the case for the areas of endemism in Amazonia. The hyperdiverse host and vector communities within areas of endemism provide ample opportunities for host switching and subsequent speciation without significant cospeciation, as supported by the infrequent movement of haemosporidian lineages between areas of endemism over evolutionary time (Fig. 2–3). However, some evidence of cospeciation does exist within Amazonia, perhaps reflecting the frequent splitting of avian host lineages. Although movement of lineages between areas occurs infrequently (Fig. 2–3), it does occur. For these lineages, cospeciation (host specialization) could result from the movement of parasites between areas with their dispersing hosts, followed by geographic isolation of both. The higher Amazonian diversity of *Plasmodium* as compared to *Parahaemoproteus*, may be a consequence of *Plasmodium* having the higher rates of codivergence, duplication, and sorting along with high rates of host switching. Additional cophylogenetic analyses that look at specific host/parasite associations would be helpful in understanding the variation in coevolutionary events within Amazonia.

Conclusions

Amazonia harbors the highest biodiversity on Earth, in part because major rivers limit dispersal and promote geographic differentiation of animal and plant populations. Here, we document correspondingly high diversity in a clade of avian parasites that readily disperse across major rivers, challenging the riverine barrier hypothesis as the primary mechanism underlying diversification for this group of organisms. Diversification within areas of endemism (duplication) has been the major mechanism of cladogenesis in avian haemosporidian parasites throughout Amazonia. Dispersal of avian hosts between areas of endemism is not only a major force in avian diversification, but has also influenced the diversification of their haemosporidian parasites. High avian diversity in Amazonia would function to increase the potential for successful host switching (colonization and diversification) due to high numbers of closely related avian hosts (Poulin 2007, Hayakawa et al. 2008, Fecchio et al. 2017a). Within Amazonia, avian hosts with high levels of niche partitioning would also promote retention of newly evolved lineages, thus

maintaining or even increasing overall haemosporidian diversity. Our results also confirm a marked difference between *Pararahaemoproteus* and *Plasmodium* with respect to their host usage: *Plasmodium* is more host-generalist and widespread over space and across host families than *Parahaemoproteus*. These findings accord with patterns emerging from regional studies that have revealed the broad variability in host specificity exhibited by these pathogens (Beadell et al. 2004, 2009, Križanauskienė et al. 2006, Fecchio et al. 2017a). A comprehensive analysis of ecological and evolutionary factors that influence the distribution of haemosporidian parasites in samples from biomes adjacent to Amazonia will shed additional light on how lineages are able to disperse across biomes in South America.

Acknowledgments – We thank Alexandre Aleixo from Museu Paraense Emílio Goeldi, who loaned part of the samples used in this study, and Ivan Pedro from Laboratório de Evolução e Biogeografia for help with the Fig. 1.
Funding – This work was funded by the Fundação de Amparo à Pesquisa do Estado do Amazonas - FAPEAM and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Process number 61.0012/2008.7-DCR/CNPq/FAPEAM) to AF, SISBIOTA/FAPEAM and SISBIOTA/CNPq to I.P.F., and US National Science Foundation grants DEB-1503804 to JDW and DEB-1120734 to VVT. During the project, AF was supported by a postdoctoral fellowship from CNPq (process no. 350140/20120 and 201275/2014-7) and he is currently supported by a PNPD scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Supplementary material (available online as Appendix oik-05115 at <www.oikosjournal.org/appendix/oik-05115>). Appendix 1–7.