MOLECULAR ECOLOGY

Molecular Ecology (2012) **21**, 1438–1452 doi: 10.1111/j.1365-294X.2012.05483.x

The role of immigration and *in-situ* radiation in explaining blood parasite assemblages in an island bird clade

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Abstract

Parasite communities on islands are assembled through multiple immigrations and/or *in-situ* diversification. In this study, we used a phylogenetic approach to investigate the role of such processes in shaping current patterns of diversity in *Leucocytozoon*, a group of haemosporidian blood parasites infecting whites eyes (*Zosterops*) endemic to the Mascarene archipelago (south-western Indian Ocean). We found that this parasite community arose through a combination of multiple immigrations and *in-situ* diversification, highlighting the importance of both processes in explaining island diversity. Specifically, two highly diverse parasite clades appear to have been present in the Mascarenes for most of their evolutionary history and have diversified within the archipelago, while another lineage apparently immigrated more recently, probably with human-introduced birds. Interestingly, the evolutionary histories of one clade of parasites and Indian Ocean *Zosterops* seem tightly associated with a significant signal for phylogenetic congruence, suggesting that host–parasite co-divergence may have occurred in this system.

Keywords: avian malaria, *in-situ* diversification, *Leucocytozoon*, Mascarene *Zosterops*, multiple colonization, parasite communities

Received 11 August 2011; revision received 20 December 2011; accepted 30 December 2011

Introduction

The need to understand the processes that determine the emergence of novel diseases has never been greater (Peterson 2008). Yet despite many hypotheses and a considerable amount of literature, we still know relatively little about what causes movements of parasites across hosts and geographic areas through time. While phylogenetic analyses of the present-day distribution of parasites across hosts and through space provide a powerful framework to reconstruct biogeographical history (Ronquist 1998), this performance relies upon the ability to estimate divergence times, usually from fossil data (e.g. Vilhelmsen 2004; Murphy *et al.* 2008). For most parasites and micro-organisms, fossil data are insufficient (Martín-González *et al.* 2009) and the common practice has been to use the time frame of hosts

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instead, provided that the evolutionary histories of hosts and parasites can be linked through the identification of co-divergence events (e.g. Hafner & Page 1995; Etherington *et al.* 2006; Storfer *et al.* 2007; Light *et al.* 2010; Shah *et al.* 2010). There are at least two caveats associated with this approach. First, synchronous co-divergence between hosts and parasites depends on strong host specificity of the parasites (Brooks 1988; Reed & Hafner 1997; Clayton *et al.* 2004; Nieberding & Olivieri 2007). Second, processes other than co-divergence can explain topological congruence between parasites and hosts, such as a series of host-switches mirroring the hosts' phylogeny (Charleston & Robertson 2002; de Vienne *et al.* 2007). In this context, haemosporidian blood parasites (Order: Haemosporida) of vertebrates are particularly problematic because they show little specificity, are highly vagile and are prone to host switching (Ricklefs *et al.* 2004; Szymanski & Lovette 2005; Garamszegi 2009). In addition, their fossil record is extremely scarce (Poinar 2005; Poinar & Telford 2005), making it difficult to estimate absolute divergence times using fossil calibration. As a consequence, the origin and evolution of these parasites, including how and when they radiated and spread to novel hosts, remains largely unknown and subject to debate. This is even the case for well-studied human parasites (e.g. Holmes 2010; Liu *et al.* 2010; Prugnolle *et al.* 2010 and Valkiūnas *et al.* 2011 illustrate recent debate over the assumed origin of *Plasmodium falciparum*).

It has been suggested that in Haemosporida of the genera *Haemoproteus* and *Leucocytozoon,* host and geographic fidelity (i.e. phylogenetic association) can persist over evolutionary timescales (Ricklefs & Fallon 2002; Hellgren *et al.* 2007). However, host fidelity is still expected to decrease as the time frame under consideration lengthens, with host specificity being mostly restricted to the tips of the parasite phylogeny (Ricklefs & Fallon 2002). Thus, phylogeographical studies of such haemosporidian blood parasites are especially valuable for examining the processes shaping contemporary diversity.

In this paper, we examine the evolutionary dynamics surrounding contemporary host and geographic distributions of *Leucocytozoon* parasites of Mascarene white-eyes (Genus: *Zosterops*). *Leucocytozoon* is the most host-specific and least vagile haemosporidian genus (Hellgren *et al.* 2007), with a complex life cycle involving one intermediate avian host where asexual multiplication occurs, and one final dipteran host (black flies, Genus: *Simulium*) in which it sexually reproduces (Valkiūnas 2005; Martinsen *et al.* 2008). As such, it provides an ideal system in which to examine the relative importance of processes such as multiple colonizations, *in-situ* diversification and co-radiation in explaining the diversity of parasite assemblages. Furthermore, Mascarene white-eyes represent an excellent host system for studying parasite evolutionary dynamics, in large part because they have undergone a relatively recent (<2 Ma, Warren *et al.* 2006) evolutionary radiation in an archipelago where contemporary infections by *Leucocytozoon* have been reported to be frequent (Peirce *et al.* 1977).

We use molecular methods to describe *Leucocytozoon* diversity in Mascarene *Zosterops* and perform phylogenetic analyses to infer how contemporary diversity in blood parasites has arisen. We included in our analyses data obtained from other local birds as well as all publicly available *Leucocytozoon* DNA sequences, to provide the basis for comparisons in a broad phylogeographical context. We first estimate the number of independent colonizations that may account for contemporary diversity by determining the number of monophyletic groups of parasites harboured by Mascarene *Zosterops*. Parasite colonizations may have occurred either in evolutionary times, mediated by black flies or avian hosts as they immigrated to the archipelago, or more recently with massive human-associated introductions of nonindigenous birds (Cheke & Hume 2008). Our second objective is thus to estimate the likelihood of parasite diversification within the archipelago after nonindigenous birds were introduced by humans, i.e. within the last 413 years (Cheke & Hume 2008). Patterns of parasite distribution across host species can also provide information about which hosts may have brought parasites into the archipelago. Finally, we examine whether some parasites could have co-radiated with *Zosterops* hosts over the Indian Ocean. Such a process, be it associated with strict co-divergence or not, is expected to result in relatively concordant host and parasite geographic distributions and phylogenies.

Methods

Samples and sites

Twelve *Zosterops* species are currently present in the south-western Indian Ocean, four of which occur in the Mascarenes: *Z. borbonicus*, *Z. mauritianus*, *Z. chloronothos* and *Z. olivaceus* (Warren *et al.* 2006). One of us (BHW) obtained blood samples from the eight non-Mascarene species and *Z. chloronothos* during a previous study (see Warren *et al.* 2006 for permits and procedures). We obtained blood samples from the three other Mascarene *Zosterops* species and twelve other bird species from Réunion by mist-netting in 2007–2010 during the austral summer (capture dates ranged from October 28th to May 17th). In total, our sample consisted of 55 non-Mascarene *Zosterops*, 852 Mascarene

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Zosterops and 89 Réunion non-*Zosterops* birds. Blood samples were collected by gently puncturing the subbrachial vein and were then 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 CRBPO Museum d'Histoire Naturelle (Paris, France) and a collection permit from the Government of Mauritius. Per-species sample sizes are indicated in Table 1, and the location of *Zosterops* species in Fig. 1.

DNA extraction, PCR screening and sequencing

DNA was extracted from blood samples using Qiagen DNeasy kits (Qiagen, Courtaboeuf, France), according to the manufacturer's protocol. Parasites were screened by amplifying a 476-bp fragment of the cytochrome *b* (cyt-*b*) gene, following the nested-PCR protocol described in Hellgren *et al.* (2004), with the *Leucocytozoon*-specific primer pair (HaemFL/HaemR2L) in the second round. To detect false positives, a negative control (distilled water) was included with every five samples. When false positives were observed, the entire PCR plate was re-run. Bi-directional sequencing was performed on an AB1 PRISM 3130 using the same primers as for the second round of PCR. Sequences from both strands were aligned using Sequencher v.4.9, and the chromatograms were visually screened for double peaks (multiple infections). Most of the sequences we obtained were of sufficiently good quality to allow accurate identification of double peaks, which were recorded with ambiguous base codes. We did not attempt to record double peaks for sequences with background noise. Once the local pool of haplotypes was known from the single infections, we searched for matches between ambiguous sequences (multiple infections) and pairs of haplotypes. A double infection was considered solved only when it yielded a match with a single pair of previously identified haplotypes and no double peaks were left unexplained. All unsolved multiple infections were withdrawn from the data set. In this study, we refer to haplotypes for unique cyt-*b* sequences and to lineages for phylogenetically distinct groups (either single haplotypes or clades of haplotypes).

Phylogenetic and divergence time analyses

To analyse our findings in a global phylogeographic context, all sequences obtained from Indian Ocean *Leucocytozoon* were aligned with the totality of sequences of sufficient length available in GenBank and Malavi (Bensch *et al.* 2009) databases. Phylogenetic analyses were performed from this alignment that consisted of 257 cyt-*b* haplotypes once redundant sequences were removed (see Table S1, Supporting information, for details of these sequences). The scarcity of nuclear and plastid DNA fragments from *Leucocytozoon* organisms in GenBank (ten entries altogether) prevents the use of markers other than cyt-*b* for global studies like this one. Thus, our analyses rely upon the assumption that phylogenetic hypotheses obtained using cyt-*b* are reasonable estimates of organism (i.e. multi-genome) phylogeny in *Leucocytozoon*, as was strongly suggested by previous studies (Rathore *et al.* 2001; Beadell *et al.* 2006; Perkins *et al.* 2007). After aligning all sequences with BioEdit v.7.0.5.3, we used MODELTEST v.2.3 (Posada & Crandall 1998) to determine which substitution model best described the data (minimum AIC). MRBAYES v.3.0.1 (Ronquist & Huelsenbeck 2003) was used for Bayesian phylogenetic inference with different substitution models for each codon position. This analysis consisted of two runs of 30 million generations with sampling every 1000 generations, each with one cold and three heated chains. Burn-in trees were discarded, and the consensus phylogeny and associated branch support values were determined from the remaining trees. PhyML-aLRT (Anisimova & Gascuel 2006) was used for maximum likelihood (ML) phylogenetic analysis, without partitioning into codon positions. Bootstrap support values were calculated as the minimum of SH-like and χ^2-based statistics. A consensus of the maximum sum of clade credibilities from the Bayesian and ML trees was obtained with TreeAnnotator v.1.5.3. This procedure firstly permitted us to test whether all parasite haplotypes recovered from *Zosterops* hosts represent a monophyletic entity or if they have multiple origins (polyphyly). Secondly, it allowed us to identify lineages of endemic parasites represented by single colonization events followed by within-island diversification.

Lineages having diversified *in-situ* (i.e. forming clades) may have colonized the archipelago with recently introduced birds, or earlier, through natural immigration. To test the origin of such clades, we assumed that recent anthropogenic introduction can be ruled out for a particular parasite clade if it can be shown with confidence that it is older than first human arrival (413 years ago). Therefore, we sought to provide a lower bound for each clade's age through dating analyses. Given that no substitution rate and no fossil calibrations are known for *Leucocytozoon* parasites, we used a conservative substitution rate for phylogeny dating. As we were interested in minimum ages of clades, we used the highest substitution rate proposed for some avian Haemosporida, i.e. 1.2% sequence divergence per million years (as estimated by Ricklefs & Outlaw 2010). Node age estimates were obtained with BEAST v.1.5.3 (Drummond & Rambaut 2007) under a strict molecular clock and from a subtree comprising all the lineages of

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Table 1 Number of occurrences of each lineage or clade in each host species. The detail of lineages within Clades A′ and B′ can be found in Table S2, Supporting information

Host species	Island*	Archipelago	Host type†	Tested	Infected	Sequencing success‡	Sequences§	Lineages**									
								Clade A′	Clade B′	B	H	X	Y	Z	A2	B2	C2
Streptopelia picturata	Réu	Mascarenes	?	4	1	1/1	1	—	—	—	—	—	—	1	—	—	—
Terpsiphone bourbonnensis	Réu	Mascarenes	I	10	0	—	0	—	—	—	—	—	—	—	—	—	—
Saxicola tectes	Réu	Mascarenes	I	10	7	6/7	6	—	—	2	—	4	—	—	—	—	—
Hypsipetes borbonicus	Réu	Mascarenes	I	21	17	11/17	12	—	—	1	—	5	4	—	—	2	—
Geopelia striata	Réu	Mascarenes	NI	1	0	—	0	—	—	—	—	—	—	—	—	—	—
Leiothrix lutea	Réu	Mascarenes	NI	1	0	—	0	—	—	—	—	—	—	—	—	—	—
Estrilda astrild	Réu	Mascarenes	NI	2	0	—	0	—	—	—	—	—	—	—	—	—	—
Acridotheres tristis	Réu	Mascarenes	NI	4	0	—	0	—	—	—	—	—	—	—	—	—	—
Pycnonotus jocosus	Réu	Mascarenes	NI	8	5	5/5	5	—	—	3	—	—	—	—	2	—	—
Foudia madagascariensis	Réu	Mascarenes	NI	10	8	7/8	8	—	—	6	—	—	—	—	—	—	2
Ploceus cucullatus	Réu	Mascarenes	NI	8	8	8/8	9	—	1	8	—	—	—	—	—	—	—
Passer domesticus	Réu	Mascarenes	NI	10	10	9/9	9	—	—	9	—	—	—	—	—	—	—
Zosterops borbonicus	Réu	Mascarenes	Z	783	431	133/145	221	56	153	2	10	—	—	—	—	—	—
Z. olivaceus	Réu	Mascarenes	Z	33	33	9/10	9	2	6	—	1	—	—	—	—	—	—
Z. mauritianus	Mau	Mascarenes		34	20	15/16	24	—	24	—	—	—	—	—	—	—	—
Z. chloronothos	Mau	Mascarenes		2	2	2/2	3	—	3	—	—	—	—	—	—	—	—
Z. maderaspatanus	Mad	—		16	16	14/16	19	—	19	—	—	—	—	—	—	—	—
Z. comoriensis	Moh	Comoros		7	7	5/6	7	—	7	—	—	—	—	—	—	—	—
Z. anjouanensis	Anj	Comoros		4	4	4/4	5	—	5	—	—	—	—	—	—	—	—
Z. kirki	GC	Comoros		11	0	—	0	—	—	—	—	—	—	—	—	—	—
Z. mayottensis	May	Comoros		6	0	—	0	—	—	—	—	—	—	—	—	—	—
Z. modestus	Con	Seychelles		3	0	—	0	—	—	—	—	—	—	—	—	—	—
Z. mouroniensis	GC	Comoros		6	0	—	0	—	—	—	—	—	—	—	—	—	—
Z. abyssinicus	Soc	—		2	2	2/2	3	—	3	—	—	—	—	—	—	—	—

*Réu, Réunion; Mau, Mauritius; Mad, Madagascar; Moh, Mohéli; Anj, Anjouan; GC, Grande Comore; May, Mayotte; Con, Conception; Soc, Socotra.

†For Réunion birds, assignment to three categories according to Barré & Barau (1996). NI: Nonindigenous (i.e. introduced on Réunion by man), I: Indigenous (i.e. non-*Zosterops* naturally occurring before the arrival of man) and Z: *Zosterops* (i.e. *Zosterops* indigenous species). ?: it is unknown whether *S. picturata* is indigenous or was introduced.

‡Number of individuals for which a usable sequence was obtained/Number of individuals for which sequencing was attempted.

§Number of sequences might be higher than number of sequenced individuals because of double infections.

**The absolute prevalence of each haplotype in a host species can be estimated as $(x/y)(w/z)$, where x is the number of birds infected by the haplotype, y is the number of infected birds that were sequenced, w is the number of birds infected by any haplotype, z is the number of birds that were screened.

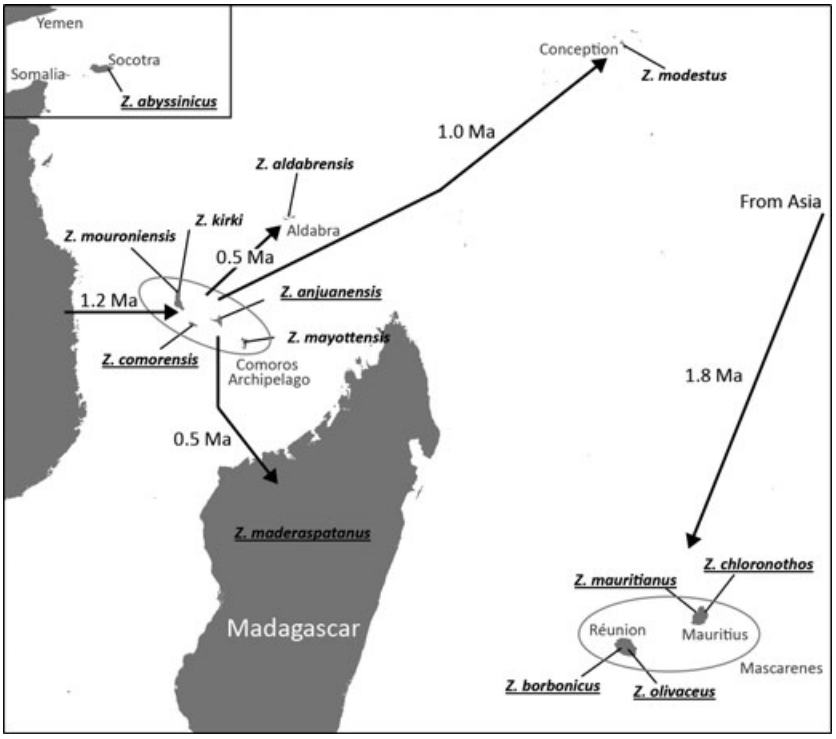

Fig. 1 Distribution of sampled species of *Zosterops* in the south-western Indian Ocean. Indian Ocean *Zosterops* species are in black, locations in grey. *Zosterops* species found infected are underlined. Black arrows were redrawn from Warren *et al.* 2006 and indicate the most likely colonization routes of *Zosterops* in the Indian Ocean. It is noted that *Z. mouroniensis*, unlike all other *Zosterops* species from the Comoros, colonized the Indian Ocean anciently. However, it is unconfirmed whether this species is a product of the same ancestral Indian Ocean colonist as the Mascarene clade, or whether its ancestor colonized the Indian Ocean independently.

interest. The analysis consisted of two independent runs of 10 million generations with sampling every 1000 generations. After discarding burn-in generations (first 10%), the results of the two runs were combined and examined with TRACER v.1.5. Convergence was confirmed by ESSs exceeding 200 for all parameters. A consensus chronogram of the maximum sum of clade credibilities was obtained with TreeAnotator v.1.5.3.

Distribution across hosts

We investigated the distribution of each parasite lineage across hosts by testing for differences in prevalence between three host reservoirs: *Zosterops*, indigenous and nonindigenous birds (see Table 1). However, the detection of a haemosporidian lineage through amplification from a particular host's blood does not necessarily reflect host competence for transmission of this lineage, as uninfective parasite stages can occur in blood samples (Valkiūnas *et al.* 2009). Although microscopy can be used to confirm the presence of infective gametocytes in the host blood, this technique does not permit the identification of haplotypes (Valkiūnas *et al.* 2009; Palinauskas *et al.* 2010). Thus, the reasonable assumption that the parasite stages amplified from blood are in most cases infective gametocytes must be made when examining patterns of distribution across hosts.

Our sample for *Zosterops* species was much larger than for the rest of the local avifauna and comparatively more parasite haplotypes could have been undetected in non-*Zosterops* hosts. To quantify the potential bias caused by incomplete haplotype sampling, we generated rarefaction curves for the three host reservoirs on Réunion by sampling without replacement (Gotelli & Colwell 2001) and estimated true haplotype richness with a Chao (1987) estimate as well as the first order jackknife and bootstrap estimates (Smith & van Belle 1984). Curves are the average of 9999 trials. These analyses were carried out using vegan 1.15 (function specpool) in the R statistical framework (R Development Core Team 2008). Uneven sample sizes among species prevented us from constructing rarefaction curves for each species independently. Consequently, individual birds were grouped together within the three host reservoirs cited earlier.

To test for differences in prevalence between host reservoirs, we used GLMMs with binomial error with host reservoir as a fixed effect and month of capture and year as random effects. Random effects were incorporated in the models to account for temporal variation in prevalence, as such variation was previously reported in haemosporidian parasites, both within- (e.g. Barnard & Bair 1986; Cosgrove *et al.* 2008; Norte *et al.* 2009) and between-years (e.g. Bensch *et al.* 2007; Knowles *et al.* 2011). The binary dependant variable was lineage identity: each cyt-*b* sequence was attributed the value of 1 if it corresponded to the lineage of interest, and the value of 0 for any other lineage. This approach tests whether one lineage's *relative prevalence* (i.e. the proportion of infections corresponding to this lineage) differs among

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host reservoirs. We could not test for differences in *absolute prevalence per lineage* (i.e. the proportion of birds in the sample that is infected by a particular lineage) as this measure is unknown when sequences are not obtained from every infected individual. However, *absolute* and *relative* prevalences are proportional when the *total absolute prevalence* (i.e. the proportion of birds infected by any lineage) does not differ among the groups compared, as is the case for our sample (*Zosterops* vs. Indigenous, $P = 0.87$; *Zosterops* vs. Nonindigenous, $P = 0.086$; Indigenous vs. Nonindigenous, $P = 0.27$; uncorrected P-values from Fisher exact tests). Following recommendations of Bolker *et al.* (2009), significance of the fixed effect was evaluated with Wald χ^2 tests. We subsequently carried out pair-wise tests following the same procedure to obtain pair-wise significance for the effect of host reservoir, and P-values were adjusted with Bonferroni correction. Analyses were restricted to Réunion birds because non-*Zosterops* birds were not screened elsewhere. The geographic areas where *Zosterops* and non-*Zosterops* birds were sampled greatly overlap (Fig. S1, Supporting information). Therefore, differences in parasite assemblages of these two reservoirs cannot be explained by small scale spatial transmission patterns (as documented by Wood *et al.* 2007). Statistical analyses were carried out using the lme4 package (Bates & Sarkar 2007) in the R statistical framework (R Development Core Team 2008).

Co-divergence analysis

We tested the hypothesis that Indian Ocean clades of parasites infecting *Zosterops* have co-diverged with their hosts using the software ParaFit (Legendre *et al.* 2002). This procedure tests the null hypothesis of independent evolution of hosts and parasites by permuting the identity of hosts associated with each parasite. Thus, it provides a test of co-divergence for the whole data set along with tests for each host–parasite pair. It requires a matrix of host–parasite association along with two matrices of principal coordinates describing phylogenetic relationships (one for hosts and one for parasites). These matrices were constructed from LogDet genetic distance matrices (Lockhart *et al.* 1994) using the DistPcoa application (Legendre & Anderson 1999). The test was conducted using 9999 permutations.

Results

Diversity and multiple colonizations

Altogether, 26 different *Leucocytozoon* haplotypes were found in the Mascarenes (Tables 1 and S2, Supporting information), 20 of which occur in Mascarene *Zosterops* (17 in *Z. borbonicus* alone). Rarefaction curves (Fig. 2) and estimates of true haplotype richness (Table 2) show that asymptotic richness on Réunion is almost reached for the non-*Zosterops* reservoirs, despite small sample sizes. In contrast, the estimated number of missed haplotypes in *Zosterops* is approximately 6 (Table 2), indicating that about 25% of the total haplotype richness of *Leucocytozoon* infecting Réunion *Zosterops* remains undiscovered.

Phylogenetic analyses indicate that the 20 Mascarene haplotypes represent four distinct lineages: Clades A′ and B′, Haplotypes B and H (Fig. 3), implying multiple colonizations of the Mascarene archipelago followed by at least two independent evolutionary radiations. Phylogenetic resolution is too poor to determine whether or not Haplotype B clusters with Clade A′ (Fig. 3). Mascarene haplotypes within Clade B′ cluster in two groups (*Mascarenes 1* and *2*).

Timing of colonization

Clades A′ and B′ are highly diverse, with seven and 11 haplotypes in the Mascarenes, respectively. Molecular dating with a divergence rate of 1.2% Ma^{-1} places the crown age estimate of Clade A′ at 0.66 Ma (95% CI: 0.31–1.07; Fig. 4) and that of Clade B′ at 1.56 Ma (95% CI: 0.97–2.24; Fig. 4). The oldest boundary for human-mediated introductions (413 years) lies well outside the 95% confidence intervals for both clades'

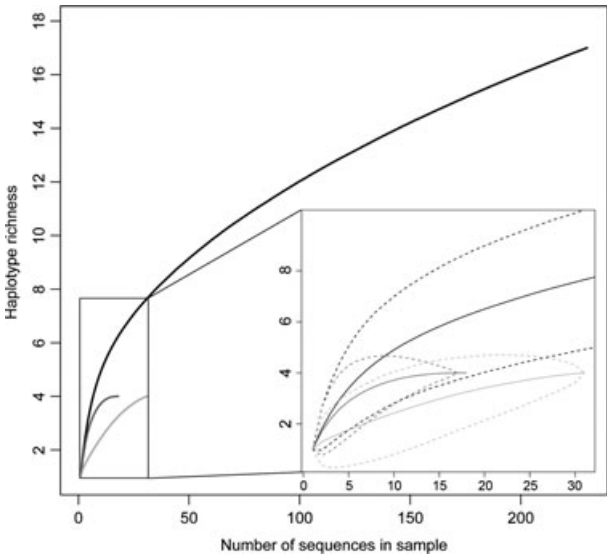

Fig. 2 Rarefaction curves for three different host reservoirs. Black: *Zosterops* reservoir, Dark grey: indigenous reservoir, Light grey: nonindigenous reservoir. Solid lines represent the average of 9999 randomly generated curves. Dashed lines are mean ± 2 times standard deviations. The smaller figure shows details of the curves from 1 to 30 haplotypes.

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Table 2 Estimated haplotype richness for the three host reservoirs on Réunion

Host reservoir	No. sequences	S_{obs}*	Chao†	Jackknife†	Bootstrap†	Average estimates	Average number of missed haplotypes
Zosterops	230	17	25.17 ± 8.28	23.97 ± 2.63	19.98 ± 1.39	23.04	6.04
Indigenous	18	4	4 ± 0	4 ± 0	4.17 ± 0.38	4.06	0.06
Nonindigenous	31	4	4.25 ± 0.73	4.97 ± 0.97	4.61 ± 0.66	4.61	0.61

*Observed haplotype richness.
†Estimated haplotype richness ± standard error obtained from three methods described in Chao (1987) and Smith & van Belle (1984).

ages, indicating that both clades have initiated their diversification before these introductions.

Distribution of lineages through hosts and space

Contrasting patterns of distribution across hosts and space were recovered between the different lineages infecting Mascarene *Zosterops*. We found that relative prevalence significantly varied among host reservoirs for Haplotype B ($\chi^2 = 57.5$, $P < 0.001$) and Clade B′ ($\chi^2 = 17.8$, $P < 0.001$), but not for Clade A′ ($\chi^2 = 2.1e^{-4}$, $P = 1$). Haplotype H was not analysed because of the small number of infections. Further information about these analyses is available in Table S3, Supporting information. Haplotype B primarily occurs in nonindigenous birds with an absolute prevalence of 63%, which is much higher than the absolute prevalence observed in indigenous (10%) or *Zosterops* (0.8%) reservoirs (Fig. 5a). It appears to be a generalist lineage as it was found in seven of nine infected species on Réunion (Table 1) and also occurs in Africa, Europe and the Comoros in five other passerine species (Table S4, Supporting information). By contrast, Clade A′ was only recovered from *Zosterops* (Table 1, Fig. 5b) and seems to be endemic to Réunion. Haplotypes from that clade were not recovered from any *Zosterops* species from outside the Mascarenes. Clade A′ was found to be nested within Clade A, which is composed of 24 additional haplotypes infecting 28 other avian species (Fig. 3, Table S4, Supporting information). Hosts of Clade A are mainly species residing in Africa and the Middle-East or European birds that winter in Africa, suggesting a within-Africa diversification of that clade. Clade A also contains three haplotypes found on Réunion: Haplotypes B, Y (found in *Hypsipetes borbonicus* only) and Z (found in *Streptopelia picturata* only). Clade B′, the most diverse clade in Mascarene *Zosterops* with a total of 11 haplotypes in the archipelago (seven haplotypes endemic to Réunion, three to Mauritius, one on both islands), seems to be restricted to *Zosterops* hosts, except for one exceptional occurrence in *Ploceus cucullatus* (Table 1, Fig. 5c). It is found in the four Mascarene *Zosterops* species and is the only lineage occurring in *Zosterops* found outside Réunion. It is also the most prevalent parasite lineage of *Zosterops*: 37% absolute prevalence on Réunion, 69% on Mauritius, 100% on Madagascar, 39% on the Comoros and 100% on Socotra. Also noteworthy in the phylogeny is the fact that Clade B′ is nested within Clade B, which contains 18 additional parasite haplotypes found mostly in Asian and European species (Fig. 3, Table S4, Supporting information). Haplotype H was found to infect only the two Réunion *Zosterops* species with low absolute prevalence (4.1% *Z. borbonicus*; 11% *Z. olivaceus*) and was not recovered from the local avifauna (Table 1, Fig. 5d). Its closest relative was found in Europe, infecting *Garrulus glandarius* (Haplotype GAGLA01). No *Leucocytozoon* parasites were recovered from *Z. mouroniensis*, *Z. modestus*, *Z. kirki*, *Z. mayottensis* and *Z. aldabrensis* (Fig. 1, Table 1).

Co-divergence of Leucocytozoon and Zosterops

Co-divergence analyses rejected the null hypothesis of independent evolution of *Leucocytozoon* and *Zosterops* for Clade B′ ($P = 0.035$), suggesting that parasite lineages belonging to Clade B′ co-diverged with their *Zosterops* hosts (Fig. 6).

Discussion

We found that *Leucocytozoon* contemporary diversity in Mascarene *Zosterops* originated through a combination of multiple colonizations and *in-situ* diversification events. We also were able to infer that the parasite lineages having undergone within-island evolutionary radiations immigrated to the Mascarenes well before the arrival of humans and concomitant introductions of nonindigenous birds. Strikingly, one of these lineages is strongly associated with *Zosterops* hosts across the entire western Indian Ocean, with a clear pattern of congruence between host and parasite evolutionary histories. Thus, our results suggest that hosts on remote islands can harbour highly diverse parasite assemblages that

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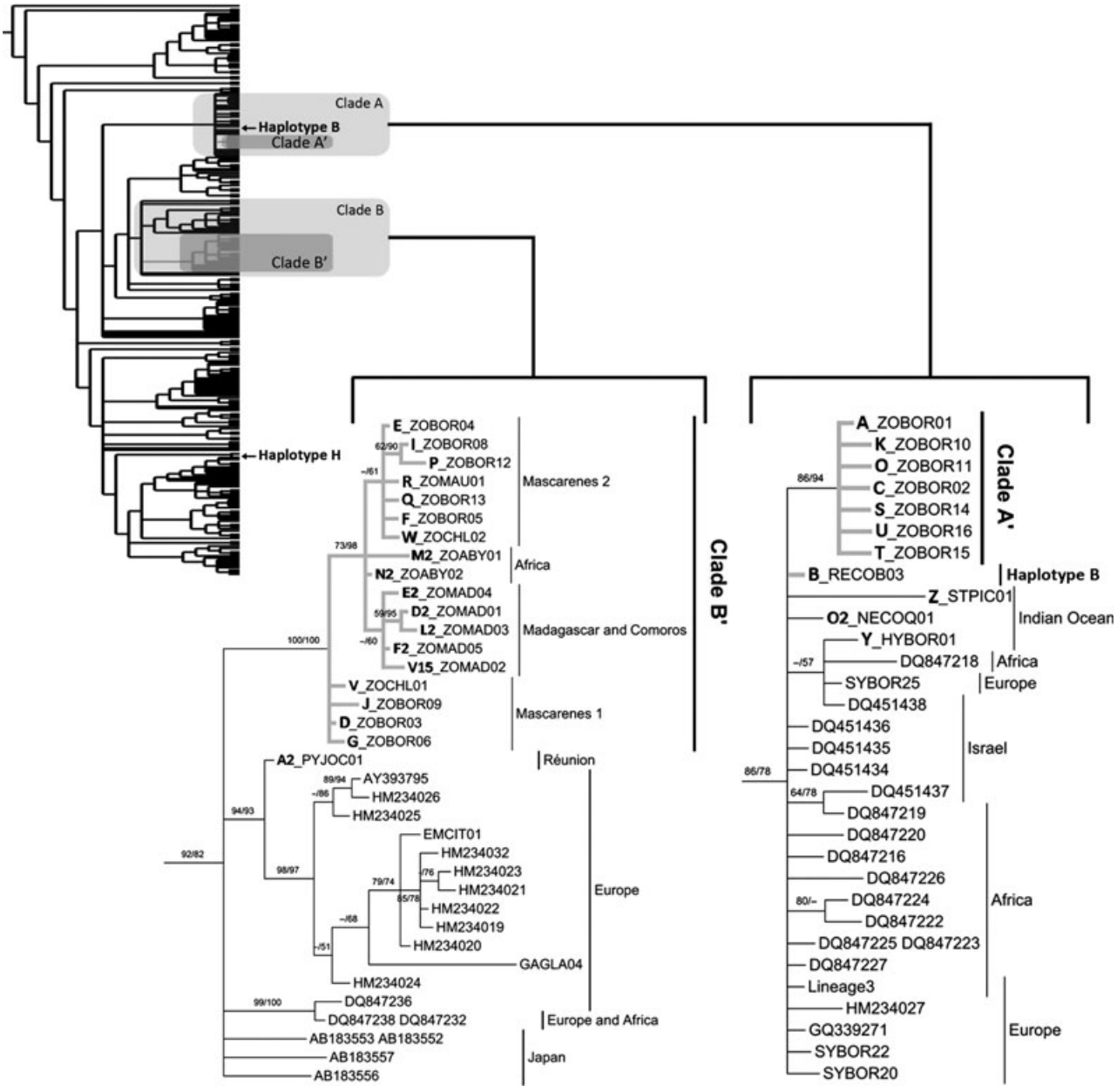

Fig. 3 Consensus phylogeny of *Leucocytozoon* lineages. The phylogeny was generated with all published cyt-*b* sequences and those of this study (altogether 257 sequences). The four lineages found in *Zosterops* are indicated in the global tree (top-left): Clade A', Clade B', Haplotype B and Haplotype H. Their polyphyly illustrates multiple origins. At the bottom, the topologies within Clades A (right) and B (left), that encompass, respectively, Clades A' and B', are detailed. For each node, support is indicated with bootstrap on the left and BBS on the right. '-' indicates that the node does not exist in the corresponding phylogeny. The lineages harboured by *Zosterops* are indicated by thick grey branches. The haplotypes found in the Mascarenes are shown in bold case (corresponding to the names in Table 1 and S2, Supporting information) plus the haplotype name in classical format (e.g. ZOBOR01). The detailed and complete consensus, maximum likelihood (ML) and Bayesian phylogenies are available in Figs S2–S4, Supporting information.

have gained new lineages through either immigration or *in-situ* diversification over evolutionary time. This stands in stark contrast to the evidence for reduced diversity of other haemosporidian parasites in several species of birds on remote islands of the Pacific Ocean (e.g. Beadell *et al.* 2006; Ishtiaq *et al.* 2010).

A total of 17 haplotypes were discovered in a single host (*Z. borbonicus*), which is, to our knowledge, the highest number of *Leucocytozoon* haplotypes ever recovered from a single host species. This figure may reflect a greater sampling effort for *Z. borbonicus* (221 sequences), which led to the detection of rare

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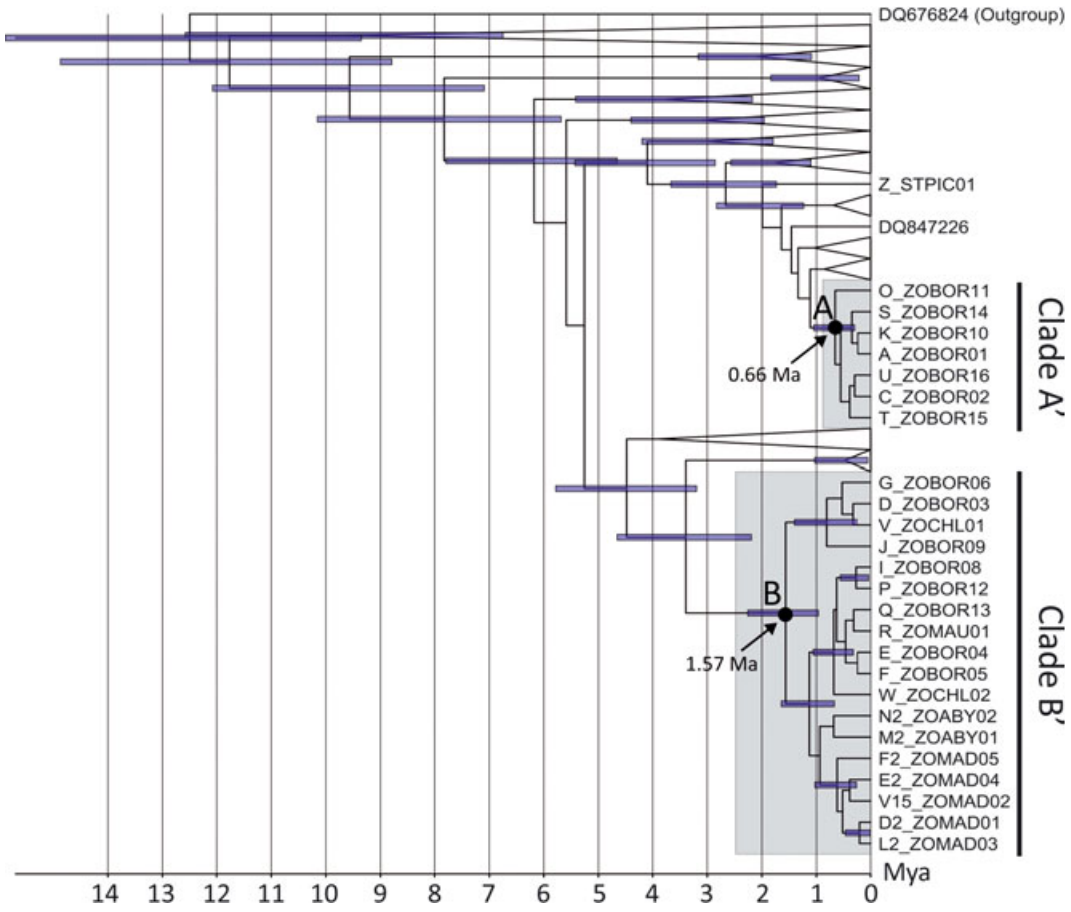

Fig. 4 Chronogram of *Leucocytozoon* lineages. The chronogram was obtained with a sequence divergence rate of 1.2% per million years, providing an approximate age of diversification events. Time before present (Ma) is on the *x* axis. Node bars indicate 95% confidence intervals for node ages.

haplotypes (12 of the 17 haplotypes occurred at a relative prevalence <4%). Our rarefaction analyses indicate, however, that approximately 25% of the true haplotype richness still remains unobserved in Réunion *Zosterops*, in spite of a considerable sample size. While available data do not allow rigorous comparisons with other species, *Leucocytozoon* contemporary diversity in Mascarene *Zosterops* may be considered exceptionally high for birds living on remote islands, and perhaps stands out among birds in general.

The inclusion in our analyses of a large sample of parasites found in other host species enabled us to place our results in a broad phylogeographic context and to assess the specificity of parasite lineages to their *Zosterops* hosts. Exact estimates of host specificity necessitate intensive sampling and are thus hard to obtain. However, the quasi-total absence of Clades A′ and B′ in non-*Zosterops* hosts (a single occurrence in this reservoir), combined with the fact that rarefaction analyses indicated that our sampling is unlikely to have missed many or any parasites in non-*Zosterops*, suggests that these parasites preferentially infect *Zosterops*. However, some Réunion landbird species were not included in our sample and therefore host specificity cannot be firmly concluded with the data at hand. It should also be noted that analyses of absolute prevalence would have been more suitable for determining whether each lineage preferentially infects certain host reservoirs but the nature of our data set compelled us to use relative prevalence instead (see Methods section). Such specificity would be compatible with the findings of previous studies that revealed some degree of host fidelity for certain haemosporidian lineages (Bensch *et al.* 2000; Ricklefs & Fallon 2002; Križanauskiené *et al.* 2006; Sehgal *et al.* 2006; Hellgren *et al.* 2007).

Conducting a comprehensive phylogenetic analysis permitted us to test a multiple colonization scenario versus *in-situ* diversification only. The nonmonophyly of *Leucocytozoon* haplotypes infecting Mascarene *Zosterops* indicates that the community was assembled in part

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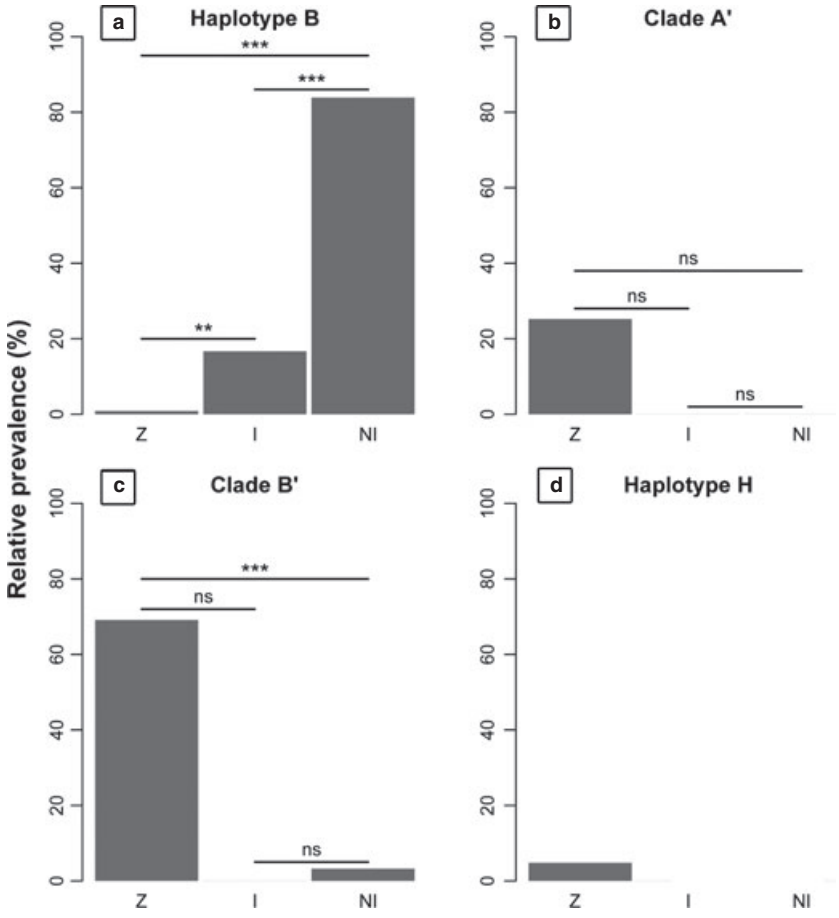

Fig. 5 Prevalence on Réunion of the four different lineages recovered from Mascarene *Zosterops*. Differences in relative prevalence between the three avian reservoirs have been tested with pair-wise GLMMs with Bonferroni correction. ns: $P > 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Z: *Zosterops* ($n = 230$), I: indigenous non-*Zosterops* ($n = 18$), NI: nonindigenous birds ($n = 31$).

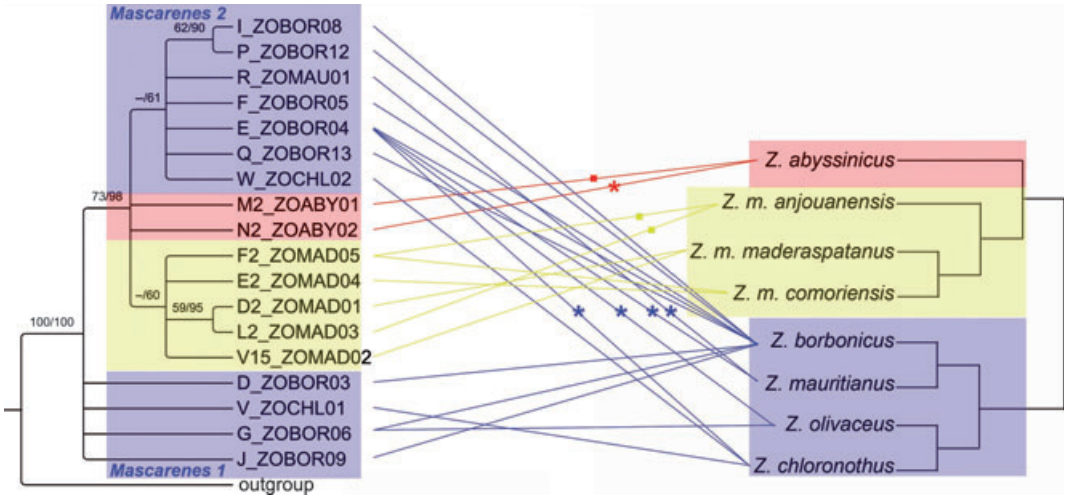

Fig. 6 Co-phylogeny of infected Indian Ocean *Zosterops* species and Clade B′. The parasite tree represented here is the consensus of maximum likelihood (ML) and Bayesian trees. Node support values read as in Fig. 3. The host tree is a representation of the topology obtained by Warren *et al.* (2006) using ML and Bayesian methods. Blue: Mascarene haplotypes, Yellow: Madagascar and Comoros haplotypes, Red: Socotra Island haplotypes. Lines represent host–parasite associations whose individual significance in topological congruence was assessed with ParaFit. Star: $P < 0.05$, Dot: $P < 0.10$.

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through multiple colonizations. The haplotypes recovered represent at least three distinct evolutionary lineages that have independently immigrated to the Mascarene archipelago. While the diversity constituting Clades A′ and B′ suggests that postcolonization *in-situ* diversification has taken place, the absence of close relatives to Haplotype B in Mascarene avian hosts may indicate either a more recent origin of that particular lineage, or different dynamics of diversification and extinction among lineages. Although our use of small DNA fragments (476 bp) may limit phylogenetic resolution, the nonmonophyly of Clade A′, Clade B′ and Haplotype H is well supported as they emanate from highly divergent branches of the global phylogeny presented in Fig. 3, and with strong bootstrap support. Moreover, the monophyly of Clade A′ and that of Clade B′ are strongly supported, as indicated by node support values in Fig. 3.

Haplotype B occurs in Europe, Africa and the Indian Ocean, and it was found in a total of 11 host species belonging to eight genera (Table 1 and S1, Supporting information), suggesting that it is a generalist parasite capable of long-distance dispersal. It is also by far the most prevalent parasite infecting nonindigenous birds (Fig. 5a), thus representing a good example of a 'jack-of-all-trades but master of some'; that is, a parasite with the capacity of maintaining both a broad host range and high prevalence in some host species (Hellgren *et al.* 2009; Jenkins & Owens 2011). Despite its generalism, Haplotype B does not seem able to achieve high prevalence in Réunion's indigenous birds (Fig. 5a). Thus, nonindigenous birds are Haplotype B's primary reservoir and thereby the most likely carrier of this parasite to the Mascarenes. The latter conclusion favours recent human-assisted introduction over a low net diversification rate in explaining the absence of diversity in this lineage.

We found that Clade A′ most probably diversified within Réunion island. Parasite cyt-*b* haplotypes diverging by as little as 0.2% may often represent distinct, nonrecombining lineages (Bensch *et al.* 2004). It is therefore possible that some mitochondrial haplotypes recovered within Clade A′ correspond to different biological species, although this remains to be confirmed with information from nuclear genes obtained from the same parasites. We estimated an older divergence time for the ancestor of this clade than the time of the earliest bird introductions, which suggests that the evolutionary diversification of Clade A′ was associated with indigenous birds well before human arrival in the Mascarenes at the end of the 16th century (Cheke & Hume 2008). This result appears to be robust to possible deviations of the cyt-*b* substitution rate in *Leucocytozoon* compared with other Haemosporida (Outlaw & Ricklefs 2011), as the 95% confidence interval for the age of Clade A′ would still not include 413 years, even with a much higher rate (Fig. 4). Colonization of Réunion through nonindigenous birds would still be possible if Clade A′ had diversified *ex-situ* and had subsequently been brought in its entirety by nonindigenous hosts. However, this would leave unexplained the current absence of Clade A′ from nonindigenous birds, their original reservoir under this scenario. The diversification of Clade A′ does not appear to have been promoted by co-radiation with *Zosterops* hosts, because all members of the clade only infect Réunion *Zosterops*. In addition, if co-radiation had driven diversification, massive sorting—that is extinction, 'miss-the-boat' events (i.e. when hosts migrate but leave their parasites behind, MacLeod *et al.* 2010) or severe sampling failure—would be needed to explain the absence of Clade A′ in all species of *Zosterops* outside Réunion.

Conservative estimates for divergence times indicate that Clade B′ has also diversified prior to bird introductions into the Mascarenes (Fig. 4), with this clade being nearly absent from the nonindigenous reservoir (Fig. 5c). As elaborated in the previous paragraph, this situation renders unlikely a recent origin from nonindigenous birds. The clade is found on the islands of Réunion, Mauritius, Madagascar, Mohéli, Anjouan and Socotra, in eight *Zosterops* species (Fig. 1) and such congruence between distributions of hosts and parasites may be indicative of co-radiation where hosts have carried their parasites during colonization events. The absence of Clade B′ from *Zosterops* of Grande Comore, Mayotte, Aldabra and Conception could be due to extinctions, miss-the-boat events or failure to establish due to vector absence or incompetence. Interestingly, the lineages infecting *Zosterops* outside the Mascarenes appear to be nested within the Mascarene lineages (Figs 3 and 6). Three scenarios can explain this pattern: (i) migration occurred from the Mascarenes to other islands and Africa, (ii) the Mascarenes were colonized twice, (iii) the divergence between groups *Mascarenes 1* and *2* (Figs 3 and 6) occurred prior to colonization of the Mascarenes, both groups being carried over when *Zosterops* migrated to the archipelago. A migration from the Mascarenes to Madagascar and Africa seems unlikely because this migration route does not correspond to any known *Zosterops* (Fig. 1) or vector movements. Although little is known about the biogeography of vectors, Giudicelli (2008) identified four species of black fly on Réunion that belong to the *Simulium ruficorne* species group, widely distributed in Africa. This suggests that Réunion black fly lineages colonized the Mascarenes from Africa and not the other way around. Moreover, the relative size of continents versus volcanic islands makes a migration from a continent to islands seem

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more likely than the reverse (but see Bellemain & Ricklefs 2008). The second possibility, a double colonization of the Mascarenes by Clade B′, also necessitates a transport of parasites that does not correspond to proposed *Zosterops* movements (i.e. from Africa or Madagascar to the Mascarenes, Fig. 1). Vectors could have transported one of *Mascarenes 1* or *2* groups to the Mascarenes or, despite the apparently high specificity of Clade B′ to *Zosterops*, these parasites may have been brought by another avian host. Regardless of which scenario took place, if the group *Mascarenes 2* is ignored, the topology within Clade B′ is strikingly similar to that of the hosts, with the divergence of Mascarene lineages from continental lineages predating the divergence between those of Madagascar and Africa (Fig. 6). Furthermore, the significant signal for co-divergence indicates that such topological congruence is unexpected under independent evolution of hosts and parasites. The divergence between Mascarene and Africa/Madagascar *Zosterops* was estimated at *c.* 1.82 Ma (maximum age) by Warren *et al.* (2006), and here, the divergence of *Mascarenes 1* and Africa/Madagascar parasites was estimated at 1.57 Ma (Fig. 4). These two estimates are relatively close, and the *Zosterops* migration to the Mascarenes might correspond to the origin of *Mascarenes 1* parasites. However, uncertainties in the substitution rate used for calibration and for divergence time estimates prevent us from definitively concluding temporal congruence. Also, a number of host–parasite links are not significant for co-divergence (Fig. 6), suggesting that while co-divergence is the predominant pattern, some deviations (unlinked divergences) also occurred. The inclusion of continental *Zosterops* and their parasites will be needed to fully link *Zosterops* and Clade B′ evolutionary histories.

In this study, we sampled parasites from birds only, thus obtaining partial information on the tripartite interaction between *Leucocytozoon*, birds and black fly vectors. Hellgren *et al.* (2008) demonstrated how patterns of host or vector specificity and vector feeding preference can affect transmission patterns of *Leucocytozoon* parasites. In island systems, vectors can mediate parasite colonization. This appears plausible for Clade A′ which is specific to *Zosterops* but whose colonization is unlikely to have been mediated by these hosts, as non-Mascarene *Zosterops* were infected by parasites phylogenetically unrelated to Clade A′. Clade A′ parasites or their ancestor may have colonized the Mascarenes with black flies and then successfully established in *Zosterops* only. In this case, the colonization of *Zosterops* by Clade A′ would represent a host-switch from an allochtonous, yet unknown, avian source.

Biogeographical studies of Haemosporida parasites are still scarce because the vagility and lack of host specificity of these parasites generally prevent accurately retracing their evolutionary history. However, as shown in Hellgren *et al.* (2007) and Ricklefs & Fallon (2002), *Leucocytozoon* and *Haemoproteus* parasites exhibit some degree of host and geographic fidelity. This host fidelity is well illustrated by our findings, as some *Leucocytozoon* parasites harboured by *Zosterops* were found to be highly specific to these hosts. Also, by taking advantage of the isolation of insular systems and placing our results in a broad phylogeographic context, our study provides partial reconstruction of the evolutionary processes accounting for the current diversity of *Leucocytozoon* parasites of Mascarene white-eyes. Notwithstanding the uncertainty in calibration of both parasite and host phylogenies, phylogenetic patterns and distribution of parasites across hosts and space indicate that parasite diversity in the Mascarenes is best explained by multiple colonizations combined with *in-situ* radiations, illustrating the roles of both processes in shaping regional parasite assemblages.

Acknowledgements

Guillaume Gélinaud, Dominique Strasberg, Juli Broggi, Magali Thierry, René-Claude Billot, Jean-Michel Probst, Isabelle Henry, Vincent Leconte, Marc Salamolard, Benoît Lequette, Vikash Tatayah, and field biologists and staff at the Mauritius Wildlife Foundation provided valuable help with fieldwork and logistics. We gratefully acknowledge the Mauritius National Parks and Conservation Services and the Réunion National Park for permission to conduct fieldwork. We also thank Monique Burrus and Émeline Lhuillier for many advices in the lab, and Joris Bertrand and Yann Bourgeois for sharing their expertise in phylogenetics. JC was supported by a MESR (Ministère de l'Enseignement Supérieur et de la Recherche) PhD scholarship. The research was supported by French National Research Agency (ANR) grants (ANR-05, NT05-3_42075 and ANR-08-0295-01) to PH, Institut Français de la Biodiversité (IFB) and ANR Biodiversity Program grants to CT, and the ''Laboratoire d'Excellence'' TULIP (ANR-10-LABX-41).

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This work formed part of J.C.'s PhD thesis. J.C. has a broad interest in biogeography, evolutionary biology and community ecology, focusing mainly on host-parasite systems. A.B. is an MSc student who worked on co-phylogenetic analyses. B.H.W's research focuses on diversification patterns in insular environments. A.L. studies the role of social systems in the extinction risks in primates. P.M. studies host-microorganism interactions. T.D. is a doctor of veterinary medicine interested in ornithology and conservation biology. B.M. is interested in phylogeography and speciation mechanisms in birds. C.T. investigates the mechanisms driving evolutionary and ecological diversification. P.H. is interested in the role played by parasites in the evolutionary biology of their hosts.

Data accessibility

DNA sequences: GenBank accessions JN032593–JN032658 (see also Table S1).

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 *Leucocytozoon* lineages included in phylogenetic analyses with hosts, locations and GenBank accession numbers.

Table S2 Number of occurrences of each lineage in each host species, detailed.

Table S3 Complete summaries of statistical models.

Table S4 Hosts and sites of Clades A and B lineages.

Fig. S1 Map of sampling locations of *Zosterops* and non-*Zosterops* birds.

Fig. S2 Detailed consensus phylogeny.

Fig. S3 Detailed maximum likelihood phylogeny.

Fig. S4 Detailed Bayesian phylogeny.

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