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Distinct biogeographic processes and areas of endemism contributed differentially to *Plasmodium* and *Parahaemoproteus* community assembly on Marajó Island

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ABSTRACT

Amazonia is the primary source of haemosporidian diversity for South American biomes. Yet, our understanding of the contribution of each area of endemism and the biogeographical processes that generated such diversity in this group of vector transmitted parasites remains incomplete. For example, a recently formed fluvial island in the Amazon delta - Marajó Island, is composed of avian lineages from adjacent Amazonian areas of endemism, but also from open habitats, such as Cerrado. This raises the question: Is the parasite assemblage found in avian hosts on this island formed by parasite lineages from adjacent Amazonian areas of endemism or Cerrado? Here, we assessed the spatiotemporal evolution of *Plasmodium* and *Parahaemoproteus* parasites. Our biogeographic analysis showed that dispersal dominated *Plasmodium* diversification, whereas duplication was more frequent for the genus *Parahaemoproteus*. We show that the Inambari area of endemism was the primary source for *Plasmodium* diversity on Marajó Island, but that this island received more *Parahaemoproteus* lineages from Cerrado than any Amazonian area of endemism. The unique patterns of dispersal for each parasite genus coupled with their propensity to shift hosts locally may have facilitated their diversification across Amazonia, suggesting that differences in deep evolutionary history may have constrained their colonization of Marajó Island.

1. Introduction

Molecular phylogenetic analysis of parasites can reveal the biogeographic history and historical movements of their hosts. For example, a *Trypanosoma* parasite lineage that infects bat hosts, has indirectly indicated recent intercontinental dispersal of Old Word bats to the Neotropical region (Hamilton et al., 2012) and a phylogenetic study of an ectoparasitic louse lineage from the genus *Penenirmus* that infests woodpeckers, also suggests a historical intercontinental dispersal of hosts from North America to Africa (Johnson et al., 2021). Moreover, if host biogeography has a significant effect on the parasite phylogeny, this may indicate a lack of host-parasite cospeciation and the importance of either host switching or ongoing parasite dispersal in a given parasite assemblage (Weckstein, 2004). This view highlights the importance of integrating historical biogeography of vertebrate hosts with different dispersal capacities to uncover how evolutionary and ecological process have shaped parasite assemblages.

Amazonia harbors the most diverse avifauna on Earth (ca 1,300 species) and is also known for its astonishing levels of bird endemism (Silva et al., 2005). Yet, the ecological and evolutionary processes that generate such diversity and endemism are still debated (Smith et al., 2014; Silva et al., 2019). The Riverine Barrier Hypothesis (RBH; Wallace, 1852) has long been famous and proposes that the Amazon River and its larger tributaries acted as barriers to avian dispersal, thus promoting allopatric speciation. Whereas many studies have shown that the large tributaries of the Amazon Basin act as barriers to gene flow (e.g.,

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Thom and Aleixo, 2015; Ferreira et al., 2017), others failed to demonstrate that larger rivers have been the main factor responsible for allopatric speciation in Amazonian avian taxa (e.g., Naka et al., 2012; Smith et al., 2014). Although wider portions of Amazonian rivers (e.g., the Negro and Tapajós rivers can exceed 10 and 20 km in width, respectively) might be effective barriers to avian dispersal, their headwater regions narrow to a few hundred meters where they may no longer function as dispersal barriers (Naka et al., 2012; Weir et al., 2015). A recent biogeographical study on avian haemosporidian parasites in Amazonia showed that these protozoans can readily disperse across the eight areas of endemism delimited by the Amazon River and its larger tributaries, demonstrating that these rivers are weak barriers for parasite dispersal (Fecchio et al., 2018). This study on parasite lineages does not support the RBH as the primary mechanism underlying diversification and reinforces a recent idea that variation in the ability of an organism to disperse, rather than barriers to gene flow, may be the core driver of speciation in some widespread Amazonian lineages (Smith et al., 2014).

Haemosporidians from the genera *Plasmodium* and *Parahaemoproteus* comprise a diverse group of vector-transmitted parasites that infect birds worldwide (Galen et al., 2018). Whereas the evolutionary history of these parasites is characterized by frequent host switching events and intercontinental dispersal (Ellis et al., 2018), avian haemosporidians are generally phylogenetic host specialists, with most lineages of *Plasmodium* and *Parahaemoproteus* infecting hosts that are phylogenetically clustered within their avian host community (Fecchio et al., 2019a). Possible specialization within vector hosts may be expected by the dependence of these parasites on different groups of hematophagous dipteran vectors for sexual reproduction. Species from the genus *Plasmodium* are transmitted by mosquitos, whereas *Parahaemoproteus* is transmitted by biting midges (Santiago-Alarcon et al., 2012).

Marajó Island, the World's largest fluvial island, is situated in the Xingú area of endemism (Silva et al., 2019). The avian composition of this island, located in the Amazon River delta, is influenced by avian lineages from adjacent Amazonian areas of endemism such as Belém and Guiana, but also harbors avian lineages from the Cerrado, a Neotropical Savanna located in the Brazilian Shield (Henriques and Oren, 1997). The unique geographic location and recent split from the continent (ca 10,000 years ago) make this island an intriguing model to understand how biogeographic events have influenced the diversification of avian haemosporidian parasites in this portion of Amazonia. Here, we used sequences of the mitochondrial cytochrome-*b* (cyt-*b*) gene to identify parasite lineages from the genera *Parahaemoproteus* and *Plasmodium*. Then, we investigated the spatiotemporal evolution of these diverse groups of blood parasites by inferring and quantifying four biogeographical processes (vicariance, extinction, duplication, and dispersal) among Marajó Island, Cerrado, and Amazonian areas of endemism. We employed the Statistical -Dispersal-Extinction-Cladogenesis (S-DEC) model (Ree et al., 2005; Ree and Smith, 2008) to depict these processes in parasite phylogenies. We also detailed the specific source and sink for parasite dispersal ratios among areas.

2. Material and methods

2.1. Avian host and parasite sampling

Our analyses are based on 7,061 blood and tissue samples from 596 avian species collected across eight Amazonian areas of endemism, Cerrado, and Marajó Island (Fig. 1). This dataset includes 86 localities surveyed in Brazil, Ecuador, and Peru (see raw data in Supporting Information Table S1). The majority of these samples were published by Lacorte et al. (2013), Moens and Pérez-Tris (2016), Fecchio et al. (2018, 2019b) and Bosholn et al. (2020) with parasite lineage location and identity available in the MalAvi database (Bensch et al., 2009). To cover all Amazonian areas of endemism, including Marajó Island, and patches of Neotropical Savanna within Amazonia, we included 1,042 additional unpublished blood samples from the Brazilian Amazon. We collated 851 *Plasmodium* sequences and 189 *Parahaemoproteus* sequences from published studies or the MalAvi database (secondary data). Details on primers, reaction protocols, and cycling conditions used to generate the secondary dataset can be found in Hellgren et al. (2004), Lacorte et al. (2013), and Bell et al. (2015).

In addition, we included 136 unpublished *Plasmodium* sequences and 35 *Parahaemoproteus* sequences (primary data generated by the authors

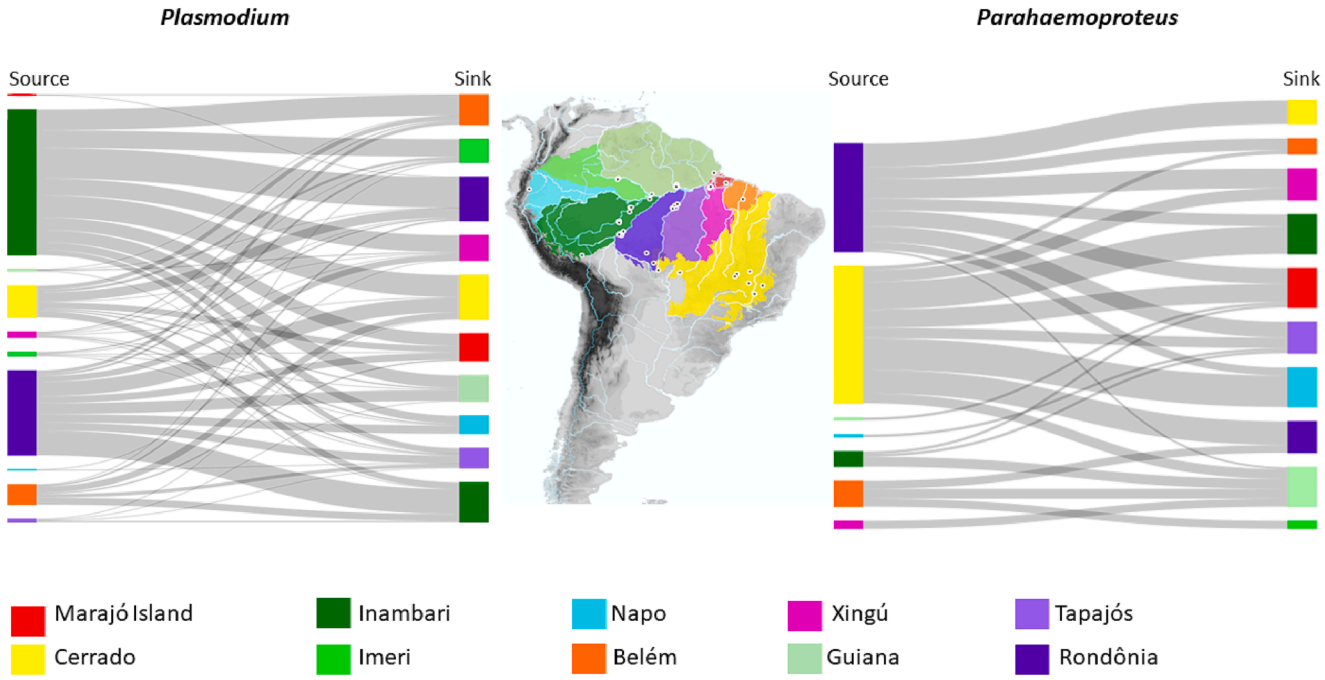

Fig. 1. Sankey diagram depicting the frequency of parasite lineage dispersal among Amazonian areas of endemism, Cerrado, and Marajó Island. Colors indicate the biogeographic region that serves as the source or sink for the dispersal events inferred for *Plasmodium* and *Parahaemoproteus*. Color scheme matches the inserted map containing the sampling sites across Amazonia and Cerrado.

for the present study). Newly collected blood samples from Brazil (primary data) were screened for haemosporidian parasites using a standard molecular barcoding approach (Hellgren et al., 2004). Briefly, DNA extracted from host tissues underwent nested PCR-based detection targeting a 477 bp barcoding fragment of the cyt-*b* gene from the haemosporidian mitochondrial genome. Sequence chromatograms with overlapping peaks were considered as co-infections and individual haplotypes were delineated following one of following two methods. First, co-infections with overlapping peaks that differed consistently in both sequencing directions were manually delineated following the protocols of Galen et al. (2019). Second, in co-infections where haplotypes could not be identified manually, we used the program PHASE 2.1.1 (Stephens et al., 2001; Stephens and Donnelly, 2003) following the protocol of Harrigan et al. (2014). As evidence indicates that avian haemosporidian haplotypes differing by one cyt-*b* nucleotide may be reproductively isolated entities (Bensch et al., 2004), we used the conventional practice of referring to each unique cyt-*b* haplotype as a unique parasite lineage following the standard naming for this group of parasites (Bensch et al., 2009).

2.2. Phylogenetic reconstruction

Assembled sequences of unique haplotypes were used to infer molecular phylogenies. We divided avian haemosporidian parasites into two separate alignments, one for the genus *Parahaemoproteus* and one for the genus *Plasmodium*. For both alignments, *Leucocytozoon fringillarum* (GenBank accession no. FJ168564) was used as the outgroup. We used the GTR + I + G model of nucleotide substitution in each alignment as determined by jModelTest (Guindon and Gascuel, 2003; Darriba et al., 2012). We reconstructed the phylogenetic relationships for each parasite lineage using Bayesian inference implemented in BEAST v. 1.10.4 (Suchard et al., 2018) on the CIPRES Science Gateway (Miller et al., 2010). We generated a non-calibrated time tree for each parasite genus alignment using the Bayesian relaxed clock model (Drummond et al., 2006). The analyses were conducted for 102 lineages of *Parahaemoproteus* and 328 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 10,000 generations, and 10% of generations discarded as burn-in. Convergence and performance of runs were inspected using Tracer 1.6 (https://beast.bio.ed.ac.uk/Tracer), to ensure that ESS (effective sample size) values exceeded 200. The maximum clade credibility (MCC) tree for each haemosporidian group was generated using TreeAnnotator. The resulting trees were visualized in FigTree ver. 1.4.2 (https://tree.bio.ed.ac.uk/software/figtree/).

2.3. Biogeographic inference

To infer biogeographic processes among Marajó Island, Amazonian areas of endemism, and Cerrado, we reconstructed ancestral areas on internal nodes of the avian haemosporidian parasite trees using the software RASP 4.3 (Yu et al., 2020). We used 100 trees randomly sampled from the posterior probability distribution of the BEAST runs for each parasite genus alignment to take into account topological uncertainty (Drummond et al., 2012). The S-DEC (Statistical Dispersal, Extinction, and Cladogenesis) method was used to infer the ancestral area distribution at each node. We considered 10 geographic areas: Belém, Guiana, Imeri, Inambari, Marajó Island, Napo, Tapajós, Xingú, and Cerrado. Parasite lineage distributions were coded as present or absent in each of the 10 areas. We set the maximum number of areas to six and pruned the outgroup from the parasite tree prior to analysis. Analyses were implemented without constraints. We then computed biogeographic events (vicariance, dispersal, duplication, and extinction) for each parasite alignment. Dispersal events among areas were plotted into a Sankey diagram using the networkD3 package (https://christophergandrud.github.io/networkD3/) in R v. 4.2.0 (R Core Team 2018) to detail the source and sink of dispersal ratios among areas.

3. Results

In total, we collated 987 sequences of *Plasmodium* and 224 sequences of *Parahaemoproteus* from the biomes Amazonia and Cerrado. Co-infections with *Plasmodium* and *Parahaemoproteus* were found in 79 individuals.

Dispersal (46.9%) followed by duplication (34.7%) were the most frequent events at ancestral nodes for *Plasmodium* (Fig. 2). However, for *Parahaemoproteus* we found the opposite pattern (Fig. 2), with duplication (45.6%) followed by dispersal (34.9%) being the most important events. Vicariance events occurred with similar frequency for *Plasmodium* (18%) and *Parahaemeoproteus* (19.5%). Extinction was rarely present in the *Plasmodium* phylogeny, and not seen for *Parahaemoproteus*.

Through dispersal events, Inambari and Rondônia areas of endemism were the most important regions for the radiation of *Plasmodium* within Amazonia, acting as the primary sources of parasite lineage dispersal to Marajó Island (Fig. 1). Furthermore, for *Plasmodium*, the Cerrado biome was a more important source of lineage dispersal to Marajó Island than were the three neighboring Amazonian areas of endemism: Xingú, Guiana, and Belém. In contrast, the Cerrado was the primary source for *Parahaemoproteus* dispersal for four Amazonian areas of endemism: Guiana, Inambari, Napo, and Rondônia. Specifically, Marajó Island received the highest number of *Parahaemoproteus* lineages from the Cerrado biome, followed by Rondônia, Guiana, and Inambari (Fig. 1).

4. Discussion

We present the most comprehensive biogeographical analysis of avian haemosporidians in the Amazon. Our results showed that the Inambari area of endemism was the primary source for *Plasmodium* diversity on Marajó Island, whereas the Cerrado biome was the main source for *Parahaemorpoteus* diversification into this newly formed fluvial island in the Amazon river delta.

The *Plasmodium* clade includes several lineages infecting bird hosts across multiple areas of endemism, demonstrating that this genus has a higher dispersal capacity than *Parahaemoproteus*, a mechanism previously proposed to explain the widespread distributions of some avian haemosporidian parasites across Amazonian birds (Fecchio et al., 2018). Our results further strengthen a previous study that *Plasmodium* parasite assemblages were likely shaped by many instances of host range expansion of parasite lineages into newly colonized areas of endemism (Fecchio et al., 2018). This reinforces that lineage dispersal limitation may play a role in determining the *Plasmodium* assemblage on Marajó Island. In addition, geographic barriers are known to affect host specificity by limiting the movement of specialist parasite lineages (Gupta et al., 2019). Since the main tributaries of the Amazon River act as geographic barriers, limiting both avian host and parasite gene flow, areas delineated by these larger rivers are expected to be dominated by generalist *Plasmodium* lineages, a hypothesis that deserves further investigation. *Plasmodium* lineages with their low degree of avian host specificity could potentially shift between phylogenetically unrelated avian host species after dispersion and eventually colonize syntopic host species (e.g., with similar habitat usage in the forest stratum).

In contrast, *Parahaemoproteus* diversification was dominated by duplication, most of which occurred within the Cerrado. Geography has been shown to play a major role in the diversification and distribution of *Parahaemoproteus* in Amazonia (Fecchio et al., 2018), with host switching between dispersing hosts being the major force of speciation for this genus (Ricklefs et al., 2014). Initially, parasite speciation within an area of endemism and host parasite coevolution take place following host range expansions with secondary sympatry resulting in local shifting of lineages across hosts. However, the underlying mechanisms of how speciation (i.e., duplication events within an area) occurs

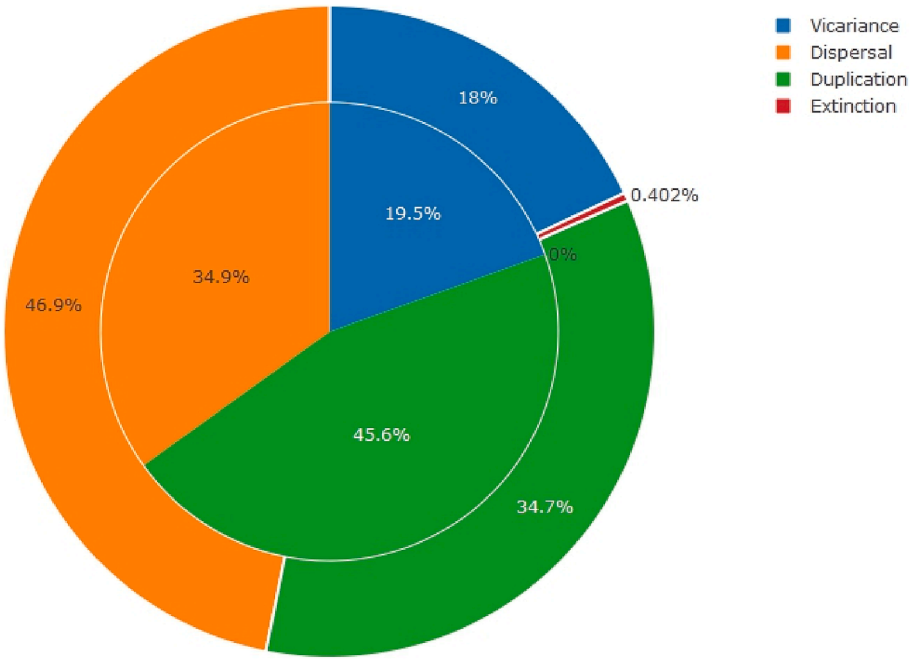

Fig. 2. Frequency of occurrence for the four biogeographic events inferred by S-DEC in Amazonian areas of endemism, Cerrado, and Marajó Island. The outer circle depicts biogeographic events in *Plasmodium* lineages, and the inner indicates events for *Parahaemoproteus*.

following host switching remains unclear, as we currently do not know the vector species responsible for the transmission of *Parahaemoproteus* lineages in Amazonia. Although radiation of *Parahaemoproteus* lineages does not entirely match the evolutionary history of their avian hosts (i.e., co-speciation), compatibility between *Parahaemoproteus* parasite lineages and avian hosts after dispersal may indicate that localized adaptations of naïve host populations have stronger effects in shaping host-parasite associations than dispersal limitations for this parasite genus.

The parasite assemblage on Marajó Island is a result of two main biogeographic processes, namely dispersal and duplication. However, the evolutionary history of *Plasmodium* and *Parahaemoproteus* was marked by different frequencies in these biogeographic processes leading to parasite diversification within Marajó Island and across Amazonia. Importantly, the eight Amazonian areas of endemism and Cerrado contributed differentially to the *Plasmodium* parasite assemblage, but surprisingly, the Cerrado biome was the primary source of *Parahaemoproteus* diversity on Marajó Island. Finally, the underlying macroevolutionary factors that contributed to the diversity and distribution of phylogenetically related groups of parasites varied drastically between the two parasite genera. Thus, suggesting that emergence and spread of these vector transmitted parasites may respond differentially to recent modification in the landscape, such as habitat fragmentation and deforestation.

Some limitations and potential biases in our study should be recognized. We used a single molecular marker to reconstruct phylogenetic relationships, divergence time, and dispersal patterns for a diverse and widespread group of parasites. Recent multi-locus analyses of avian haemosporidian parasites have raised concerns that the cyt-*b* barcoding region may be saturated, thus reducing its power to infer complex biogeographic processes accurately (Galen et al., 2018). Saturation occurs because faster evolutionary rates usually increase the likelihood of multiple mutations at any given nucleotide site and may erase the evolutionary history at deeper nodes in the phylogeny (Davalos and Perkins, 2008; Liu et al., 2014). In the past two decades single and short molecular markers were broadly applied to detect and delineate species limits for haemosporidian parasites with the majority of sequences deposited in open access curated databases allowing researchers to test ecological and evolutionary hypotheses (Bensch et al., 2009). To move the field forward, it is ultimately imperative to generate multi-locus data for representative parasite lineages from local and regional studies to improve the taxonomic resolution and reconstruct robust phylogenetic relationships of this highly speciose group of avian parasites (Galen et al., 2018; Pacheco et al., 2018). This would allow researchers to further understand the complex macroecological processes and macroevolutionary patterns within this diverse group of parasites.

CRediT authorship contribution statement

Alan Fecchio: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing. **Henrique Batalha-Filho:** Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing. **Janice H. Dispoto:** Data curation, Methodology, Software, Visualization, Writing – review & editing. **Jeffrey A. Bell:** Conceptualization, Data curation, Methodology, Software, Validation, Visualization, Roles/Writing – original draft, Writing – review & editing. **Jason D. Weckstein:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Roles/Writing – original draft, Writing – review & editing.

Data Accessibility Statement

The raw dataset will be uploaded as Supporting Information. All DNA sequences will be available in Genbank (see Supplementary Table 1 for accession numbers) and MalAvi.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2023.107828.

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