# Timing and Number of Colonizations but Not Diversification Rates Affect Diversity Patterns in Hemosporidian Lineages on a Remote Oceanic Archipelago

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abstract: Parasite diversity on remote oceanic archipelagos is determined by the number and timing of colonizations and by in situ diversification rate. In this study, we compare intra-archipelago diversity of two hemosporidian parasite genera, *Plasmodium* and *Leucocytozoon*, infecting birds of the Mascarene archipelago. Despite the generally higher vagility of *Plasmodium* parasites, we report a diversity of *Plasmodium* cytochrome *b* haplotypes in the archipelago much lower than that of *Leucocytozoon*. Using phylogenetic data, we find that this difference in diversity is consistent with differences in the timing and number of colonizations, while rates of diversification do not vary significantly between the two genera. The prominence of immigration history in explaining current diversity patterns highlights the importance of historical contingencies in driving disparate biogeographic patterns in potentially harmful blood parasites infecting island birds.

*Keywords:* colonization, diversification rate, parasite diversity, avian malaria, Mascarenes, island biogeography.

## Introduction

Understanding the processes driving the buildup of species diversity in ecological communities remains a central theme of evolutionary biology and ecology. In oceanic islands that were never connected to the continent, the diversity of a given taxon is the product of the combined effects of immigration and in situ diversification (Losos and Schluter 2000; Wiens and Donoghue 2004; Whittaker et al. 2008). Thus, the insular diversity of a taxon relates to several biogeographical features: immigration rate, in situ diversification rate (speciation minus extinction), diversity on the mainland source, and the history of island colonization (MacArthur and Wilson 1967). To the extent that it controls the time that has been available for the taxon to diversify in situ, the sequence of colonization may directly influence insular diversity. All other things being equal, a taxon may be more diverse than another if it arrived earlier, allowing more time for cladogenetic lineages to arise through in situ diversification (McPeek and Brown 2007; Ricklefs 2007; Kozak and Wiens 2012).

Such biogeographical features of a taxon may also be influenced by a series of taxon-specific characteristics, such as dispersal ability, size of the source population, niche breadth, and competitiveness (Loreau and Mouquet 1999; Gillespie and Baldwin 2010). For parasites, generalist versus specialist strategies of infection may influence diversity and biogeographical patterns through various mechanisms. For example, in specialist parasites, host switching provides opportunities for speciation (Zięłara and Lumme 2002; Sorenson et al. 2003; Favret and Voegtlin 2004; Meinilä et al. 2004) and may thus have played an important role in diversification dynamics (Hoberg and Brooks 2008). In contrast, generalist parasites may have fewer opportunities to speciate if their populations continue to interbreed after infecting different hosts (e.g., Sehgal et al. 2001; Archie and Ezenwa 2011). However, generalist parasites are also predicted to occupy larger geographic ranges because of their capacity to exploit a variety of hosts and environments (niche breadth hypothesis; Krasnov et al. 2005), and such large geographic ranges may promote speciation (Maurer and Nott 1998; but see Jablonski and Roy 2003), although this may ultimately depend on both

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dispersal abilities (Birand et al. 2012) and the spatial scale (Kisel and Barraclough 2010).

For most parasites, dispersal is usually host mediated, and infecting various hosts may increase the immigration rate into new regions by providing more migration opportunities (Gómez-Díaz et al. 2012). This is especially likely in avian parasites, such as hemosporidians, that can benefit from their hosts' long-distance colonizations of remote areas (Pérez-Tris and Bensch 2005; Hellgren et al. 2007). Successful establishment in a new region should also be more probable for generalist than for specialist parasites, as the pool of competent hosts at destination should be larger for generalists (MacLeod et al. 2010). However, this effect could interact with infection prevalence (percent infection of a host population) to influence dispersal patterns. Higher proportions of infected birds increase the dispersal ability of parasites, because more prevalent parasites are more likely to be brought along with their hosts as they move (Clayton et al. 2003; MacLeod et al. 2010). Generalist parasites may achieve low average infection prevalence in individual host species if they parasitize several or many suboptimal reservoirs in a given locality (dilution effect; Schmidt and Ostfeld 2001; Keesing et al. 2006). This could cause reductions in parasite transmission at both local and regional scales, depending on patterns of host diversity and distribution. However, in hemosporidians, generalists are not necessarily less prevalent than specialists (Hellgren et al. 2009), and the large host range of most generalists may counterbalance their lower prevalence in individual host species as a result of a greater number of susceptible hosts (amplification effect; Keesing et al. 2006).

Host specificity and dispersal ability should thus be linked, and both may influence colonization and diversification dynamics on remote islands. In this study, we compare the history of community development of two genera of vector-borne hemosporidian parasites, *Plasmodium* and *Leucocytozoon*, that parasitize birds in the Mascarene archipelago (Indian Ocean). Differences between these genera in a number of attributes make this system suitable for investigating the role of historical and evolutionary processes in driving species buildup. The two genera differ in vagility and host specificity (Ricklefs and Fallon 2002; Hellgren et al. 2007). *Plasmodium* has been shown to be more generalist and more vagile than *Leucocytozoon* (Hellgren et al. 2007), with some lineages being distributed almost globally (Beadell et al. 2006). In contrast, *Leucocytozoon* mostly contains species with small geographic ranges, with concentrations of recently diverged lineages in certain regions (Hellgren et al. 2007). This led us to make two predictions. First, *Plasmodium* should have colonized the islands more frequently than *Leucocytozoon*. Second, *Leucocytozoon* should show higher in situ diversification rates than *Plasmodium* because of the effects of host switching in promoting speciation at the local scale. However, greater specialization of *Leucocytozoon* may also entail smaller population sizes and a greater susceptibility to host population dynamics. This, in turn, may increase the vulnerability to extinction of these parasites on islands and reduce their in situ diversification rate. On the whole, the diversity of parasite assemblages should therefore result from the combined dynamics of colonization and in situ diversification.

In a previous study (Cornuault et al. 2012), we found that contemporary *Leucocytozoon* diversity in the Mascarene archipelago originated through a combination of multiple colonizations and in situ diversification events. However, we did not evaluate quantitatively the dynamics of colonization and diversification, and our study was restricted to *Leucocytozoon* infecting just one bird genus (*Zosterops*). Here, we develop a quantitative framework to assess the relative contribution of immigration and in situ diversification events in shaping present diversity in both *Leucocytozoon* and *Plasmodium* found in Mascarene passerine birds. We then explore the interplay between immigration history and diversification by examining whether observed differences in the number of cladogenetic lineages (i.e., lineages that originated through in situ cladogenesis) between the two genera are influenced by the number and timing of colonizations and/or different diversification rates. Finally, we analyze our results in light of our hypotheses on factors that may influence diversity and biogeographical patterns in hemosporidian parasites.

## Methods

### *Samples and Sites*

Birds were mist-netted between 2007 and 2010, and blood samples were collected by gently puncturing the subbrachial vein and stored in lysis buffer until freezing at −20°C. Birds were ringed, measured, and released unharmed. All manipulations were conducted under a ringing permit issued by the Centre de Recherches sur la Biologie des Populations d'Oiseaux–Museum d'Histoire Naturelle (Paris) and a collection permit from the Government of Mauritius. In total, 987 Mascarene birds from 16 species were screened for blood parasites (fig. 1). Very similar sets of samples were screened for *Plasmodium* and *Leucocytozoon* parasites, with 826 individual birds screened for both parasites, 49 for *Plasmodium* only, and 112 for *Leucocytozoon* only. Unbalanced host taxon sampling can introduce bias in the analysis of diversity and diversification rates. The fact that the two parasite genera were mostly detected from the same samples suggests that such sampling bias may have a limited effect on our estimates, especially since we

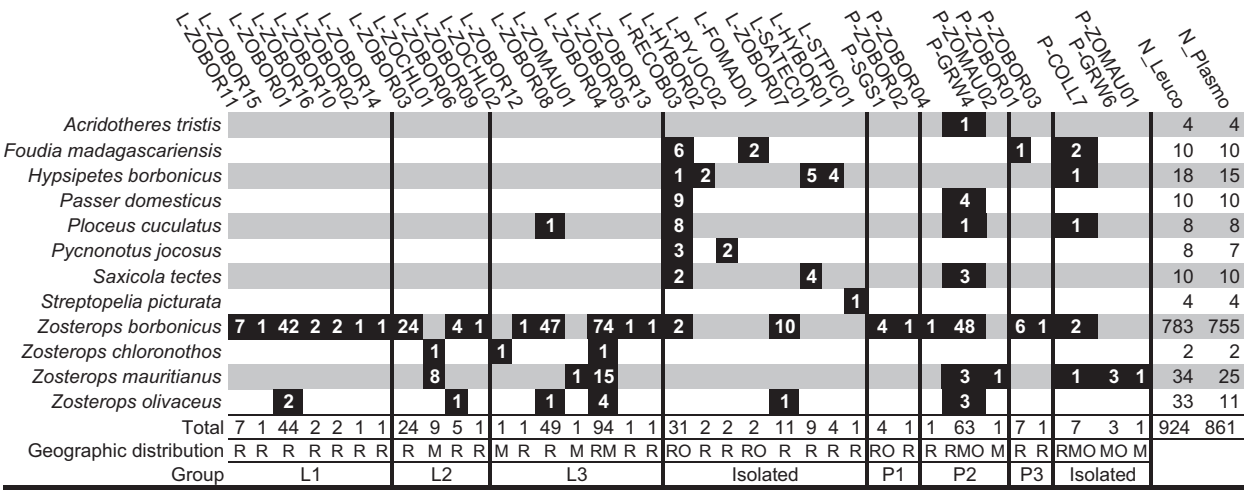



**Figure 1:** Number of occurrences of the different *Leucocytozoon* and *Plasmodium* haplotypes per host species in the Mascarenes. All bird species were sampled on Réunion, except *Zosterops mauritianus* and *Zosterops chloronothos*, which are endemic to Mauritius. The geographic distribution of lineages is indicated: R for Réunion, M for Mauritius; and O for outside the Mascarenes. Four species were screened but were not infected: *Terpsiphone bourbonensis* ($n = 10$), *Estrilda astrild* ($n = 2$), *Leiothrix lutea* ($n = 1$), *Geopelia striata* ($n = 1$). N_Leuco and N_Plasmo indicate the number of birds screened per host species and parasite genus.

see no reason why the two genera would be unequally represented in un- or undersampled host species.

### *Haplotype Identification and Diversity*

Parasite haplotype richness was estimated as the number of different cytochrome *b* (cyt *b*) haplotypes. Since most molecular studies of hemosporidian parasites have examined genetic diversity at the cyt *b* locus only (e.g., Belo et al. 2011; Marzal et al. 2011; Svensson-Coelho and Ricklefs 2011; Szöllősi et al. 2011), sequences in public databases are almost exclusively cyt *b* haplotypes, and thus a global phylogeographic context, needed for the estimation of the number of colonizations and dating analyses, cannot be provided for other markers. Analyses of the genetic diversity of hemosporidian parasites increasingly suggest that species described on the basis of morphological characters are actually species complexes (e.g., Sehgal et al. 2006). In the absence of objective criteria to define species limits in hemosporidian parasites, intra- and interspecific diversities cannot be teased apart. Bensch et al. (2004), however, suggested that cyt *b* haplotypes differing by as little as 0.5% in sequence divergence (representing two substitutions in a 476-bp fragment) may represent separately evolving entities that do not recombine. The potential causes of such a slow cyt *b* nucleotide substitution rate in hemosporidians are detailed in Bensch et al. (2013). Haplotype identification was carried out through PCR amplification, as described in Hellgren et al. (2004). Bidirectional sequencing was then carried out on an Applied Biosystems PRISM 3130, yielding 476- and 478-bp sequence fragments for *Leucocytozoon* and *Plasmodium*, respectively. Amplification conditions and sequencing details followed Cornuault et al. (2012). Diversity for each genus was estimated with Simpson's index (Simpson 1949). In order to determine whether there is a significant difference in diversity between the two genera, we calculated 95% confidence intervals, following Grundmann et al. (2001).

### *Phylogenetic and Dating Analyses*

We reconstructed a phylogeny with sequences from both *Plasmodium* and *Leucocytozoon*, using BEAST v1.7.3 (Drummond and Rambaut 2007). The analysis was carried out from an alignment including all haplotypes recovered from Mascarene birds, along with allochtonous haplotypes whose cyt *b* sequences were obtained from the GenBank and MalAvi (Bensch et al. 2009) databases. For both *Leucocytozoon* and *Plasmodium*, these were retrieved from all continents except Antarctica (see supplementary table, available online, for accession numbers). While all *Leucocytozoon* lineages infect birds, a number of *Plasmodium* lineages infect other vertebrates (Valkiūnas 2005). Thus, since previous phylogenetic studies have shown that avian *Plasmodium* form multiple clades that do not comprise any nonavian *Plasmodium* lineage (Perkins and Schall 2002; Martinsen et al. 2008), we included avian *Plasmodium* lineages only in our alignment so as to provide the relevant phylogeographic context for the study of colonization and diversification dynamics. In total, the align-

ment comprised 203 avian *Plasmodium* and 236 *Leucocytozoon* DNA sequences. The model of nucleotide substitution that best fitted the data according to the minimum Akaike Information Criterion (GTR + Γ + I) was determined using Mega5 (Tamura et al. 2011). The reciprocal monophyly of *Leucocytozoon* and avian *Plasmodium* is supported by all molecular phylogenies that included these two groups (Perkins and Schall 2002; Martinsen et al. 2008; Outlaw and Ricklefs 2011). Accepting this relationship as true, we chose to use relaxed-clock rooting, as implemented in BEAST, for inferring the root position between the two groups (see also Renner et al. 2008). We assessed the possible effect of this rooting method on our results by repeating our analyses using outgroup rooting, with *Theileria annulata* (order Piroplasmida) as an outgoup. Although *Theileria* represents one of the closest known relatives of Haemosporida, it remains distantly related to this group. Molecular dating was carried out under a birth-death speciation model with incomplete taxon sampling as the tree prior (Stadler 2009) and a relaxed, uncorrelated, lognormal molecular clock (Drummond et al. 2006). We assessed the robustness to the choice of tree prior by rerunning all analyses with a standard birth-death prior (Gernhard 2008) and a Yule prior (Yule 1925; Gernhard 2008). The nucleotide substitution rate of hemosporidian parasites is still unknown, and estimates obtained with different methods differ from one another by 1 order of magnitude (Bensch et al. 2013). Consequently, the mean substitution rate across all branches of the tree (*ucld.mean* parameter) was arbitrarily set to 1, and we used relative ages in all analyses. Four independent Markov chain Monte Carlo (MCMC) runs of 30 million generations, with sampling every 5,000 steps, were combined after the first five million generations of each run were discarded as burn-in. Convergence was visually assessed in Tracer v.1.5 and confirmed by effective sample sizes greater than 200 for all parameters. All subsequent analyses were based on the whole post-burn-in posterior distribution of 20,000 trees, so as to account for phylogenetic uncertainties. To carry out these analyses, we wrote a script in R language that gathers, from each tree of the posterior distribution, the number of Mascarene clades (i.e., the number of colonization events), the timing of colonization by each clade (i.e., its stem age), and the diversity of each clade (i.e., the number of haplotypes they comprised).

### *Colonization Dynamics*

Both single- and multiple-lineage clades were included in all analyses. To estimate the number of distinct lineages that colonized the Mascarenes, we assumed that clades composed solely of Mascarene haplotypes were most likely the product of a single colonization event. Thus, our measure of the number of colonizations was the number of Mascarene clades. This measure is possibly an overestimate of the true number of colonizations, particularly when mainland haplotypes are found nested within a Mascarene clade. Such a topological arrangement may arise from either phylogenetic uncertainty or reverse colonization. Reverse colonization seems unlikely in our study system, because we focused on small and remote islands (Bellemain and Ricklefs 2008), with no evidence that reverse colonization has ever happened in any group of the potential bird hosts for which phylogenetic evidence is available (see, e.g., Warren et al. 2005, 2006; Fabre et al. 2012). Thus, we expect the potential effect of nested mainland haplotypes to be mostly due to phylogenetic uncertainty and unlikely to qualitatively change our conclusions. For each tree of the posterior distribution, we calculated the number of *Plasmodium* and *Leucocytozoon* colonizations, along with the difference between these quantities. We then computed the 95% highest posterior density (HPD) interval for this posterior distribution of the difference in the number of colonizations by the two genera. If this interval does not include 0, we can be confident that the number of colonizations was different between *Plasmodium* and *Leucocytozoon*, taking phylogenetic uncertainty into account.

Similarly, we derived the posterior distribution of the ratio of the mean ages of *Plasmodium* and *Leucocytozoon* clades. If the 95% HPD interval of this distribution does not include 1, we can be confident that *Plasmodium* and *Leucocytozoon* clades colonized the Mascarenes at different times.

### *In Situ Diversification Dynamics*

To estimate the contribution of in situ diversification to current diversity, we produced posterior distributions of the number of Mascarene haplotypes that had arisen through cladogenesis after initial island colonization for each genus and the difference in this quantity between the two genera. To do so, we calculated the number of cladogenetic lineages as the total number of Mascarene haplotypes minus the number of colonizations over trees drawn from the posterior distribution of the Bayesian MCMC analysis. We then computed the 95% HPD interval for the posterior distribution of the difference in the number of cladogenetic lineages between the two genera. If this interval does not include 0, we can be confident that in situ diversification produced different numbers of lineages in *Plasmodium* and *Leucocytozoon*, with phylogenetic uncertainty taken into account.

Examining differences in diversification dynamics between groups of organisms where fossils are absent, such as hemosporidian blood parasites, usually involves esti-

mating speciation ($\lambda$) and extinction ($\mu$) rates and subsequently diversification rates ($r$) from phylogenetic information (Nee et al. 1994*a*). This is typically done by comparing the observed distribution of a tree's node ages with the expectation under a birth-death process with given parameters. However, because the clades considered here comprise few extant taxa, this approach could not be implemented in this study, and we used instead an alternative approach based only on information about the number of extant taxa ($N$) and clade ages ($t$) for comparing clade rates. We built on previous work by Bokma (2003), who described a procedure for testing equality of diversification rates between groups of clades. This test relies on estimates of the maximum likelihood combination of parameters ($\hat{\lambda}, \hat{\mu}$) for each group individually and then for all groups combined, and it uses the likelihood ratio as a statistic for evaluating significance. Here, we modified this test so that only $r$, the net diversification rate, is estimated.

The probability that a single lineage that arose at time $t$ in the past resulted in $N$ extant lineages, conditioned on $N > 0$ and with diversification rate $r$ and relative death rate $\kappa = \mu/\lambda$, is expressed as

$$l(r, \kappa) = (1 - \beta)\beta^{N-1}, \tag{1}$$

with

$$\beta(r, \kappa) = \frac{e^{rt} - 1}{e^{rt} - \kappa} \tag{2}$$

(Raup 1985; Nee et al. 1994*b*). Generalizing for a group of $m$ clades, the likelihood function becomes

$$l_m(r, \kappa) = \prod_{i=1}^{m} (1 - \beta_i)\beta_i^{N_i - 1}. \tag{3}$$

Both $r$ and $\kappa$ parameters could be estimated through maximum likelihood. However, here we want to specifically test for differences in $r$, and we consider $\kappa$ a nuisance parameter. Therefore, we do not impose any constraint on $\kappa$ (i.e., $\kappa$ can vary within and between groups of clades), and we integrate over $\kappa$ to obtain the marginal likelihood function, depending only on $r$. The likelihood function for $m$ clades becomes

$$l_m(r) = \int \dots \int_0^1 \left[\prod_{i=1}^{m} (1 - \beta_i)\beta_i^{N_i - 1}\right] d\kappa_1 \dots d\kappa_j \dots d\kappa_m, \tag{4}$$

which is equivalent to

$$l_m(r) = \prod_{i=1}^{m} \left[\int_0^1 (1 - \beta_i)\beta_i^{N_i - 1} d\kappa_i\right]. \tag{5}$$

Fortunately, these integrals can be solved, and there is no need for numerical integration (app. A, available online):

$$\int_0^1 (1 - \beta_i)\beta_i^{N_i - 1} d\kappa_i = \frac{e^{rt_i} - 1}{(N_i - 2)(N_i - 1)} - \frac{(e^{rt_i} - 1)^{N_i - 1}(e^{rt_i})^{2 - N_i}}{N_i - 2} + \frac{(e^{rt_i} - 1)^{N_i}(e^{rt_i})^{1 - N_i}}{N_i - 1}$$

when $N_i > 2$,

$$\int_0^1 (1 - \beta_i)\beta_i^{N_i - 1} d\kappa_i = -\ln (e^{rt_i} - 1)(e^{rt_i} - 1) + (rt_i - 1)(e^{rt_i} - 1) + \frac{(e^{rt_i} - 1)^2}{e^{rt_i}}$$

when $N_i = 2$, and

$$\int_0^1 (1 - \beta_i)\beta_i^{N_i - 1} d\kappa_i = 1 + \ln (e^{rt_i} - 1)(e^{rt_i} - 1) - rt_i(e^{rt_i} - 1)$$

when $N_i = 1$.

For the null hypothesis, the likelihood is calculated for all groups of clades combined, so that $r$ is constrained to be identical in all groups. For the alternative hypothesis, $r$ is allowed to vary between groups of clades. In this study, we wanted to test for equality of rates between *Plasmodium* and *Leucocytozoon*, and we used as a statistic $T = -2 \ln (l_{\text{null}}/l_{\text{alternative}}) = 2(L_1 + L_p - L_c)$, where $L_1$, $L_p$ and $L_c$ are the maximum log likelihoods for *Leucocytozoon*, *Plasmodium*, and the two genera combined, respectively. The $T$ statistic follows a $\chi^2$ distribution whose number of degrees of freedom is the difference between the number of parameters estimated for the alternative hypothesis and the number of parameters estimated for the null hypothesis (Bokma 2003). With two groups, the alternative has two parameters and the null one, so that the distribution here has $2 - 1 = 1$ degree of freedom. The case with $r < 0$ and $\kappa > 1$ (when the death rate is greater than the birth rate) was not considered. Note that in this article, the term "diversification rate" always means "net diversification rate" ($r$).

As is, this likelihood framework applies to a single tree. However, large data sets are typically consistent with numerous alternative phylogenies that may greatly vary in

terms of clade ages and topology, and using a single consensus tree may greatly bias the estimation of diversification rates. Therefore, we adapted the likelihood function described above for fitting to all the trees of the posterior distribution of the Bayesian MCMC analysis, so as to integrate phylogenetic uncertainty. The null hypothesis of our test is the equality of diversification rates. We could fit the likelihood function in equation (5) to all trees at once so as to estimate the two genera's absolute diversification rates and then proceed to the likelihood ratio test. This procedure would constrain the absolute values of the diversification rates across the posterior distribution of trees. However, our null hypothesis of rate equality makes an assumption about the relative value of rates, not their absolute values. Consequently, we modified equation (5) so as to estimate the ratio of diversification rates $R = r_{\mathrm{P}}/r_{\mathrm{L}}$, with $r_{\mathrm{P}}$ and $r_{\mathrm{L}}$ the diversification rates of *Plasmodium* and *Leucocytozoon*, respectively. Only $R$ is constrained to be the same for all trees, while the absolute rates are estimated independently for each tree. This is important because the variation in absolute clade ages among trees may differentially affect the absolute diversification rates and their relative value.

For the $j$th tree of the posterior distribution, the likelihood function of equation (5) becomes

$$l_j(R, r_{\mathrm{L}}) = \prod_{i=1}^{m_{\mathrm{P}_j}} \left\{ \int_0^1 [1 - \beta_i(Rr_{\mathrm{L}})]\beta_i(Rr_{\mathrm{L}})^{N_i-1} d\kappa_i \right\} \times \prod_{i=1}^{m_{\mathrm{L}_j}} \left\{ \int_0^1 [1 - \beta_i(r_{\mathrm{L}})]\beta_i(r_{\mathrm{L}})^{N_i-1} d\kappa_i \right\}, \tag{6}$$

with $m_{\mathrm{P}_j}$ and $m_{\mathrm{L}_j}$ the respective number of clades of *Plasmodium* and *Leucocytozoon* in the $j$th tree. For $S$ trees, the likelihood function becomes

$$l_S(R, r_{\mathrm{L}_1}, \ldots, r_{\mathrm{L}_S}) = \prod_{j=1}^{S} \left( \prod_{i=1}^{m_{\mathrm{P}_j}} \left\{ \int_0^1 [1 - \beta_i(Rr_{\mathrm{L}_j})]\beta_i(Rr_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} \times \prod_{i=1}^{m_{\mathrm{L}_j}} \left\{ \int_0^1 [1 - \beta_i(r_{\mathrm{L}_j})]\beta_i(r_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} \right), \tag{7}$$

and the log likelihood is

$$\begin{aligned} L_S(R, r_{\mathrm{L}_1}, \ldots, r_{\mathrm{L}_S}) &= \ln \left[ \prod_{j=1}^{s} \left( \prod_{i=1}^{m_{\mathrm{P}_j}} \left\{ \int_0^1 [1 - \beta_i(Rr_{\mathrm{L}_j})]\beta_i(Rr_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} \times \prod_{i=1}^{m_{\mathrm{L}_j}} \left\{ \int_0^1 [1 - \beta_i(r_{\mathrm{L}_j})]\beta_i(r_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} \right) \right] \\ &= \sum_{j=1}^{S} \left( \sum_{i=1}^{m_{\mathrm{P}_j}} \ln \left\{ \int_0^1 [1 - \beta_i(Rr_{\mathrm{L}_j})]\beta_i(Rr_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} + \sum_{i=1}^{m_{\mathrm{L}_j}} \ln \left\{ \int_0^1 [1 - \beta_i(r_{\mathrm{L}_j})]\beta_i(r_{\mathrm{L}_j})^{N_i-1} d\kappa_i \right\} \right). \end{aligned} \tag{8}$$

This likelihood function treats the sample size (i.e., the number of independent observations of a clade's diversification) as $S \times \bar{m}_j$, where $\bar{m}_j$ is the average number of clades per tree across the two genera. However, the different trees of the posterior distribution provide different estimates of the ages and diversities of the same sample of clades and therefore are not independent replicates. Thus, summing over the $S$ trees of the posterior distribution unjustifiably multiplies the log likelihood, and consequently the likelihood ratio $T$, by a factor $S$. Therefore, $L_S$ in equation (8) must be divided by $S$ to restore the sample size to $\bar{m}_j$, the actual number of independent observations. In the null hypothesis, $R$ is constrained to 1 (equality of rates) and $r_{\mathrm{L}_j}$ can vary over the $S$ trees, so the number of free parameters is $S$. In the alternative hypothesis, the number of free parameters is $S + 1$, because $R$ is also estimated. Therefore, $T$ follows a $\chi^2$ distribution with $S + 1 - S = 1$ degree of freedom. To obtain estimates of the absolute diversification rates, we used the average estimate of $r_{\mathrm{L}}$ as an estimate of *Leucocytozoon* diversification rate and deduced that of *Plasmodium* ($\hat{r}_{\mathrm{P}} = \hat{R} \times \hat{r}_{\mathrm{L}}$).

We determined whether the difference in diversification rates estimated for the two genera could have caused the observed differences in the number of cladogenetic lineages, all else being equal. This step is important because a difference in diversification rates, even if statistically significant, could produce only a negligible difference in terms of current diversity if the time frame of diversification was too short or a very large difference if the time frame was very long. The probability density function of the number of lineages accumulated by $m$ clades diversifying over a period of time $t$ is given in equation (5).

We fixed $m$ and $t$ in this function to values representative of our system: $m = 10$ colonizations and $t$ is the mean stem age of *Leucocytozoon* clades (representing an upper temporal bound for our system, because *Leucocytozoon* colonized earlier than *Plasmodium*; see "Results"). The diversification rate $r$ was given the values obtained through maximum likelihood estimation. Thereby we obtained the probability density distributions of the number of lineages that the two genera would have accumulated in situ, given their estimated diversification rates, independently of the effects of immigration dynamics (number and timing of colonizations).

## Results

The results we obtained using outgroup rooting and alternative tree priors are qualitatively identical and quantitatively very similar in each case (see table A1, available online), and so for conciseness we present only the results using a birth-death speciation model with incomplete taxon sampling as the tree prior and relaxed-clock rooting.

### *Diversity*

In total, we recovered 10 and 26 haplotypes of *Plasmodium* and *Leucocytozoon*, respectively, from the Mascarene avifauna. Sequences were deposited in GenBank under accessions JN032593–JN032658 (*Leucocytozoon*, published in Cornuault et al. 2012) and JX276926–JX276947 (*Plasmodium*, this study). Haplotype abundances and distributions can be found in figure 1. For *Leucocytozoon*, none of the Mascarene haplotypes had been previously reported outside the archipelago, except for haplotypes L-RECOB03 and L-FOMAD01. For *Plasmodium*, 4 of the 10 Mascarene haplotypes were previously reported from various regions of the world (P-SGS1, P-GRW4, P-COLL7, and P-GRW6; see supplementary table for details). Therefore, the majority of Mascarene lineages may be endemic to this archipelago. Simpson's index (decreasing with increasing diversity) offers an estimation of the diversity of the two samples and equals 0.52 (95% confidence interval [CI]: 0.39–0.64)] and 0.16 (95% CI: 0.14–0.18) for *Plasmodium* and *Leucocytozoon*, respectively. Nonoverlapping 95% CIs indicate that *Leucocytozoon* is significantly more diverse than *Plasmodium* in the Mascarenes.

### *Number of Colonizations*

The consensus phylogeny shows that the diversity of both genera is the consequence of multiple colonizations, as the haplotypes recovered in the Mascarenes are not monophyletic for either genus (fig. 2). For *Leucocytozoon*, haplotypes L-SATEC01, L-FOMAD01, L-PYJOC02, L-HYBOR02, and L-ZOBOR07 have no close relatives in the Mascarenes and are therefore the products of five distinct colonizations. The monophyly of both group L1 and group L2 is well supported, suggesting that the haplotypes they comprise accumulated through in situ diversification. The haplotypes of group L3 form a polytomy together with allochtonous sequences, preventing us from concluding a single colonization event for this group. For *Plasmodium*, haplotypes PGRW6, P-ZOMAU01, and P-COLL7 and groups P1, P2, and P3 are unrelated and thus represent six colonization events (fig. 2). We are unable to infer the number of colonization events that gave rise to P1, P2 and P3, since all three received poor support as monophyletic groups (fig. 2). The average number of substitutions among haplotypes within Mascarene groups was 1.8 for *Leucocytozoon* (groups L1, L2, and L3) and 1.1 for *Plasmodium* (groups P1, P2, and P3). A detailed summary of genetic distances among haplotypes of this study has been deposited in the Dryad Digital Repository (http://dx.doi.org/10.5061/dryad.fj5d0; Cornuault et al. 2013).

The posterior distribution of the number of colonizations was estimated by counting the number of monophyletic Mascarene groups in each tree. The mean number of colonizations is 11.57 (95% HPD interval: 11–13) for *Leucocytozoon* and 9.20 (95% HPD interval: 8–10) for *Plasmodium*, providing very strong support for a scenario of multiple colonizations by the two genera. Also, the posterior distribution of the difference of the number of colonizations (fig. 3*A*) has a mean of 2.37 (95% HPD interval: 1–4). The 95% HPD interval excludes 0, indicating that *Leucocytozoon* colonized the Mascarenes more often than *Plasmodium* since the archipelago's origin, in contrast to our initial prediction.

### *Timing of Colonizations*

The analysis of the posterior distribution of the ratio of the mean ages of *Leucocytozoon* and *Plasmodium* clades revealed that *Leucocytozoon* clades are on average 2.46 (95% HPD interval: 1.22–3.81) times as old as *Plasmodium* clades (fig. 3*B*), indicating a strong difference in the timing of colonization events between the two genera.

### *In Situ Diversification*

The maximum likelihood (ML) estimator of the ratio of diversification rates ($\hat{R}$) is 0.22 (95% CI: 0.003–3.30; fig. 4*A*) and does not differ from 1, according to the likelihood ratio test ($T = 1.06$, $P = .30$), indicating that the null hypothesis of equal diversification rates cannot be rejected. The ML estimator of the *Leucocytozoon* diversification rate averaged across all trees ($\hat{r}_L$) is 27.16 (95% CI: 10.76–34.09). The ML estimator of the *Plasmodium* diversifica-

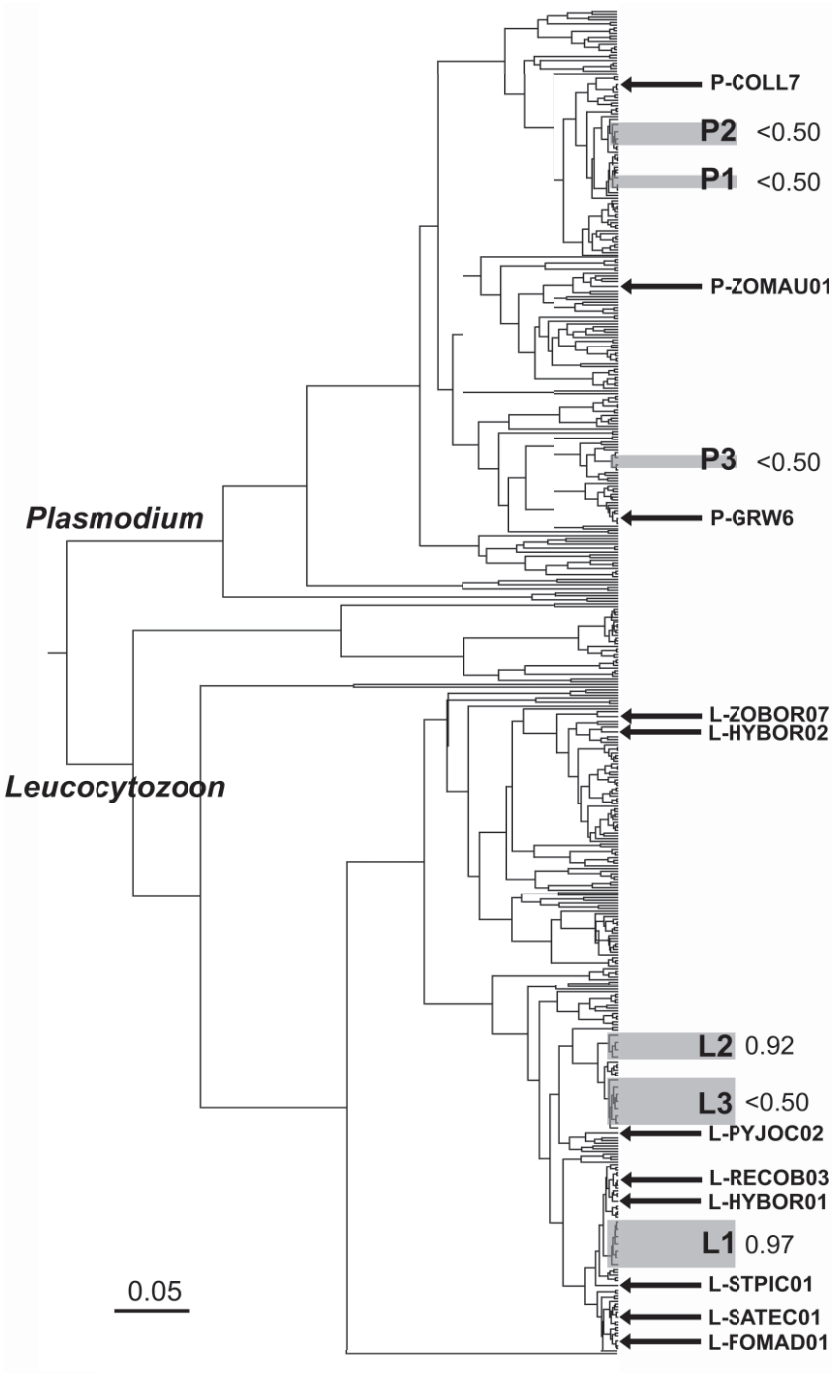



**Figure 2:** Phylogenetic position of Mascarene lineages. A single Bayesian phylogeny including both genera was constructed with BEAST. This figure shows the maximum clade probability consensus tree. The scale is by time unit and is arbitrary, because we carried out relative dating with an arbitrary value for the substitution rate. The position of Mascarene haplotypes in the phylogeny is indicated: those that may form monophyletic clades are represented in gray (Groups P1, P2, P3, L1, L2, and L3), with their posterior probability and isolated haplotypes indicated by arrows. For both genera, the disparate positions of different haplotypes indicate multiple colonizations.

tion rate, deduced from $\hat{r}_P = \hat{R} \times \hat{r}_L$, is 6.08 (95% CI: 0.10–35.50).

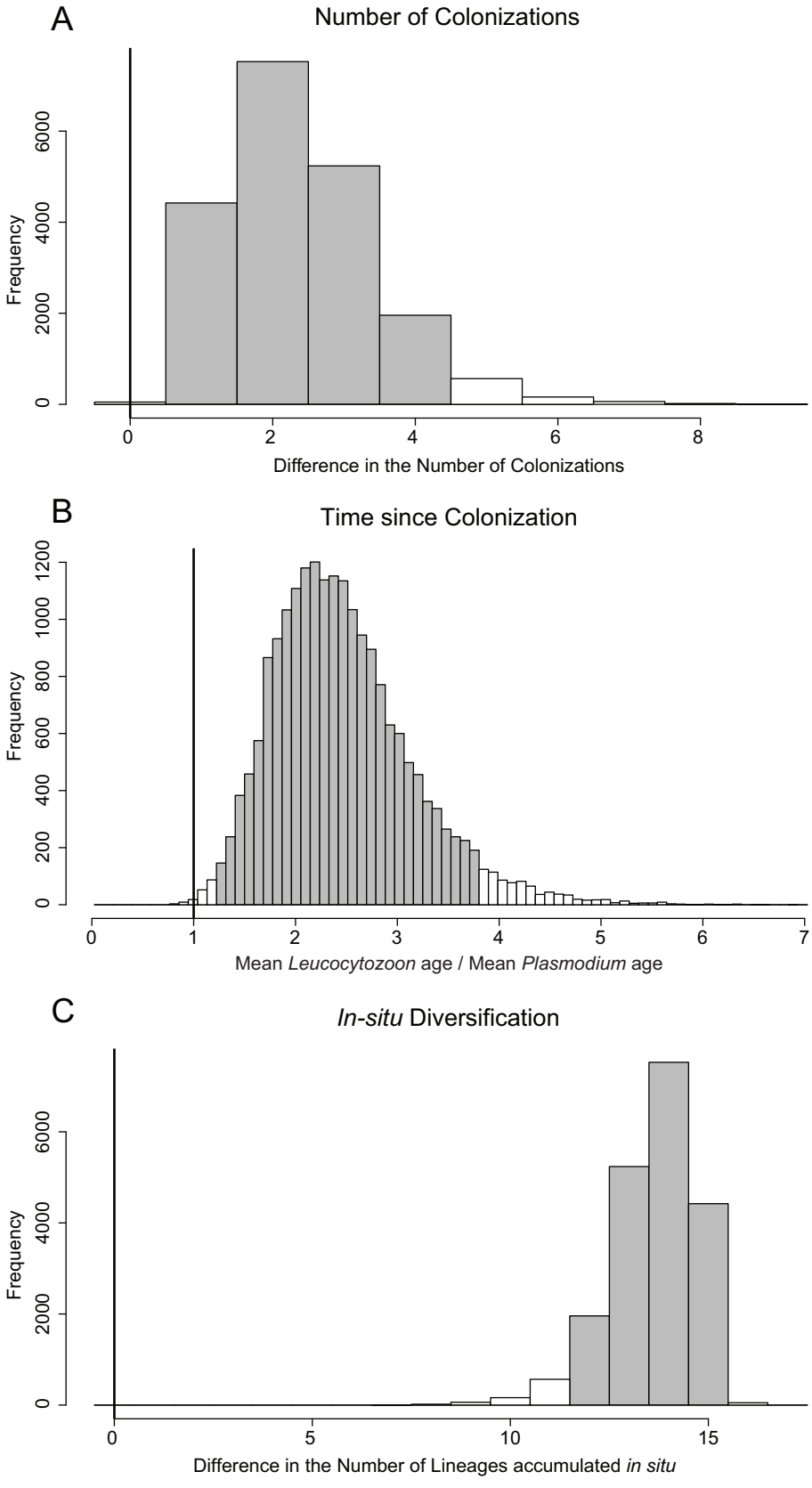
The posterior distribution of the number of cladogenetic haplotypes has a mean of 14.43 (95% HPD interval: 13–15) for *Leucocytozoon*, 0.80 (95% HPD interval: 0–2) for *Plasmodium*, and 13.63 (95% HPD interval: 12–15) for the difference between genera (fig. 3*C*). These results indicate that in situ diversification produced many more haplotypes in *Leucocytozoon* than in *Plasmodium*, both in terms of absolute values (14.43 and 0.80, respectively) and relative to the total haplotype richness (55.5% and 8%, respectively). We derived the probability density functions of the number of lineages that would accumulate with the two estimated diversification rates over the same period of time ($t$ is the mean stem age of *Leucocytozoon* clades) and with the same number of colonizations ($m = 10$; fig. 4*B*). The expected values of these distributions are 31.1 (95% CI: 10–74) and 15.2 (95% CI: 10–24) for *Leucocytozoon* and *Plasmodium*, respectively. The confidence intervals largely overlap, which means that even if our data could provide more confidence in our estimates of diversification rates, the number of lineages accumulated in the time period considered would still not differ much more between the two genera than expected under a stochastic model of a birth-death diversification process.

## Discussion

Greater diversity on islands can result from more numerous colonizations, earlier colonizations (leaving more time for in situ diversification), or a higher in situ diversification rate (MacArthur and Wilson 1967; Stephens and Wiens 2003; Ricklefs 2007; Wiens 2011; Kozak and Wiens 2012). Here, we investigated the relative contribution of these three processes in explaining the difference in diversity between two genera of Mascarene bird blood parasites. In doing so, we extended previously existing likelihood methods for the estimation of diversification rates (Bokma 2003), integrating out the relative extinction rate, which is particularly challenging to estimate from phylogenies (Rabosky 2010). We also used a Bayesian phylogenetic approach to generate a posterior distribution of trees, which we used to estimate differences in colonization number, colonization timing, and diversification rates between genera, incorporating phylogenetic uncertainty into the analyses. Integrating over all alternative phylogenies is a critical step in biogeographical studies, because a majority of phylogenetic trees suffer from poor node support and large dating uncertainties, which could greatly affect conclusions based on a single consensus tree (Sanmartín et al. 2008). Our approach can therefore be used to evaluate the relative importance of colonization and diversification dynamics in any group of organisms and is especially appropriate for dealing with uncertainties in both relative extinction rate and phylogeny.

We found that the diversity of *Leucocytozoon* in the Mascarenes is significantly (2.6 times) greater than that of *Plasmodium*. The estimated number of colonizations appears to be higher for *Leucocytozoon* than for *Plasmodium*, suggesting that the number of colonizations may be one of the factors explaining the higher diversity of *Leucocytozoon*. This was not expected from published studies, because *Plasmodium* is considered to be more vagile and a greater generalist than *Leucocytozoon*, which should have increased opportunities for colonization as well as the probability of successful establishment in the archipelago (MacLeod et al. 2010). The difference in the number of colonizations is in the range of 1–4, representing a marginal cause for the higher diversity of *Leucocytozoon*. However, we note that repeated immigrations by the same taxa cannot be detected from the phylogenetic information available. Thus, our estimates of the number of colonizations may reflect just the gross immigration rate (i.e., immigration of taxa that are not yet present on the island), which should vary positively with mainland diversity (MacArthur and Wilson 1967). If so, the higher number of colonizations by *Leucocytozoon* relative to *Plasmodium*, which contradicts our initial hypothesis of a higher immigration rate for *Plasmodium*, might reflect a greater continental diversity of *Leucocytozoon* rather than differences in their dispersal biology. While appropriate data are currently lacking to evaluate this hypothesis, Savage et al. (2009), in a detailed morphological survey of avian hemosporidian blood parasites in Madagascar (the source pool nearest to the Mascarenes), reported a large difference in diversity between *Leucocytozoon* (15 species, of which five were considered endemic to Madagascar) and *Plasmodium* (five species, of which one was considered endemic to Madagascar).

The immigration rate of a parasite is also a function of its prevalence on the continent, as a higher prevalence should decrease the frequency of miss-the-boat events (Clayton et al. 2003; MacLeod et al. 2010). Valkiūnas (2005) summarized prevalence data for *Plasmodium* and *Leucocytozoon*, showing that *Leucocytozoon* is globally more prevalent than *Plasmodium*. The relative prevalences of the two genera in the continental biogeographic zones closest to the Mascarenes are quite similar, but those of *Leucocytozoon* are usually slightly higher: 1.9% and 9.4% in Madagascar, 3.2% and 4.6% in the Ethiopian region, and 0.8% and 2.9% in Southeast Asia for *Plasmodium* and *Leucocytozoon*, respectively (Valkiūnas 2005; Savage et al. 2009). Data on the prevalence of hemosporidians in southern Africa, another potential source of parasites for the Mascarenes, are currently lacking, and such data will help


A
Number of Colonizations
Frequency
0
2000
4000
6000
0
2
4
6
8
Difference in the Number of Colonizations
B
Time since Colonization
Frequency
0
200
400
600
800
1000
1200
0
1
2
3
4
5
6
7
Mean Leucocytozoon age / Mean Plasmodium age
C
In-situ Diversification
Frequency
0
2000
4000
6000
0
5
10
15
Difference in the Number of Lineages accumulated in situ

in further assessments of the effect of prevalence on immigration dynamics of hemosporidians.

The diversity that cannot be explained by multiple colonizations must have arisen through in situ diversification (Wiens 2011). We estimated that 13–15 *Leucocytozoon* haplotypes arose through cladogenesis after colonization, while the number of cladogenetic haplotypes of *Plasmodium* was very small (0–2 haplotypes). Thus, the difference in diversity between *Plasmodium* and *Leucocytozoon* is largely due to *Plasmodium* having barely diversified in the Mascarene archipelago when compared to *Leucocytozoon*. Such a pattern can be explained by an earlier arrival of *Leucocytozoon* in the archipelago and/or a higher diversification rate (Ricklefs 2007).

*Leucocytozoon* clades are on average 2.46 times as old as *Plasmodium* clades. This implies that more time has been available for *Leucocytozoon* colonist lineages than for *Plasmodium* colonist lineages to diversify in the Mascarene archipelago. This finding highlights the importance of considering the interplay between immigration history and diversification when attempting to understand current diversity (Losos et al. 1998; Gillespie 2004; Wiens and Donoghue 2004; McPeek and Brown 2007; Ricklefs 2007); in the case of this study, the greater diversity of *Leucocytozoon* relative to *Plasmodium* is largely explained by the mere fact that *Leucocytozoon* colonist lineages became established in the Mascarene archipelago earlier (time-for-diversification effect; Stephens and Wiens 2003) and more often.

The maximum likelihood estimator of the ratio of diversification rates was 0.22, which represents a rate five times as high for *Leucocytozoon* as for *Plasmodium*. The null hypothesis of equality of diversification rates, however, cannot be rejected. The large confidence interval for the ratio of diversification rates (95% CI: 0.003–3.30) indicates that we lack statistical power and that we therefore cannot conclude that there is equality of diversification rates. Moreover, our use of a constant-rate tree prior (as currently implemented in BEAST) could lead to homogeneous diversification rates across the tree, which may then artificially reduce the estimated difference between diversification rates. Even though we cannot definitely accept the equality of diversification rates, we can assess the expected impact of a five-fold difference in diversification rates on relative diversity patterns, after fixing the other parameters (i.e., number and time of colonizations). Having done that, we found that the difference in diversity expected when imposing a five-fold difference in diversification rates is not sufficient to produce the contrasted diversity patterns we observed in *Leucocytozoon* and *Plasmodium*. This latter result suggests a greater role for a time-for-speciation effect than for differences in diversification rates in our system.

Our estimation of diversification rates rests on a birth-death model with a time-homogeneous diversification rate. There has been considerable debate about the constancy of diversification rates through time, notably in relation to the possible existence of a carrying capacity for the number of species constituting a clade (Ricklefs 2007; Rabosky 2009; Wiens 2011). Under the ecological-limits hypothesis, saturation of an island by species from a given clade may cause a gradual reduction of the clade's net diversification rate toward 0 (Rabosky 2009; Rabosky and Glor 2010). Unfortunately, our data cannot address the hypothesis of constant diversification rates because all clades that we recovered in this study are of low diversity, with a majority of single-lineage clades and a maximum of seven lineages for the most diverse clades. If diversification rates did vary temporally in our study system, then our method would still estimate the average diversification rate over the period from the time of colonization to the present. Thus, a lower rate for one genus could be indicative of a generally slower pace of lineage accumulation through time and/or a slowdown of lineage accumulation as the genus reaches carrying capacity.

In summary, this study demonstrates that the difference in diversity between *Leucocytozoon* and *Plasmodium* lineages in the Mascarene archipelago is due to between-parasite variation in colonization history (number and timing of colonizations), while in situ diversification seems to result in the accumulation of insular lineages at similar rates in both genera, although this latter result must be taken with caution. Studies of continental hemosporidian communities suggest that *Plasmodium* parasites are more vagile and more generalist than *Leucocytozoon* (Hellgren et al. 2007), which should drive *Plasmodium* to exhibit high immigration rates and low diversification rates on islands. Our results contradict these predictions, as *Plasmodium* colonized the Mascarenes less often and did not

**Figure 3:** Posterior distributions of between-genera differences in the number and timing of colonizations and the number of cladogenetic haplotypes. All distributions were calculated from the set of 20,000 trees of the posterior distribution. Gray bars represent the 95% HPD intervals. *A*, Posterior distribution of the difference between the number of colonizations by *Leucocytozoon* and the number of colonizations by *Plasmodium*. The 95% HPD interval is above 0, indicating that *Leucocytozoon* colonized more often. *B*, Posterior distribution of the ratio of the mean stem age of *Leucocytozoon* clades to the mean stem age of *Plasmodium* clades. The 95% HPD interval is above 1, indicating that *Leucocytozoon* colonized earlier. *C*, Posterior distribution of the difference between the number of *Leucocytozoon* cladogenetic haplotypes and the number of *Plasmodium* cladogenetic haplotypes. The 95% HPD interval is above 0, indicating that *Leucocytozoon* accumulated more haplotypes through in situ diversification.

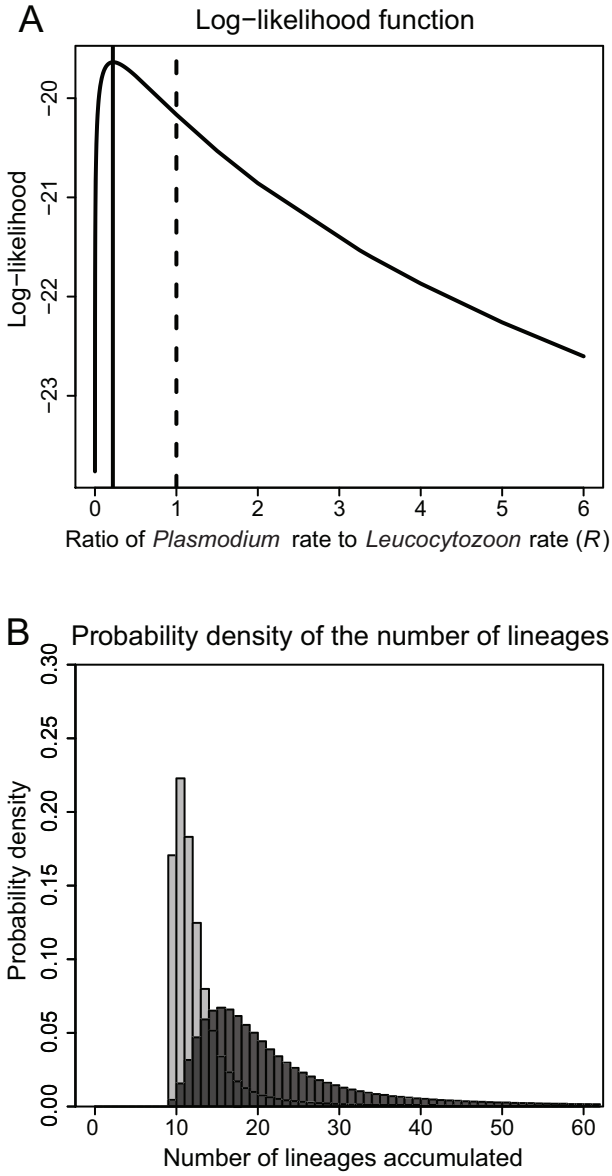



**Figure 4:** Maximum likelihood estimation and the expected number of cladogenetic haplotypes. *A*, Shape of the log-likelihood function. The solid vertical line indicates the maximum likelihood estimator of the ratio of diversification rates ($\hat{R} = 0.22$), and the dashed line indicates the value of this ratio under the null hypothesis ($R = 1$). *B*, Probability density functions of the number of lineages expected after a diversification process with $m = 10$ clades, $t$ is the mean stem age of *Leucocytozoon* clades, and $r = 6.08$ (estimated rate for *Plasmodium*, light gray) or 27.16 (estimated rate for *Leucocytozoon*, dark gray).

present a significantly slower diversification than *Leucocytozoon*. Much of the difference in diversity between *Plasmodium* and *Leucocytozoon* in the Mascarene archipelago is explained by differences in immigration history. Chance events happening at large spatial and temporal scales likely influence the order and timing of colonizations, possibly resulting in highly singular, idiosyncratic diversity patterns, rather than diversity patterns that are predictable on the basis of the biology of organisms (see also Fukami et al. 2007). Although making generalizations regarding the effect of parasite life-history traits on biogeographical dynamics will require further research across numerous replicated systems, our study highlights the potential of an integrative approach for inferring predominant processes that drive the buildup of species diversity in ecological communities.

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