Species formation by host shifting in avian malaria parasites

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Contributed by Robert E. Ricklefs, August 31, 2014 (sent for review April 4, 2014)

The malaria parasites (Apicomplexa: Haemosporida) of birds are believed to have diversified across the avian host phylogeny well after the origin of most major host lineages. Although many symbionts with direct transmission codiversify with their hosts, mechanisms of species formation in vector-borne parasites, including the role of host shifting, are poorly understood. Here, we examine the hosts of sister lineages in a phylogeny of 181 putative species of malaria parasites of New World terrestrial birds to determine the role of shifts between host taxa in the formation of new parasite species. We find that host shifting, often across host genera and families, is the rule. Sympatric speciation by host shifting would require local reproductive isolation as a prerequisite to divergent selection, but this mechanism is not supported by the generalized host-biting behavior of most vectors of avian malaria parasites. Instead, the geographic distribution of individual parasite lineages in diverse hosts suggests that species formation is predominantly allopatric and involves host expansion followed by local host–pathogen coevolution and secondary sympatry, resulting in local shifting of parasite lineages across hosts.

emerging infectious disease | *Haemoproteus* | host switching | *Plasmodium* | species diversification

Cospeciation of symbionts with their hosts has been recognized in parasites with strong vertical transmission (1, 2), viruses that spread by direct contact (3), and bacterial and viral symbionts passed from mother to offspring through the egg (4). Species formation in parasites that are transmitted between hosts by vectors is less well-understood (5, 6). Poor matching between the phylogenetic trees of vector-borne hemosporidian (malaria) parasites and their North American avian hosts suggests a predominance of host shifting compared with cospeciation (7) (reviewed in a broader context in ref. 8). Whether host shifting occurs primarily between closely related hosts and in geographic sympatry, and whether rates of host shifting followed by species formation are sufficient to explain the contemporary diversity of hemosporidian parasites, have not been determined.

Many species of hemosporidian parasites have been described and named based primarily on the microscopic morphology of meronts and gametocytes in blood smears (9). The more recent discovery of hundreds of lineages based on DNA sequence variation in the mitochondrial cytochrome *b* gene (*cyt b*) (5, 10, 11) requires, however, a different species concept based on analysis of independent components of the genome (12–16). Recent estimates of the rate of molecular evolution in hemosporidian mitochondrial genes imply that the contemporary malaria parasites of vertebrates might have descended from a common ancestor within the past 20 (17) or 40 Ma (18) or, perhaps, a longer time period (19). Although an appropriate calibration for the rate of hemosporidian evolution remains unsettled (20, 21), host shifting almost certainly has been frequent, likely across great host distances at times, over the recent history of the group.

Speciation in sympatry (i.e., in the absence of geographic barriers to gene flow through local host specialization) might follow host shifting if mating between parasites was assortative with respect to vertebrate host or if different hosts imposed strong disruptive selection on parasites (22). However, despite some documented feeding preferences (23–25), dipteran vectors of avian malaria parasites do not seem to be sufficiently specialized to isolate populations of parasites on different hosts (26–29). In addition, many parasite species and many parasite lineages distinguished by DNA sequence variation occur locally across broad ranges of hosts without apparent differentiation, at least in the mitochondrial cytochrome *b* gene (30–32) and several nuclear markers (12, 14). Alternatively, host shifting in one allopatric population of a parasite species could be followed, after sufficient host–pathogen coevolution and evolutionary differentiation to produce reproductive incompatibility, by secondary sympatry, thereby increasing local parasite diversity.

Here, we examine recent nodes in an mtDNA-based phylogeny of New World hemosporidian parasites to determine the degree to which lineage formation is associated with host shifting. Although our phylogenetic reconstruction is based on a single mitochondrial gene (*cyt b*), phylogenies based on genes from the mitochondrial, nuclear, and apicoplast genomes are broadly consistent for the relatively recent nodes considered in this analysis (6, 12–14, 33–35). In addition, analyses of avian hemosporidian parasites based on multiple independent markers have distinguished mtDNA-defined lineages on the basis of significant linkage disequilibrium (13).

We distinguish as species the lineages that differ in their mtDNA cytochrome *b* gene sequence (by as few as 2 nt) and, for the most part, occur in either different hosts in the same local

Significance

Emerging infectious diseases pose threats to humans and livestock, but little is known about the general propensity of parasitic organisms to shift between hosts or the role of host shifting in the diversification of parasite lineages. The malaria parasites of contemporary vertebrate species descended from a common ancestor, likely after the diversification of their major host taxa, requiring rapid speciation and shifting between hosts across large host–taxonomic distances. Examination of sister lineages of avian malaria parasites in the New World suggests that such host shifting is common and often leads to the origin of new evolutionary lineages of parasites.

Author contributions: R.E.R. and D.C.O. designed research; R.E.R., D.C.O., M.S.-C., M.C.I.M., V.A.E., and S.L. performed research; R.E.R. analyzed data; and R.E.R., D.C.O., and M.S.-C. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper are listed in Table S2.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1416356111/-/DCSupplemental.

area or the same or different hosts in different geographic areas (32, 36). In some cases, closely related lineages occur in the same host locally. Sister lineages in this analysis differ by an average of about 1% sequence divergence, although some sequences separated by as little as a single nucleotide can exhibit consistent host or geographic differences. Inference concerning the mode of species formation is based primarily on host and geographic distributions of these hemosporidian mtDNA lineages. However, the correspondence between lineages and reproductively isolated species is poorly resolved (13, 37, 38). Each node was designated as either sympatric or allopatric depending on whether the descendant lineages occurred on the same West Indian islands or in the same regions within larger continental areas. The status of closely related parasite lineages occurring locally in the same host species is ambiguous, but these lineages might reflect genetic variation within a parasite species.

Previous analyses have suggested that host shifting, rather than codivergence, predominates species formation in the hemosporidian parasites of birds (5, 7). We find this most frequently to be the case in this analysis, and we discuss whether species formation by host shifting occurs primarily in sympatry or allopatry.

Results

We analyzed 181 genetic (mtDNA) lineages of hemosporidian [*Plasmodium* spp. (81 lineages) and *Haemoproteus* spp. (100 lineages)] parasites identified among 3,849 sequenced individual parasites from *ca.* 16,000 host individuals of primarily passerine birds (Aves: Passeriformes) from North and South America, including the West Indies (*SI Text*, section S1). Phylogenetic relationships of the parasite lineages (*SI Text*, section S2) are displayed in Fig. S1. We analyzed 58 superficial (terminal and subterminal) branch points, or nodes, with bootstrap support exceeding 50% (average ± SD = 76 ± 17%; raw *cyt b* genetic distance ± SD = 0.0111 ± 0.0067) (*SI Text*, section S3 and Fig. S1). Host distributions of parasite lineages on either side of each of these nodes were scored for allopatry (27 nodes) vs. sympatry (31) and for the taxonomic level of host distinction, which ranged from none (same host species = 16) to different species (8), genera (15), families (14), and orders (5) (Table 1). Thus, 28% of parasite sister lineages occurred within the same host species, whereas 26% occurred in different host genera, and 24% occurred in different host families. The taxonomic level of host difference was unrelated to the distinction between allopatry and sympatry ($G = 1.2$, df = 4, $P = 0.87$) and did not differ between *Haemoproteus* (39 nodes) and *Plasmodium* (19 nodes) lineages ($G = 2.0$, df = 3, $P = 0.58$). Genetic distances between sister lineages did not differ between allopatric and sympatric lineages ($F = 0.10$, df = 1, 52, $P = 0.94$) and did not vary with level of host taxonomic distinction, ranging from none to order ($F = 0.44$, df = 4, 52, $P = 0.78$) (Fig. 1).

If parasite lineages shifted to individuals of new host species at random within a local host community, most of these shifts would involve host species in different families of birds (Fig. 2). We calculated probabilities, under random host shifting, that sister parasite lineages would infect hosts related at different taxonomic levels for 757 potential host individuals of 42 species

Table 1. Sympatry and degree of host taxonomic distinction

	Host distinction				
	None	Species	Genus	Family	Order
Sympatric	8	5	9	6	3
Allopatric	8	3	6	8	2
Percentage of total	27.6	13.8	25.9	24.1	8.6

Each of 58 nodes was classified with respect to taxonomic distinction between hosts and allopatry vs. sympatry of occurrence.

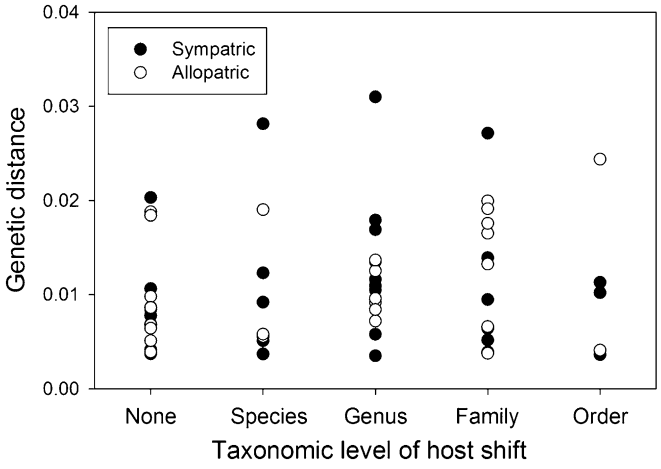

Fig. 1. Genetic distance between 58 sister lineages of avian malaria parasites. Distances, which are the unweighted proportions of nucleotide differences, are plotted as a function of taxonomic level of host difference; the distinction of sister lineages as sympatric (●) or allopatric (○) is indicated. Genetic distance is unrelated to taxonomic level of host switch ($P > 0.05$) whether treated as a class variable or a continuous variable from none = 1 to order = 5.

sampled at a field site in southern Missouri (32) and 2,433 potential host individuals of 101 species at Tiputini in Amazonian Ecuador (36). Compared with such random shifting in the North American locality, no differences and species-level host differences are overrepresented, and family-level host differences are underrepresented (Fig. 2) ($G = 11.5$, df = 4, $P = 0.002$). The result for North America is consistent with lower barriers to shifting between more closely related hosts. In the South American locality, family-level host differences are overrepresented at the expense of genus-level host differences, but the overall distribution does not differ significantly from the expected random distribution ($G = 8.5$, df = 4, $P = 0.07$).

Our sample of nodes ($n = 58$) included eight cases of divergent parasite lineages that occur locally within the same host species. In five of these cases, one of the paired lineages was a unique sequence and therefore, not confirmed in additional hosts. One of the remaining three nodes (node 21) (*SI Text*, section S3) unites NA04 [recovered from 23 northern cardinals (*Cardinalis cardinalis*) in the Chicago area (19) and southern Indiana (4)] with OZ03 [recovered from 5 northern cardinals in Missouri (4) and southern Indiana (1)] as well as individuals of 14 additional species sampled from Connecticut to New Mexico [and one recovery from an antbird (Thamnophilidae: *Thamnophilus murinus*) in Ecuador]. The second node (node 22) unites lineage CHI20PA [four white-throated sparrows (*Zonotrichia albicollis*) and one red-winged blackbird (*Agelaius phoeniceus*) in the Chicago area] with NA04 (42 occurrences, including two red-winged blackbirds in the Chicago area) and OZ03 (not recovered from either species). The third node (33) links two lineages [one recovered commonly (LA22) and the other recovered rarely (NA13; one instance) from the vermillion cardinal *Cardinalis phoeniceus* in northern Venezuela].

Discussion

Our analysis of the host distributions of malaria lineages suggests that most cases of lineage splitting in hemosporidian parasites follow host shifting, often across large host taxonomic distances. Although repeated shifting back and forth between closely related hosts can produce the appearance of codivergence (8, 39), such back-shifting to the original host apparently does not occur commonly among hemosporidian parasites of birds. Indeed, we

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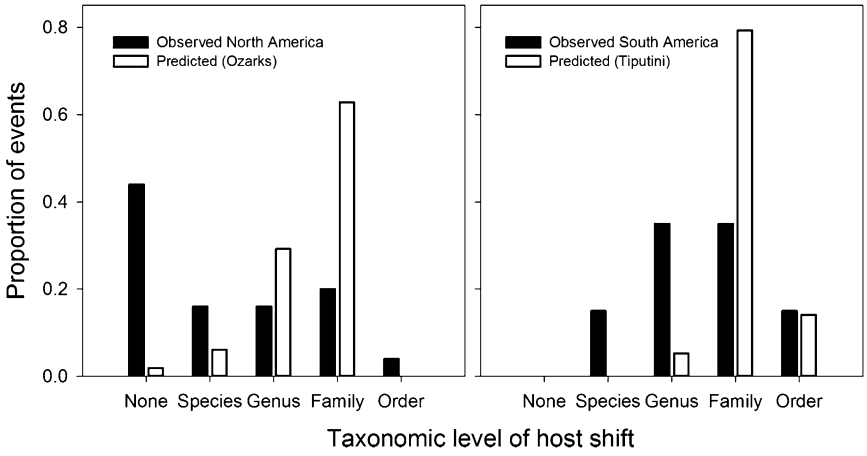

Fig. 2. Proportions of host shifting associated with different taxonomic levels of host difference. The comparisons involve a sample of (*Left*) 25 North American parasite lineages and (*Right*) 23 South American parasite lineages compared with random host shifting among individuals captured in the Ozark field site in southern Missouri and the Tiputini field site in Amazonian Ecuador. Predicted ordinal shifts were not calculated for the Ozark site, because only about 1% of captures were of species other than Passeriformes (nine individuals of the order Piciformes: the downy woodpecker, *Picoides pubescens*).

were not able to reject a model of random shifting to available hosts, regardless of species, from data obtained in one location in South America (Fig. 2).

Regardless of the taxonomic difference between their hosts, closely related lineages of parasites—putatively sister species—are about evenly split between those exhibiting allopatric distributions (27) and those presently occurring in sympatry (31). With respect to related parasite lineages occurring in sympatry, it is difficult to imagine how local host expansion could result in interrupted gene flow between parasite populations on different hosts and lead to sympatric parasite species formation. This possibility would require (*i*) a preadaptation that suited an individual parasite to a new host while preventing its continued infectivity in the old host (in which case host species would differ), (*ii*) the presence of vectors that show strong (but not perfect) host specialization, such that gene flow between the two host populations of a particular parasite was substantially reduced, or (*iii*) strong diversifying selection (22). Mutations that would preadapt a parasite to a new host (at the same time, reducing adaptation to the original host) are difficult to envision, and such cases presumably would not be detected readily in samples of local parasite–host communities. More frequently, a particular parasite lineage is distributed across several to numerous hosts, sometimes achieving high prevalence in one or more of them. However, although certain *Anopheles* mosquitoes seem to be host-restricted—to human hosts, for example (25)—most vectors of hemosporidian parasites are not sufficiently specialized on individual host species to prevent gene flow between parasite populations on different hosts locally (26, 28, 29, 40).

An alternative mechanism of species formation would involve parasites adapting to different host populations in allopatry (i.e., through gaining new hosts and losing old hosts in isolated areas) followed by secondary sympatry after parasite populations had been apart long enough to evolve reproductive incompatibility. Such host shifting could also involve coevolution of resistance factors by the host populations. If this mechanism were operating, one would recognize the initial stages of this process as a particular parasite lineage infecting different hosts in different localities. Indeed, this pattern is frequently observed, particularly in our samples from the West Indies, where the isolation of island populations reflects unambiguous barriers to dispersal. Fig. 3, for example, shows the West Indian host and island distributions of a clade of *Haemoproteus* spp. parasites representing nodes 23–29 in Fig. S1. Sister lineage pairs JA02/DR03, LA07/LA14, and DR02/LA27 are all allopatric, and most of the lineages exhibit substantial gaps (disjunctions) across continuous distributions of their principal hosts.

Table 2 examines parasite distributions from the point of view of a single host, the bananaquit (*Coereba flaveola*)—the most abundant species of bird in the West Indies. No parasite lineage of this host has a continuous distribution throughout the archipelago, and the primary parasite lineages infecting the bananaquit shift from island to island and often, back again. Each of the parasite lineages also appears in different host species on the islands where it is absent from bananaquits. For example, lineage OZ04, a common parasite of bananaquits ($n = 62$) on Jamaica and St. Lucia, infects the plumbeous warbler (*Setophaga plumbea*; $n = 9$), Lesser Antillean bullfinch (*Loxigilla noctis*; $n = 6$), and black-faced grassquit (*Loxigilla bicolor*; $n = 5$) on Dominica, where the bananaquit is common and infected, instead, with lineage OZ21 ($n = 9$).

Lineage OZ21 (*Haemoproteus coatneyi*) exhibits high prevalence in both the bananaquit ($n = 105$) and the Lesser Antillean bullfinch (*L. noctis*; $n = 118$) on most of the Lesser Antilles but is apparently absent from the bananaquit on Martinique and rare in bullfinches on Guadeloupe, where both hosts are common. Lineage OZ02 infects primarily the bananaquit ($n = 64$) in the Greater Antilles, including the Cayman Islands (except that the parasite lineage is absent from Jamaica), but also, the Puerto Rican bullfinch (*Loxigilla portoricensis*; $n = 15$) on Puerto Rico, the green-tailed warbler (*Microligea palustris*; $n = 13$) and black-crowned palm tanager (*Phaenicophilus palmarum*; $n = 59$; and four bananaquits) on Hispaniola, the northern cardinal (*C. cardinalis*; $n = 14$) in southern Mexico, and the scarlet tanager (*Piranga olivacea*; $n = 8$) in the Missouri Ozarks.

Although sister parasite lineages within the same host might follow a series of shifts locally from one host to another and back again, with loss of the intermediate parasite lineage in the alternate host (8), lineage divergence within a host might also occur in allopatric host populations followed by secondary sympatry. The possibility of sympatric speciation has been raised in some studies, particularly the work by Pérez-Tris et al. (41), in reference to closely related lineages of *Haemoproteus* in populations of European blackcap warblers (*Sylvia atricapilla*), with some infections occurring in the closely related garden warbler (*Sylvia borin*) and the African hill babbler (*Sylvia abyssinica*), with which the blackcap overlaps on its wintering range. Although

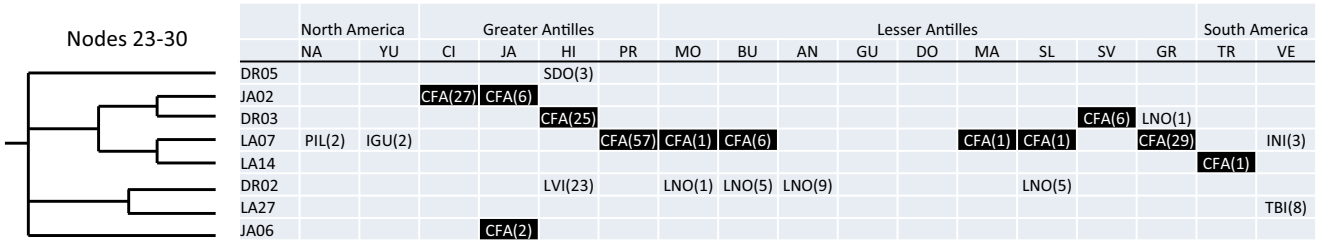

Fig. 3. Distribution of a clade of hemosporidian parasites (*Haemoproteus*), representing nodes 23–30 in *SI Text*, section S2, from North and Central America through the West Indies to northern South America. Darkened boxes represent recoveries (numbers of infected individuals in parentheses) from island populations of the bananaquit (*C. flaveola*; CFA), the principal host of this clade of parasites, along with the related Lesser Antillean bullfinch (*L. noctis*; LNO) and Greater Antillean bullfinch (*Loxigilla violacea*; LVI). Hosts in continental localities are unrelated. Island populations of bananaquits lacking or nearly lacking parasites from this clade have been found to be infected with (Table 2): Antigua, OZ21 (4); Guadeloupe, LA21 (13); Dominica, OZ21 (11); St. Vincent, OZ21 (34); and Montserrat, five parasites (all from different lineages). Island populations of bullfinches lacking this clade are infected with: Puerto Rico, OZ21 (21) and OZ02 (15); Jamaica, OZ04 (3), JA05 (2), and JA04 (1); Montserrat, OZ21 (4); Guadeloupe, OZ04 (3) and OZ21 (2); Dominica, OZ04 (6) and OZ21 (7); Martinique, OZ21 (13); and St. Vincent, OZ21 (20). Locations: AN, Antigua; BU, Barbuda; CI, Cayman Islands; DO, Dominica; GR, Grenada; GU, Guadeloupe; HI, Hispaniola; JA, Jamaica; MA, Martinique; MO, Montserrat; NA, North America; PR, Puerto Rico; SL, St. Lucia; SV, St. Vincent; TR, Trinidad; VE, northern Venezuela; YU, Yucatan Peninsula, Mexico.

some of these lineages might represent genetic variation within a single parasite population, others exhibit clear species distinction shown by linkage disequilibrium between mitochondrial and nuclear genes (13).

We suggest that such examples of sympatric occurrence of closely related parasite lineages might represent earlier allopatric speciation in isolated host populations. The average genetic distance of sister lineages in our analysis (1.1% sequence divergence) represents a minimum interval of 0.92 Ma based on the most rapid evolutionary rate estimated for *cyt b*. This minimum interval includes most of the major advances and retreats of ice sheets in Europe and North America. The average uncorrected pairwise genetic distance (p) between 17 parasite lineages in the blackcap clade in the work by Pérez-Tris et al. (figure 2b in ref. 41) is 0.018 ± 0.010 (range = 0.002–0.033), corresponding to an average of at least 1.5 Ma. Thus, opportunity for isolation and differentiation of parasite lineages in host populations inhabiting ice-free refugia during the height of glacial advances or other previously isolated host populations could explain the co-occurrence of closely related lineages in a single contemporary host population without having to resort to a hypothesis of sympatric species formation.

A similar parasite lineage swarm occurs in the widespread North American red-eyed vireo (*Vireo olivaceus*), which includes the related lineages LA26, NA10 (node 14), OZ05 (node 13), OZ10, OZ12, OZ13 (node 15), OZ17, and OZ28 (node 11), at least six of which co-occur locally in southern Missouri (32), and other lineages in additional species of *Vireo*. The average pairwise genetic distance between 11 lineages in this clade is 0.031 ± 0.017 (range = 0.002–0.065), corresponding to an average time interval of at least 2.6 Ma. The most common of these lineages also occurs in the related black-whiskered vireo (*Vireo altiloquus*), which is distributed within the wintering range of the migratory red-eyed vireo in the West Indies and in resident populations (*Vireo chivi*) in northern South America. One of the lineages (LA26; four examples from Trinidad) has not yet been identified in North America. Thus, origin in allopatry followed by subsequent spread and secondary sympatry remains a possible scenario for this clade.

The parasite lineages included in our sample span the most basal node (i.e., most recent common ancestor) in the avian malaria phylogeny uniting the genera *Plasmodium* and *Haemoproteus* (11, 12, 42) and represent a basal dichotomy occurring as little as 9 Ma ago, according to one calibration (17). A maximum rate of lineage splitting can be estimated for a constant rate process from the lengths of terminal branches in the phylogeny, although several sources of bias can influence such estimates (43). For 42 terminal comparisons, the genetic distance between parasite sister lineages averaged 0.0093 ± 0.0060 SD (range = 0.0035–0.0310). This distance represents the average time looking backward to a lineage splitting event, which is the inverse of the speciation rate. Using the highest estimated rate of genetic divergence of 0.012 Ma^{-1} (17), 0.0093 sequence divergence represents a lineage doubling time of 0.775 Ma. At this rate and ignoring the unknown rate of extinction of parasite lineages, a single ancestral lineage could produce *ca.* $2^{9/0.775}$ = 3,132 descendant lineages in 9 Ma. Slower rates of *cyt b* divergence would produce the same number of descendants over correspondingly longer periods.

This estimate considerably exceeds the number of parasite lineages identified in the host species included in this study.

Table 2. Principal hemosporidian lineages of the bananaquit (*C. flaveola*) through its range in the West Indies

Lineages (more than nine infections)	Greater Antilles							Lesser Antilles									
	GC	LC	CB	JA	DR	PR	EY	MO	BU	AN	GU	DO	MA	SL	BA	SV	GR
DR03					19*											6	
JA02	20*	4	3	6													
LA07						49*		1	6*				1	1	2		29*
OZ02	5	21*	23*		4	11*											
OZ04				35*	2	2	1				3		1	14*			3
OZ19					2		10*										
OZ21	2					16*		1	1	4	13*	9*		6	18*	34*	

AN, Antigua; BA, Barbados; BU, Barbuda; CB, Cayman Brac; DO, Dominica; DR, Dominican Republic (Hispaniola); EY, El Yunque montane forest, Puerto Rico; GC, Grand Cayman; GR, Grenada; GU, Guadeloupe; JA, Jamaica; LC, Little Cayman; MA, Martinique; MO, Montserrat; PR, Puerto Rico; SL, St. Lucia; SV, St. Vincent.
*Principal parasite populations.

However, the estimate might approximate the diversity of avian malaria parasites globally considering that the number of recognized lineages roughly parallels the number of host species sampled thus far [the MalAvi database (mbio-serv2.mbioekol.lu.se/Malavi/) lists 1,545 haplotypes of hemosporidian *cyt b* obtained from 855 host species (44); our database (in part) (*SI Text*, section S2) contains 211 lineages recovered from 318 species]. Also, because we have broadly sampled small birds in local avifaunas, our local estimate of the net rate of recent lineage splitting might well approach the overall diversification rate of malaria species in the global avifauna.

Haemosporidian (malaria) parasites of birds have diversified across the *ca.* 10,000 species in the class Aves, perhaps now numbering as many parasite species as their hosts in as little as *ca.* 10 Ma and plausibly within several multiples of this period (almost certainly after the diversification of major host taxa). Closely related lineages of parasites are most frequently associated with different hosts that are often separated by large host phylogenetic distance. Species formation evidently follows on host shifting, although the underlying mechanisms (including the role of vector behavior) are not understood. Spatial heterogeneity in the host associations of individual parasite lineages suggests that host–parasite coevolution and, perhaps, local interactions among parasites through host immune systems can cause parasites to shift among host species locally, which might promote disruptive selection and speed the evolution of reproductive incompatibility between allopatric populations. Secondary expansion of differentiated parasite populations to ancestral localities would increase local parasite diversity. The key to rapid evolutionary diversification and species formation would seem to be strong selection on parasites by evolving host defenses. This dynamic coevolutionary interaction of a parasite lineage with a broad range of hosts might explain how hemosporidian parasites could diversify so rapidly as to spread across all terrestrial vertebrates in a short period.

Methods

Samples and Phylogenetic Analysis. Malaria parasites were characterized by variation in mitochondrial cytochrome *b* gene sequences obtained from blood samples of birds at sites throughout the Americas under appropriate governmental permits and Institutional Animal Care and Use Committee protocols and imported under permits from the Animal and Plant Health Inspection Service and United States Fish and Wildlife Service. Birds were caught opportunistically by mist nets in representative habitats to provide a broad, albeit not complete, sample of local host species and parasite diversity. Because we used mist nets at ground level, we sampled large species and canopy birds poorly. Our samples, therefore, represent mostly passerine birds (Passeriformes) and doves (Columbiformes) that feed in open habitats and the understory of forests.

Approximately 5–10 μL blood was drawn from the brachial vein on the undersurface of the wing and stored in 300 μL Longmire's or PureGene lysis buffer. All subjects were released unharmed, generally within 30 min of capture.

In the laboratory, DNA was extracted by alcohol precipitation. DNA samples were initially screened by PCR with primers that target a conservative 154-bp sequence of parasite mitochondrial ribosomal RNA (31, 32, 45). For samples that screened positive, we sequenced part of the mitochondrial cytochrome *b* gene or its entirety using a variety of primer combinations (31, 32, 46). Chronic infections not present in the peripheral blood are likely to be missed (47). In some cases, a detected parasite represented a sporozoite that did not develop into a mature gametocyte, which is the stage that transmits infections to the dipteran vector (48). Mixed infections were not commonly apparent, but they were resolved by phasing (49) where possible.

Parasite Lineages. Parasite lineages (species?) were distinguished by cytochrome *b* gene sequence divergence combined with differences in distribution among hosts (32, 36). Phylogenetic relationships of the parasite lineages were reconstructed based on between 239 and 1,031 nt (average = 537 ± 204 SD nt) mitochondrial cytochrome *b* gene sequence. Phylogenetic reconstruction was accomplished in the RAxML blackbox (50, 51) by maximum likelihood using the default GTR + γ-model of nucleotide substitution with 100 bootstrap replications to quantify support for individual nodes in the tree. Maximum likelihood uses all of the information in an alignment, regardless of sequence length, and therefore, including short sequences does not limit phylogenetic inference.

Statistical Analyses. The maximum likelihood tree (*SI Text*, section S2 and Fig. S1) was inspected for nodes to be included in the analysis. They were generally terminal nodes or nodes within small clades with >50% bootstrap support. We devised a weight for each comparison that included bootstrap support (b) for the node and samples sizes (n_1 and n_2) of each sister parasite lineage or clade of descendant lineages:

$$W = b\left(\frac{n_1}{n_1+1}\right)\left(\frac{n_2}{n_2+1}\right),$$

which is equal to a minimum of $0.25b$ when only one example of each lineage has been obtained and a maximum of b. As the number of infected individuals increases, one has more confidence that an infection is not merely adventitious—a spillover infection—which might not be transmittable to other individuals of the same host species. Additional statistical analyses are described in *Results*.

We used general linear models to ask whether the level of phylogenetic support for a node, the weight for the comparison including the sample size, and the percent sequence divergence were related to (*i*) allopatry (yes or no) or (*ii*) taxonomic level of host shift (Fig. 1). There were no significant statistical interactions between these variables and either allopatry or taxonomic level (all $P > 0.05$), and none of the independent variables was significantly related to allopatry or taxonomic level of host shift (all $P > 0.05$).

We calculated the probability under the null hypothesis that, if a parasite from any single host individual were to shift at random to another host individual in the same locality, the shift would occur at the taxonomic level of the same species, a species in the same genus, a genus in the same family, a family in the same order, or a different order. We made this calculation separately for the total captures of host individuals in the Ozarks of southern Missouri (32) and at Tiputini, Ecuador (36). For each host species at the two locations, we calculated the proportion of host individuals among those captured that were members of the same species (p_S), genus (p_G), family (p_F), and order (p_O) in the data. At the species level, this proportion is $p_S = \Sigma p_i^2$, where p_i is the proportion of species i among potential hosts in the area. The calculation is the same at the genus level and above, except that one removes the proportion of matches at lower nested taxonomic levels. That is, if a shift occurs between individuals of the same species, it also occurs within the same genus, family, and order, and cannot be counted two times. We tested the correspondence between the null distribution and the observed distribution of the number of species at each level of taxonomic difference by G test, with 0.5 species added to each count to eliminate counts of 0 species.

Host taxonomy was based on Taxonomy in Flux (jboyd.net/Taxo/List.html) and the South American Classification Committee (www.museum.lsu.edu/~Remsen/SACCBaseline.htm).

ACKNOWLEDGMENTS. We thank Peter Marra, Eldredge Bermingham, and Sylvia Fallon for providing blood samples from the Caribbean Basin; Alan Fecchio, Adriana Rodriguez, and Nayara Belo for providing blood samples and parasite sequences from Brazil and Venezuela; John Blake and Bette Loiselle for providing blood samples from Ecuador; and Bethany Swanson and Julia Gray for assisting in the laboratory. Most of our field work could not have been carried out without the support and encouragement of local government personnel and natural resources and conservation agencies. We also thank Matt Pennell, Luke Harmon, Ananias Escalante, Steven Rich, and several anonymous reviewers for helpful comments on the manuscript. Field and laboratory work was supported by grants from the National Geographic Society, the National Science Foundation, and the Curators of the University of Missouri.

1. Demastes JW, Hafner MS (1993) Cospeciation of pocket gophers (*Geomys*) and their chewing lice (*Geomydoecus*). *J Mammal* 74(3):521–530.
2. Page RDM, Hafner MS (1996) Molecular phylogenies and host-parasite cospeciation: Gophers and lice as a model system. *New Uses for New Phylogenies*, eds Harvey PH, Brown AJL, Maynard Smith J, Nee S (Oxford Univ Press, Oxford), pp 255–270.
3. Dimcheff DE, Drovetski SV, Krishnan M, Mindell DP (2000) Cospeciation and horizontal transmission of avian sarcoma and leukosis virus gag genes in galliform birds. *J Virol* 74(9):3984–3995.
4. Clark MA, Moran NA, Baumann P, Wernegreen JJ (2000) Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* 54(2):517–525.
5. Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites. *Proc Biol Sci* 269(1494):885–892.
6. Escalante AA, Freeland DE, Collins WE, Lal AA (1998) The evolution of primate malaria parasites based on the gene encoding cytochrome *b* from the linear mitochondrial genome. *Proc Natl Acad Sci USA* 95(14):8124–8129.

7. Ricklefs RE, Fallon SM, Bermingham E (2004) Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Syst Biol* 53(1): 111–119.
8. de Vienne DM, et al. (2013) Cospeciation vs host-shift speciation: Methods for testing, evidence from natural associations and relation to coevolution. *New Phytol* 198(2): 347–385.
9. Valkiūnas G (2005) *Avian Malaria Parasites and Other Haemosporidia* (CRC, Boca Raton, FL).
10. Bensch S, et al. (2000) Host specificity in avian blood parasites: A study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc Biol Sci* 267(1452): 1583–1589.
11. Perkins SL, Schall JJ (2002) A molecular phylogeny of malarial parasites recovered from cytochrome b gene sequences. *J Parasitol* 88(5):972–978.
12. Martinsen ES, Perkins SL, Schall JJ (2008) A three-genome phylogeny of malaria parasites (*Plasmodium* and closely related genera): Evolution of life-history traits and host switches. *Mol Phylogenet Evol* 47(1):261–273.
13. Bensch S, Pérez-Tris J, Waldenström J, Hellgren O (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: Multiple cases of cryptic speciation? *Evolution* 58(7):1617–1621.
14. Perkins SL, Sarkar IN, Carter R (2007) The phylogeny of rodent malaria parasites: Simultaneous analysis across three genomes. *Infect Genet Evol* 7(1):74–83.
15. Ramiro RS, Reece SE, Obbard DJ (2012) Molecular evolution and phylogenetics of rodent malaria parasites. *BMC Evol Biol* 12:219.
16. Outlaw DC, Ricklefs RE (2014) Species limits in avian malaria parasites (Haemosporida): How to move forward in the molecular era. *Parasitology* 141(10):1223–1232.
17. Ricklefs RE, Outlaw DC (2010) A molecular clock for malaria parasites. *Science* 329(5988):226–229.
18. Hayakawa T, Culleton R, Otani H, Horii T, Tanabe K (2008) Big bang in the evolution of extant malaria parasites. *Mol Biol Evol* 25(10):2233–2239.
19. Duval L, et al. (2010) African apes as reservoirs of *Plasmodium falciparum* and the origin and diversification of the Laverania subgenus. *Proc Natl Acad Sci USA* 107(23): 10561–10566.
20. Pacheco MA, et al. (2011) Timing the origin of human malarias: The lemur puzzle. *BMC Evol Biol* 11(1):299.
21. Bensch S, et al. (2013) How can we determine the molecular clock of malaria parasites? *Trends Parasitol* 29(8):363–369.
22. Giraud T, Gladieux P, Gavrilets S (2010) Linking the emergence of fungal plant diseases with ecological speciation. *Trends Ecol Evol* 25(7):387–395.
23. Hellgren O, Bensch S, Malmqvist B (2008) Bird hosts, blood parasites and their vectors—associations uncovered by molecular analyses of blackfly blood meals. *Mol Ecol* 17(6): 1605–1613.
24. Malmqvist B, Strasevicius D, Hellgren O, Adler PH, Bensch S (2004) Vertebrate host specificity of wild-caught blackflies revealed by mitochondrial DNA in blood. *Proc Biol Sci* 271(Suppl 4):S152–S155.
25. Besansky NJ, Hill CA, Costantini C (2004) No accounting for taste: Host preference in malaria vectors. *Trends Parasitol* 20(6):249–251.
26. Hamer GL, et al. (2009) Host selection by *Culex pipiens* mosquitoes and West Nile virus amplification. *Am J Trop Med Hyg* 80(2):268–278.
27. Kilpatrick AM, Daszak P, Jones MJ, Marra PP, Kramer LD (2006) Host heterogeneity dominates West Nile virus transmission. *Proc Biol Sci* 273(1599):2327–2333.
28. Gager AB, Del Rosario Loaiza J, Dearborn DC, Bermingham E (2008) Do mosquitoes filter the access of *Plasmodium* cytochrome b lineages to an avian host? *Mol Ecol* 17(10):2552–2561.
29. Medeiros MCI, Hamer GL, Ricklefs RE (2013) Host compatibility rather than vector-host-encounter rate determines the host range of avian *Plasmodium* parasites. *Proc R Soc Lond B Biol Sci* 280(1760):2947–2954.
30. Križanauskienė A, et al. (2006) Variation in host specificity between species of avian hemosporidian parasites: Evidence from parasite morphology and cytochrome B gene sequences. *J Parasitol* 92(6):1319–1324.
31. Fallon SM, Bermingham E, Ricklefs RE (2005) Host specialization and geographic localization of avian malaria parasites: A regional analysis in the Lesser Antilles. *Am Nat* 165(4):466–480.
32. Ricklefs RE, et al. (2005) Community relationships of avian malaria parasites in southern Missouri. *Ecol Monogr* 75(4):543–559.
33. Escalante AA, Ayala FJ (1994) Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. *Proc Natl Acad Sci USA* 91(24):11373–11377.
34. Escalante AA, Barrio E, Ayala FJ (1995) Evolutionary origin of human and primate malarias: Evidence from the circumsporozoite protein gene. *Mol Biol Evol* 12(4): 616–626.
35. Outlaw DC, Ricklefs RE (2010) Comparative gene evolution in haemosporidian (apicomplexa) parasites of birds and mammals. *Mol Biol Evol* 27(3):537–542.
36. Svensson-Coelho M, et al. (2013) Diversity, prevalence, and host specificity of avian *Plasmodium* and *Haemoproteus* in a western Amazon assemblage. *Ornithological Monogr* 76(1):1–47.
37. Beadell JS, et al. (2006) Global phylogeographic limits of Hawaii's avian malaria. *Proc Biol Sci* 273(1604):2935–2944.
38. Martinsen ES, Paperna I, Schall JJ (2006) Morphological versus molecular identification of avian Haemosporidia: An exploration of three species concepts. *Parasitology* 133(Pt 3):279–288.
39. Charleston MA, Robertson DL (2002) Preferential host switching by primate lentiviruses can account for phylogenetic similarity with the primate phylogeny. *Syst Biol* 51(3):528–535.
40. Hamer GL, et al. (2008) *Culex pipiens* (Diptera: Culicidae): A bridge vector of West Nile virus to humans. *J Med Entomol* 45(1):125–128.
41. Pérez-Tris J, et al. (2007) Within-host speciation of malaria parasites. *PLoS ONE* 2(2): e235.
42. Outlaw DC, Ricklefs RE (2011) Rerooting the evolutionary tree of malaria parasites. *Proc Natl Acad Sci USA* 108(32):13183–13187.
43. Weir JT, Schluter D (2008) Calibrating the avian molecular clock. *Mol Ecol* 17(10): 2321–2328.
44. Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: A public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Mol Ecol Resour* 9(5):1353–1358.
45. Fallon SM, Ricklefs RE, Swanson BL, Bermingham E (2003) Detecting avian malaria: An improved polymerase chain reaction diagnostic. *J Parasitol* 89(5):1044–1047.
46. Maria L, Svensson E, Ricklefs RE (2009) Low diversity and high intra-island variation in prevalence of avian *Haemoproteus* parasites on Barbados, Lesser Antilles. *Parasitology* 136(10):1121–1131.
47. Jarvi SI, Schultz JJ, Atkinson CT (2002) PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. *J Parasitol* 88(1):153–158.
48. Valkiūnas G, Iezhova TA, Loiseau C, Sehgal RNM (2009) Nested cytochrome B polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. *J Parasitol* 95(6):1512–1515.
49. Browning SR, Browning BL (2011) Haplotype phasing: Existing methods and new developments. *Nat Rev Genet* 12(10):703–714.
50. Stamatakis A (2006) RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22(21):2688–2690.
51. Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML Web servers. *Syst Biol* 57(5):758–771.

Supporting Information

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SI Text

S1. Avian Blood Samples. Avian blood samples used in this analysis were obtained in the field from the locations listed in Table S1.

S2. Lineage Circumscription. Genetically differentiated parasites recovered from different hosts in the same area were distinguished as different lineages. Genetically differentiated parasites recovered from the same host or hosts within a region were distinguished as separate lineages when other lineages from different hosts inserted between them phylogenetically. Allopatric lineages in the same host or hosts were distinguished as different lineages when the genetic difference between them was in the range of other lineage distinctions (1). In the absence of multiple independent genetic markers, one has to accept a certain level of uncertainty in these lineage designations, but there are few ambiguities in this dataset. Table S2 lists the lineages considered in this analysis. Phylogenetic relationships among these lineages are shown in Fig. S1.

S3. Nodes Analyzed in this Study. In Table S3, each node was designated as either sympatric or allopatric depending on whether the descendant lineages occurred in the same place (individual West Indian islands or larger continental areas). We also distinguished the host taxonomic difference between the descendant lineages at each node.

1. Ricklefs RE, et al. (2005) Community relationships of avian malaria parasites in southern Missouri. *Ecol Monogr* 75(4):543–559.

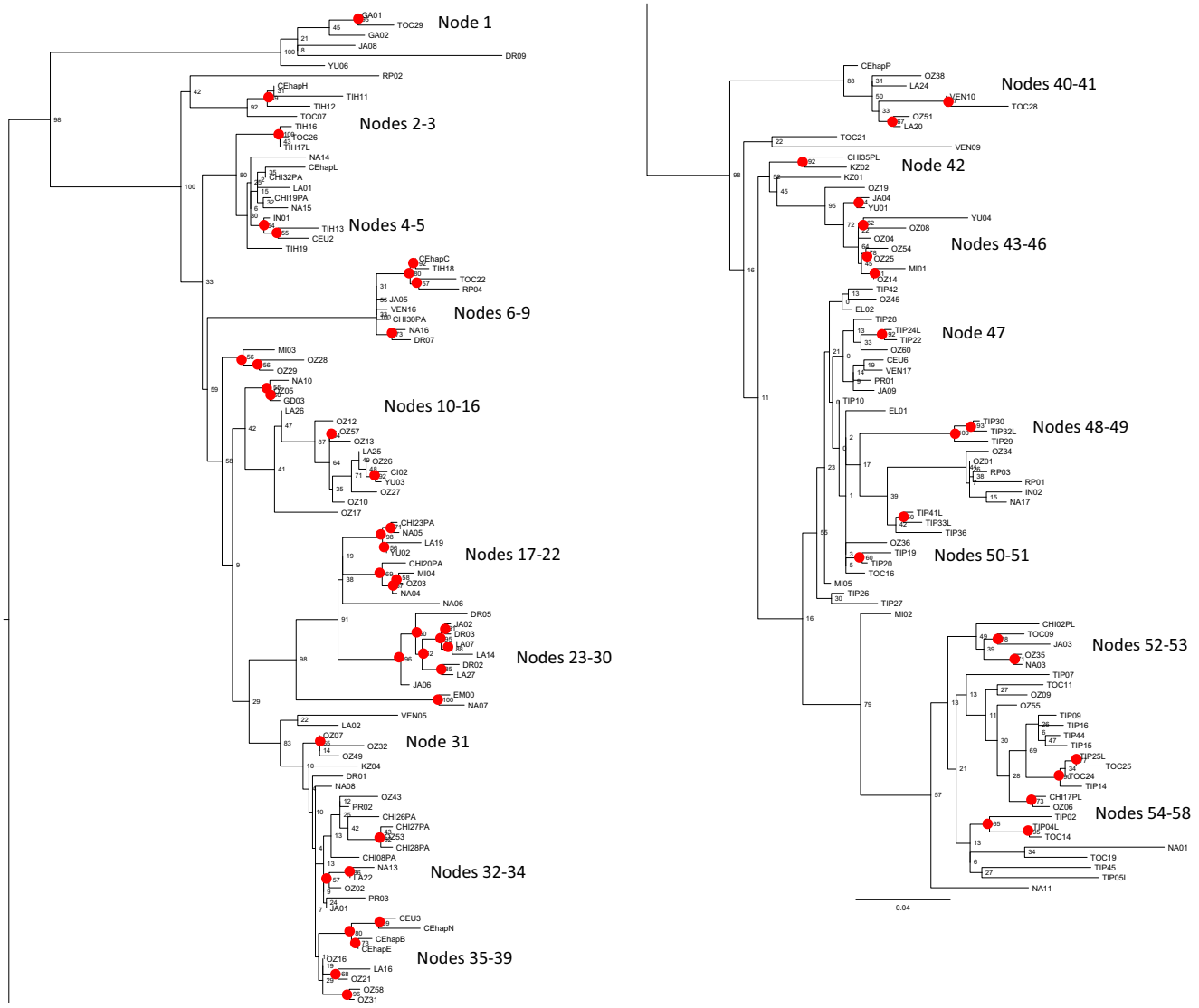

Fig. S1. Maximum likelihood phylogenetic reconstruction of relationships among the avian malaria parasite lineages considered in this analysis. The phylogeny is rooted between *Haemoproteus* and *Plasmodium*. Nodes considered in the analysis (n = 58) are indicated by red dots.

Other Supporting Information Files

Table S1 (DOCX)
Table S2 (DOCX)
Table S3 (DOCX)