Phylogeography of Caribbean lizard malaria: tracing the history of vector-borne parasites

S. L. PERKINS

Department of Biology, University of Vermont, Burlington, VT 05405, USA

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Abstract

The *Anolis* lizards of the eastern Caribbean islands are parasitized by several species of malaria parasites (*Plasmodium*). Here I focus on two species of *Plasmodium*, using molecular data (mitochondrial cytochrome *b* sequences) to recover the phylogeography of the parasites throughout the Lesser Antilles and Puerto Rico. The two parasites were originally described as a single species, *P. azurophilum*, which infects both red and white blood cells. Here the two species are termed *P. azurophilum* Red and *P. azurophilum* White based on their host cell type. Six haplotypes were found in 100 infections sequenced of *P. azurophilum* Red and six in 45 infections of *P. azurophilum* White. Nested clade analysis revealed a significant association of geographical location and clades as well as a pattern of past fragmentation of parasite populations. This is consistent with the hypothesis that vector-borne parasites such as malaria may be subject to frequent local extinctions and recolonizations. Comparison of the phylogeography of the lizard and parasites shows only weak concordance; that is, the parasites colonized the lizards in the islands, but dispersal events between islands via vectors or failed lizard colonizations were present. The two parasites had different histories, *P. azurophilum* Red colonized the islands from both the north and south, and *P. azurophilum* White originated in the central Lesser Antilles, probably from *P. azurophilum* Red, then moved to both north and south. This is the first study to examine the biogeography of a pair of sibling species of vector-borne parasites within an island archipelago system.

Introduction

Biogeography is an intrinsically historical science. A full understanding of the present geographical distribution of species requires reconstruction of events both in ecological time (dispersal rates, resource partitioning between species, etc.) and over an evolutionary time scale (changes within genes, genetic divergence among populations, speciation and origin of clades). This broad historical approach becomes more intriguing, and far more complex, when considering the distribution of species that are obligately linked over both ecological and evolutionary time scales, such as parasites and their hosts. As the hosts themselves are the 'environment' of the parasites, speciation in hosts could equate to allopatric isolation of their parasites. A comparison of biogeographical patterns and systematic relationships (phylogeography) of host and parasite lineages should thus present similar stories (Page & Charleston, 1998). However, phylogenetic concordance is disrupted if parasites switch hosts, breaking the tight historical linkage between the taxa.

Most biogeographical studies of parasites have examined large groups of parasite taxa that inhabit a wide geographical range, such as Brooks's (1979) work on the digeneans of crocodilians worldwide, Brooks *et al.* (1981) examination of the helminths of neotropical stingrays, and Hoberg's (1992) studies of the tapeworms of the Arctic seabirds and seals. Very few studies have compared biogeographical patterns of either closely

Correspondence: S. L. Perkins, Division of Invertebrates, American Museum of Natural History, Central Park West at 79th Street, New York, NY 10024, USA.
Tel.: +1212 7695639; fax: +1212 7695 277;
e-mail: sperkins@amnh.org

related parasites or those within a single species (phylogeography) of parasite. Also, little is known about the biogeography of parasites that are transmitted by vectors, such as blood-feeding insects and their vertebrate hosts. Like the above studies that involved parasites with complex life cycles (>1 host), vector-borne parasite–host systems offer an additional dimension of complexity as the distribution of the parasites will be dictated by the dispersal and availability of both hosts. The use of a blood-feeding vector may also make host switching possible because such insects are often not highly specialized for their food source (Githeko *et al*., 1994). Finally, island systems for the study of nonmarine parasite biogeography have rarely been utilized (but see Ayala & Hutchings, 1974; Staats & Schall, 1996a). As the space between islands (ocean waters) is unsuitable for either vertebrate host or vector, dispersal may be difficult and gene flow for parasites and hosts minimal.

Here, I have pursued all of these issues with a study of the historical biogeography of two species of malaria parasites (*Plasmodium*) that infect *Anolis* lizards of the eastern Caribbean islands of the Lesser Antilles. These two species of lizard malaria were only recently discovered to be part of a cryptic species complex (Perkins, 2000). *Plasmodium azurophilum sensu* Telford (1975) was described as a malaria parasite capable of undergoing both schizogony (asexual division) and gametogony (production of gametocytes or sex cells) in both erythrocytes and in two classes of white blood cells of its host. These two forms (herein referred to using the original species name followed with the host cell, i.e. *P. azurophilum* Red and *P. azurophilum* White) have since been shown to be genetically distinct, monophyletic and independent sister taxa (Perkins, 2000). The phylogeographical results presented here offer additional support to the claim that these parasites are composed of separate evolutionary lineages and not merely a plastic response of the parasites to infect a wide variety of host blood cells.

Unfortunately, the vectors for Caribbean lizard malaria remain unknown, although sanguivorous dipterans are the only insects believed to transmit *Plasmodium* (Garnham, 1966). A North American saurian malaria species is vectored by phlebotomine sandflies (Fialho & Schall, 1995) and these same insects have been observed feeding on Caribbean *Anolis* (Johnson *et al*., 1992). It is also possible that culicine mosquitoes are the vectors. In the laboratory, *Culex erraticus* has been shown to support development of another species of lizard malaria parasite found in the Caribbean, *P. floridense* (Klein *et al*., 1987), but its natural competence as a vector has yet to be demonstrated. Ectoparasites such as ticks or mites may be involved in the transmission of other blood parasites (e.g. *Hepatozoon*; Smith, 1996), but have never been shown to be capable of transmitting *Plasmodium* between vertebrates. Additional evidence that mosquitoes or other biting flies are the vectors comes from Ayala's (1975) survey of lizard haemoparasites on the western Caribbean island of San Andrés. He observed numerous *Hepatozoon*-like infections in lizards, but just one lizard infected with *Plasmodium (P. floridense)*, and this occurred in the only region of the island where mosquito-control efforts had not occurred. Thus, we assume here that the vectors of these lizard malaria species are small dipterans and not ectoparasitic arthropods that would be directly associated with vertebrates during dispersal.

Vector-borne parasites, such as these lizard malaria species, may be susceptible to extinction on small islands because they rely on stable populations of both hosts to persist. On the Lesser Antilles, volcanic eruptions, hurricanes, droughts or other environmental disturbances may cause the extinction of the parasite if the vector or host population density is greatly reduced, disrupting transmission. Lizard malaria parasites offer an opportunity to study the natural mobility of protozoan parasites because the parasites have colonized the islands via relatively rare natural dispersal events of either lizards or vectors. Although more work has been done on the factors affecting the occurrence of human malaria parasites because of their public health impact, those species have distributions recently influenced by colonization, urbanization and frequent air travel (Martens & Hall, 2000), which may obscure natural historical and evolutionary relationships.

The Lesser Antillean archipelago has long served as a model system for biogeographical and evolutionary ecology studies, particularly on the *Anolis* lizards of the region (Williams, 1969; Losos, 1994; Roughgarden, 1995). These islands have never been connected to the mainland, most of them having arisen as a result of volcanic activity. K-Ar dating has provided estimates of time since emergence that range from 10 to 37 million years for the older, eastern arc of islands to >8 million years for the western arc (Briden *et al*., 1979). Volcanic activity in many of these islands continues; 14 volcanoes have erupted repeatedly in the last 100 000 years (Wadge, 1994) with the most recent eruptions still ongoing on the island of Montserrat. The climate of these islands has also been extremely variable within both century- and millennium-length time scales (Bonatti & Gartner, 1973; Black *et al*., 1999). Thus, the lizards, vectors and parasites could have arrived in the eastern Caribbean as early as 37 million years ago, but volcanic activity and climate changes have probably altered the distribution of the parasites and their hosts throughout the history of the islands. Some of the northern Lesser Antillean islands were connected into island banks during low sea levels during the Pleistocene, approximately 15 000 years ago (Gorman & Kim, 1976), however, no islands where *Plasmodium* parasites were found in this study were ever joined to any others. Thus, we can assume that only dispersal events have contributed to the distribution of observed parasite haplotypes.

An additional feature of this system is that the biogeography of Lesser Antillean *Anolis* lizards themselves

presents a complex but fairly well resolved story. They have been divided into two groups or series, the *bimaculatus* group and the *roquet* group, based on morphological, immunological, karyological, electrophoretic and DNA sequence data (Underwood, 1959; Williams, 1969; Gorman *et al*., 1980; Williams, 1983; Losos, 1990; Roughgarden, 1995). The *bimaculatus* group is restricted to the northern islands from the Anguilla bank to Dominica and the *roquet* group to the southern islands from Martinique to Grenada. These distinct distributions suggest different geographical origins and evolutionary histories and complex colonization patterns (Gorman *et al*., 1980).

Until recently, recovering the phylogeny and historical biogeography of *Plasmodium* was hampered by the lack of informative morphological characters; this was especially true for within-species studies. Here I have used molecular genetic data in the form of sequences of the parasite cytochrome *b* gene in an attempt to explain the current distribution of lizard malaria haplotypes in this region. Using phylogenetic and nested cladistic analyses (Templeton *et al*., 1987), I propose a scenario for colonization for each of two *Plasmodium* species. Nested clade analyses have been used in several other recent phylogenetic studies, including those of viruses (Crandall, 1995), vertebrates (Templeton *et al*., 1995; Durand *et al*., 1999; Johnson & Jordan, 2000) and invertebrates (Turner *et al*. 2000), but never in a study of vector-borne parasites. My goals were to compare the results for the two species of parasites to determine if their historical reconstructions are similar (which would suggest some general conclusion on how vector-borne parasites move among islands), and to compare the histories of the parasites and the lizard hosts.

Methods

Sampling

I sampled *Anolis* lizards from 11 islands in the Lesser Antilles (Anguilla, St Martin, Saba, St Kitts, Guadeloupe, Dominica, Martinique, St Vincent, Bequia, Carriacou and Grenada), as well as Puerto Rico and Trinidad in 12 collecting trips between January 1996 and March 1999. Lizards were collected either by hand or by slip noose and a toe clip was used to obtain blood for both a thin smear as well as to blot filter paper for subsequent DNA extraction. All lizards were returned to their place of capture within 24 h as per the protocol approved by the University of Vermont Animal Care and Use Committee. Thin smears were stained with Giemsa and scanned for 6 min or more at 1000× to determine infection status and parasite species present. Lizards observed to be infected with more than one species of *Plasmodium* were not used for genetic analyses.

DNA extraction, amplification and sequencing

Genomic DNA from the blood dried on filter paper was extracted using the DNeasy extraction kit (QIAGEN), following the protocol for animal tissues but resolubilizing the DNA in only 50 μL of elution buffer. A 673-bp fragment of the mitochondrial cytochrome *b* gene was amplified using a nested PCR design. Reactions were set up in 25 μL polymerase chain reactions with Ready-to-Go PCR beads (Amersham Pharmacia) using 1.5 mM $MgCl_2$ and 2.5 μM of each primer. For the outer reaction, *Plasmodium*-specific primers DW2 (5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′, self-designed) and DW4 (5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′ = AL1356, Escalante *et al*., 1998) were used and the reactions were subjected to 4 min at 94 °C followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 1.5 min. A 0.5-μL aliquot of this product was used as a template for a nested reaction with primers from Creasey *et al*. (1993), DW1 (5′-TCA ACA ATG ACT TTA TTT GG-3′) and DW3 (5′-TGC TGT ATC ATA CCC TAA AG-3′). Amplification reactions were heated to 94 °C for 1 min followed by 40 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 30 s with a final dwell at 72 °C for 7 min. To minimize contamination that has been implicated as a problem with nested PCR designs (Adagu & Warhurst, 1999), amplification of only a single species from just one island at a time was performed. The PCR products were concentrated with Nanosep 100K columns (Pall Gelman), subjected to cycle sequencing with BigDye terminator mix (ABI) using both forward and reverse primers (DW1 and DW3) and run on an ABI Prism 377 automated sequencer (Division of Invertebrates, American Museum of Natural History, New York, NY).

Phylogenetic and nested clade analysis

The phylogenetic relationship of the parasite haplotypes was assessed using PAUP* 4.0b4 (Swofford, 1999). Unweighted parsimony using branch-breaking as a heuristic search was employed with 10 replicates of random addition sequences of taxa. However, traditional phylogenetic techniques are not always appropriate for intraspecific data as they often give poor resolution, including ambiguous placement of the root because of the effects of evolutionary stochasticity at these levels of divergence (Crandall *et al*., 1994). I instead used the methods of Templeton *et al*. (1992), created for intraspecific cladogram estimation. This approach begins with an assessment of statistical parsimony, calculated here with the program ParsProb 1.1 (written by D. Posada), which uses a Bayesian approach to provide the total number of parsimonious connections that are justified for a particular sequence length. Nested cladistic analyses were performed using the program GeoDis 2.0 (Posada *et al*., 2000) which uses as input a description of a nested cladogram. This method of analysing intraspecific genetic

data was developed by Templeton *et al*. (1987) as a means of using temporal information on allelic variation in the form of haplotype networks to distinguish between ongoing processes (such as recurrent gene flow) that affect population structure from potential historical events such as population fragmentation and range expansion. Unlike traditional *F*-statistic methods that use the frequency of alleles in a population to infer gene flow and population subdivision, the combination of molecular genetic and geographical sampling data allow the user to distinguish many more processes and can provide a better understanding of the evolutionary history of the species. A detailed description of the process is outlined in Templeton (1998); a brief summary is included here.

The procedure begins with creating a haplotype network, in this case of the variants observed within each of the lizard malaria species for the portion of the cytochrome *b* gene that was sequenced. Networks need not be rooted, however, if desired, rooting may be accomplished in one of two ways. If an appropriate outgroup is available, then the clades may be 'polarized temporally' with this taxon (Templeton, 1998). In this study, I used *Plasmodium fairchildi* (Telford, 1989) from *Anolis cupreus*, a Costa Rican species, as an outgroup. This species consistently clusters with both *P. azurophilum* Red and *P. azurophilum* White in phylogenies of lizard malaria species (S.L. Perkins & J.J. Schall, unpublished). As an alternative rooting method, Castelloe & Templeton (1994) constructed a simple heuristic algorithm using neutral coalescent theory for determining which haplotypes in an intraspecific network have high root probabilities. As this rooting method is based on the relative proportion of each haplotype, it may not be appropriate for these lizard malaria parasites because the relative frequency of each haplotype is primarily a function of parasite prevalence on the island (because of current local transmission parameters and past extinctions) and may not be an accurate estimator of haplotype age.

Once the networks are constructed, haplotypes are nested according to the rules of Templeton *et al*. (1987) and Templeton & Sing (1993) into 1-step clades, 2-step clades and so on. Next, a nested contingency analysis (Templeton & Sing, 1993) is implemented, whereby an exact permutational contingency test is performed. This allows one to accept or reject the null hypothesis that there is no association of clades with geographical location. Clades where the null hypothesis has been rejected at the 5% level are evaluated further. Using geographical data implemented by the user, such as the latitude and longitude of each sampled site, two distance measures are calculated. The first is the clade distance, D_c, which provides information on the geographical range of a given clade. This value is calculated by taking the average distance that each isolate of a given haplotype is from the geographical centre of all isolates that have haplotypes that belong to the same clade. The second distance is the nested clade distance, D_n, which yields information of how each clade is distributed in relation to clades within the same higher-level nesting category. It is calculated as the average distance that each isolate of a given haplotype from the clade of interest is from the geographical centre of all isolates that have haplotypes from the next higher-level nesting clade. The observed distances calculated as such are then determined to be significantly large or small based upon 1000 random permutations of the data which provide the null hypothesis of random geographical distribution given the same sampling regimen from each site. The output of the GeoDis program allows inspection for these significantly large or small distance values which are then followed through an inference key (Templeton, 1998) to uncover the possible genetic structuring or historical events that could explain such results.

Results

In all, 7224 anoles were captured and examined for parasites. In Table 1, for each island where *P. azurophilum*

Table 1 Host *Anolis* species, latitude and longitude, and overall prevalence of malaria parasites* for each island where *P. azurophilum* Red and *P. azurophilum* White were found.

Island	Host *Anolis* species	Latitude, longitude	N	Prev. *P. azurophilum* Red (%)	Prev. *P. azurophilum* White (%)
Puerto Rico	*A. gundlachi*	18°21′N, 65°52′W	†	†	†
Saba	*A. sabanus*	17°39′N, 63°15′W	†	†	†
St Kitts	*A. bimaculatus*	17°20′N, 62°45′W	207	7.2	1.9
	A. schwartzi		15	20.0	0
Guadeloupe	*A. marmoratus*	16°15′N, 61°35′W	162	1.2	1.2
Dominica	*A. oculatus*	15°25′N, 61°20′W	318	6.6	1.2
Martinique	*A. roquet*	14°40′N, 61°00′W	275	7.3	0.7
St Vincent	*A. trinitatus*	13°15′N, 61°12′W	312	3.5	2.6
Grenada	*A. richardi*	12°07′N, 61°40′W	208	5.8	0

*Prevalence includes all infections found on a given island, including those present in mixed-species infections. †Prevalences for parasites on Puerto Rico and Saba are unavailable as these islands are part of a long-term study of lizard malaria ecology by J. Schall *et al*. and not all individuals have been examined to date. See Staats & Schall (1996) and Schall *et al*. (2000) for additional information.

Red or White was found, the host species of *Anolis*, the prevalence of each of the parasite species, and the latitude and longitude of the island is given. A third species of lizard malaria parasite, *P. floridense*, was found on some islands but its overall prevalence was low: only one infection with this species was observed from both St Martin and Dominica and only three were found on Martinique. Because of these small sample sizes, this species was not used for phylogeographical analyses, although its distribution will be discussed briefly below. No lizards infected with *Plasmodium* were found on Anguilla, Bequia, Carriacou or Trinidad.

A total of 142 infected lizards were used for the phylogeographical analyses: 97 infected with *P. azurophilum* Red and 45 infected with *P. azurophilum* White. With the exception of Puerto Rico and Saba, where long-term studies of the parasite populations are underway (Staats & Schall, 1996b; Schall *et al.*, 2000), I utilized every infection of the two forms of *P. azurophilum* that was found on each island (provided it was not present in a mixed infection) for amplification and sequencing. A total of 601 bp of the cytochrome *b* gene PCR product from the parasites were aligned by eye (there were no insertions or deletions). There were six haplotypes of *P. azurophilum* Red and six haplotypes of *P. azurophilum* White observed. (The distributions are summarized in Table 2.) Three of the lizards infected with *P. azurophilum* Red had a mixed infection of two haplotypes as evidenced by a double peak in the electropherogram in the

Table 2 Cytochrome *b* haplotype frequencies of *P. azurophilum* Red (A–F) and *P. azurophilum* White (G–L) by collection locality.

	Haplotype											
Locality	A	B	C	D	E	F	G	H	I	J	K	L
Puerto Rico	3	10					16					
Saba			11					13				
St Kitts			8	8					2	1		
Guadeloupe			1					1				
Dominica			7		9			2				
Martinique					13						2	
St Vincent					15							8
Grenada						14						

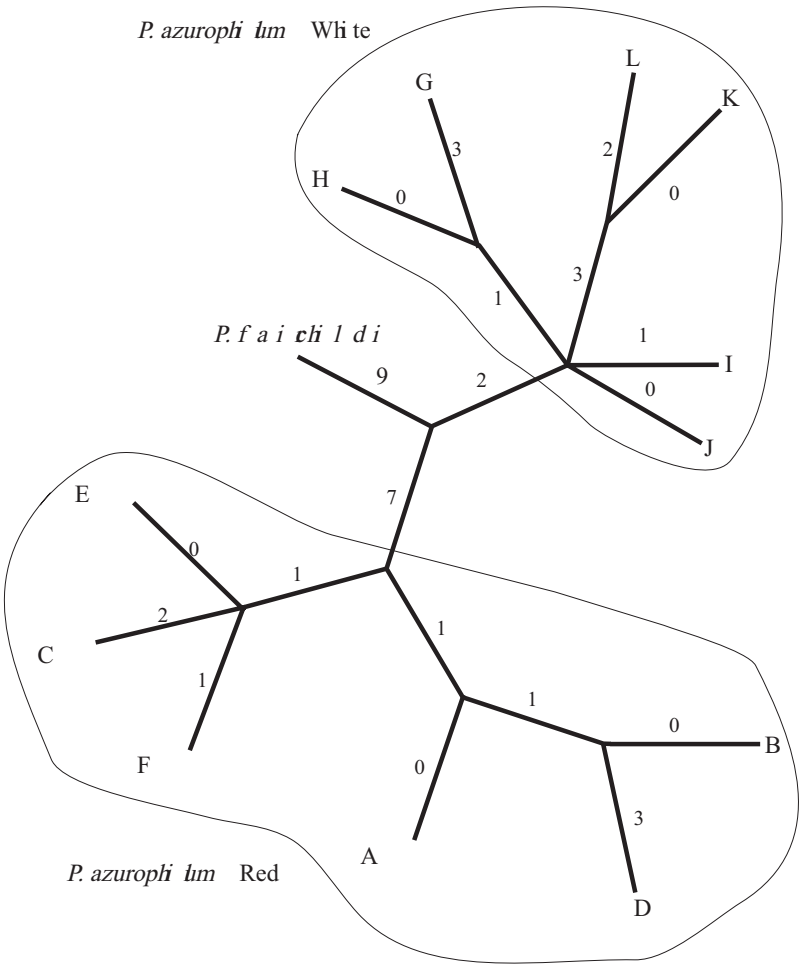

Fig. 1 Single most parsimonious phylogenetic tree of all cytochrome *b* haplotypes of *P. azurophilum* Red and *P. azurophilum* White, as well as *P. fairchildi*, a lizard malaria parasite from Costa Rica. Numbers along branches are mutational steps.

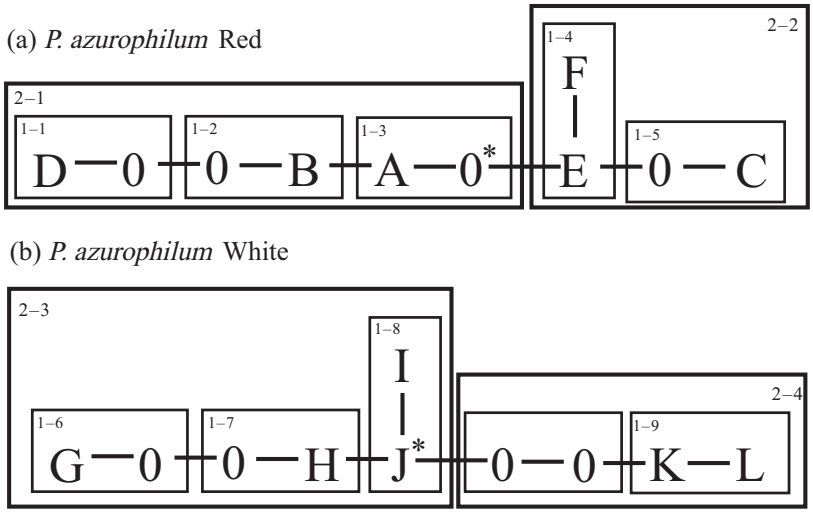

Fig. 2 Nested cladistic design of cytochrome *b* haplotypes of *P. azurophilum* Red (a) and *P. azurophilum* White (b). Each line represents a single mutational change, zeroes represent missing (unsampled) haplotypes. The asterisk represents the position of the root based on the phylogeny of all haplotypes and *P. fairchildi* presented in Fig. 1.

informative sequence positions for differentiating them. These haplotypes were scored independently, that is, each haplotype present was counted separately, bringing the total of isolates of *P. azurophilum* Red to 100.

Figure 1 presents a phylogenetic tree of all 12 haplotypes of these two species as well as *P. fairchildi*. The inclusion of this outgroup allowed determination of the root of each of these two clades. The networks and corresponding nested clades for *P. azurophilum* Red and White are shown in Fig. 2, with the asterisk depicting the probable position of the root of each clade as determined by outgroup rooting (Fig. 1). For both *P. azurophilum* Red and *P. azurophilum* White, nested contingency analyses showed significant association of geographical locations and clades, an indication that there was phylogeographical structure present in these parasites; that is, the null hypothesis that there is no association between the frequency of haplotypes and geography is rejected at the 0.05 level (Table 3). The complete results of the nested clade analyses including the two distance values calculated for these data are presented in Fig. 3 for *P. azurophilum* Red and Fig. 4 for *P. azurophilum* White. Using the inference key from Templeton (1998), a conclusion of past fragmentation of parasite populations, was inferred for each of the nested clades (Table 4).

Table 3 Exact contingency analysis of geographical associations for *P. azurophilum* Red and *P. azurophilum* White. Clades are represented in Fig. 1; clades where there is no genetic and/or geographical variation are not included as no such tests are possible.

	Clade	Permutational χ^2 statistic	Probability
P. azurophilum Red	1–4	51.00	<0.0001
	2–1	22.00	<0.0001
	2–2	39.04	<0.0001
	Entire cladogram	76.69	<0.0001
P. azurophilum White	1–9	10.00	0.0260
	2–3	70.00	<0.0001
	Entire cladogram	45.00	<0.0001

Discussion

Distribution of the Caribbean lizard malaria parasites

Three species of *Plasmodium* were found in the *Anolis* from Puerto Rico south to Grenada, *P. azurophilum* Red, *P. azurophilum* White and *P. floridense*. These results are similar to those reported by Staats & Schall (1996a), but with some key differences. Both *P. azurophilum* Red and White were both found on one additional island (Guadeloupe) and *P. floridense* was found on two additional islands, Dominica and Martinique. The latter is particularly intriguing, because Staats & Schall (1996a) noted that this species was restricted to the northern islands in *bimaculatus* anoles, but it is now seen to be present on a southern island in a lizard from the *roquet* group. The complete absence of both *P. azurophilum* Red and White observed during three collecting trips to St Martin was in sharp contrast to Schall's (1992) finding of >40% of lizards infected. In 480 lizards sampled from that island in the current study, only one was infected with *Plasmodium*, and that infection was of *P. floridense*, a species that was much rarer in previous samples. This island was hit by several major hurricanes in the years between the studies. Hurricanes have been observed to cause local extinctions of lizard and arthropod populations (Spiller *et al.*, 1998), thus these major disturbances may have caused a crash in vector or infected vertebrate populations, disrupting the life cycle of the parasites and causing an extinction of these two species (J.J. Schall *et al.*, unpublished data). These observations may have recorded an extinction event for two malaria parasites on a small island.

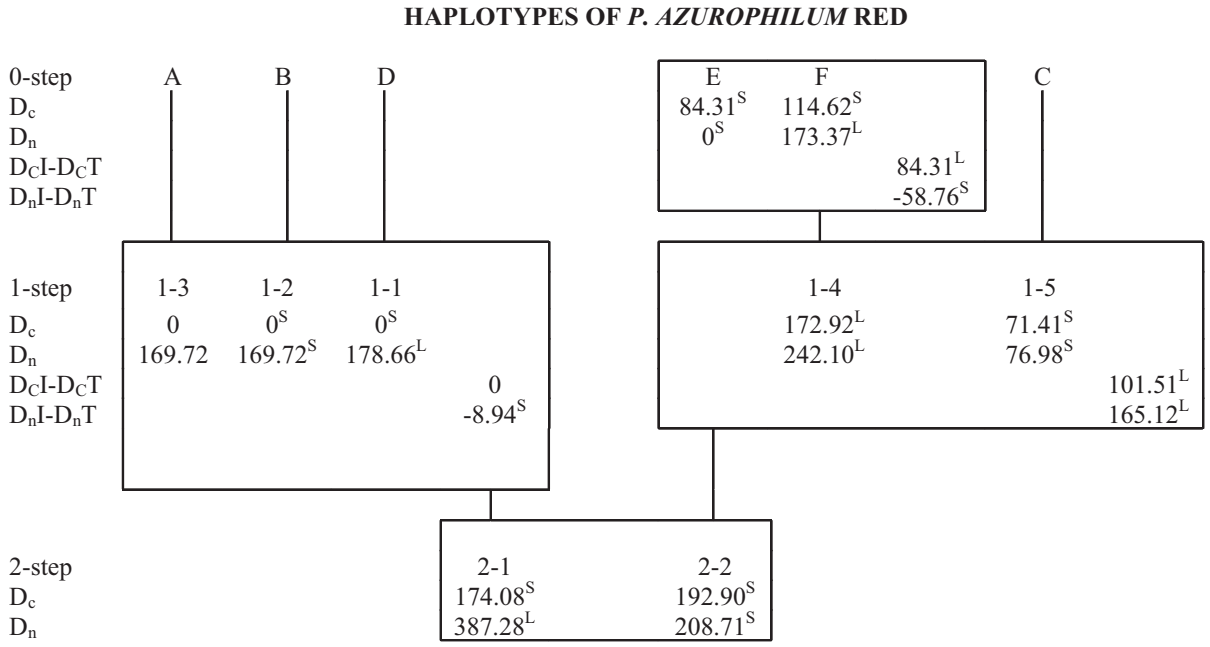

Fig. 3 Results of the nested clade analyses for the cytochrome *b* haplotypes of *P. azurophilum* Red. A superscript indicates a statistically significant small (S) or large (L) clade or nested clade distance value (values are calculated with the GeoDis program according to calculations given in Templeton *et al.,* 1995). Average differences between interior (I) and tip (T) distance values are also shown.

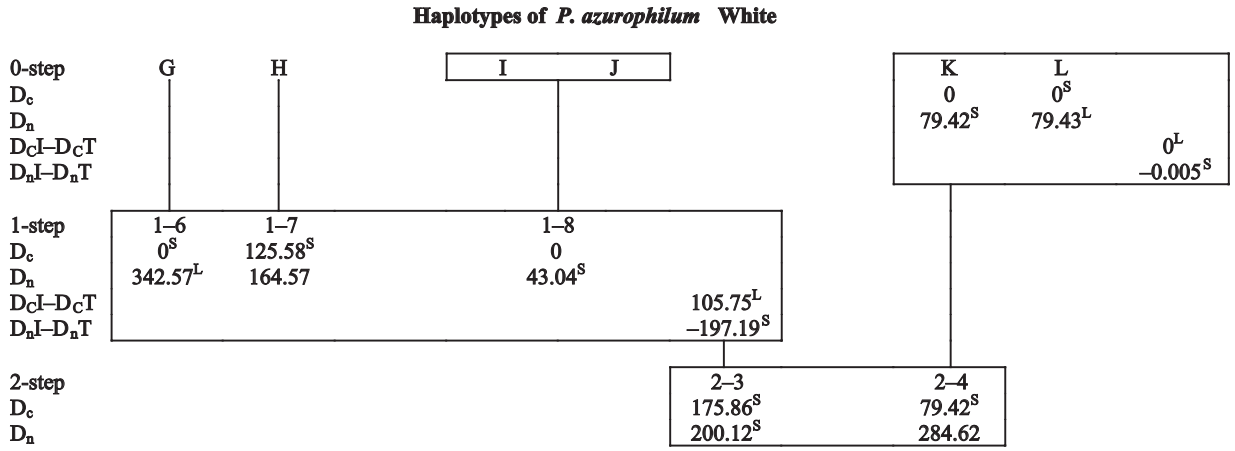

Fig. 4 Results of the nested clade analyses for the cytochrome *b* haplotypes of *P. azurophilum* White. Details as described for Fig. 2.

The colonization of the islands: in rafting vertebrates or wind-blown vectors?

Small arthropods such as flies and mosquitoes may easily be carried by prevailing winds or storms (Carlquist, 1974). However, many vectors of *Plasmodium* suffer very high mortality after taking a blood meal and producing a clutch of eggs such that very few survive to take a second blood meal and transmit the parasite (Rodriguez *et al.*, 1992; Fialho & Schall, 1995). The combined probability for the survival of an infected vector that is blown from one island to another must then be extremely small. *Anolis* lizards appear to be good dispersers because virtually every island in the Caribbean, no matter how small, has a resident population of anoles (Losos, 1996) and experiments and observations have supported the dispersal capability of lizards (Schoener & Schoener, 1984; Censky *et al.*, 1998). Even if the established lizard species prevent successful colonization by the new population (Soulé, 1966; Gorman & Atkins, 1969),

Table 4 Inferences based on the results of the nested clade analyses using the results summarized in Figs 2 and 3 and the inference key found in the appendix of Templeton (1998).

Clade	Inference
Haplotypes in 1–4	Past fragmentation
1-step clades nested in 2–1	Past fragmentation
1-step clades nested in 2–2	Past fragmentation
Haplotypes in 1–9	Past fragmentation
1-step clades nested in 2–3	Past fragmentation
1-step clades nested in 2–4	Past fragmentation

The final decision made in each chain of inference was that the different geographically concordant areas are not separated by areas that have not been sampled; presumably they are not, but it is possible that the prevalence of infections in these intermediate locations was so low that neither collections for this study nor that of Staats & Schall (1996a) were able to detect the parasites.

land-fallen lizards may serve as a means of introducing the parasites to the residents. Thus, it seems that the parasites would be far more likely to successfully colonize a new site within their lizard hosts rather than in their vectors.

Several decades ago, Gorman and coworkers (Gorman & Atkins, 1969; Yang *et al.* 1974; Gorman & Kim, 1976) used biochemical data to analyse the phylogenetic relationships of the Lesser Antillean *Anolis* lizards and to reconstruct their colonization order. Colonization for *bimaculatus* anoles was from north to south in a generally stepwise fashion. This is against the direction of generally north-west prevailing currents, so Gorman & Kim (1976) suggested that each dispersal event was a short hop between islands, not unreasonable given that the distances between these islands is fairly small (<40 km). The *roquet* group appears to have moved from south to north. The first landing of the original colonizer might have been Grenada, St Vincent, or St Lucia, with Martinique colonized last, probably from lizards on Barbados. The islands on either side of the split between the two groups, Dominica and Martinique may have been the last to acquire anoles, although they have radiated quickly to fill the diverse array of ecological niches (Gorman & Atkins, 1969). Although Underwood (1959) stated that he was 'at a loss to explain why the two groups do not overlap' given their geographical proximity and the dispersal abilities of anoles, Gorman & Atkins (1969) proposed that lizard colonization between them was perhaps hampered by competition from a well-adapted resident species (notably, with a different karyotype, preventing hybridization).

The observed distribution of the haplotypes of each of the two species of lizard malaria is shown in Table 2. Several of the haplotypes have widespread distributions throughout the island chain. Haplotypes C and E of *P. azurophilum* Red are present at four and three islands, respectively, and haplotype H of *P. azurophilum* White occurs on three islands. The anoles of these same islands, however, are distinct genetically. For example, the five *Anolis* species that are hosts of haplotype C (*A. sabanus*, *A. marmoratus*, *A. oculatus*, *A. bimaculatus* and *A. schwartzi*) show uncorrected sequence divergences for a portion of the cytochrome *b* gene of between 4.9 and 21.3% (Schneider *et al.*, 2000; S. L. Perkins, unpublished data). Also, haplotype E of *P. azurophilum* Red is present on both Dominica and Martinique, although, as stated above, the lizards on these two islands represent what are probably endpoints of separate radiations (Gorman & Atkins, 1969). Thus, presumably, some movement of the parasites, either in vectors or failed lizard colonists has occurred following the initial colonization of the Lesser Antilles by the lizards. However, it is also apparent, given the distribution of the haplotypes and the results of the nested clade analysis, that the distribution of these parasites in the Lesser Antilles is not random, i.e. neither vectors nor lizards appear to be moving about the islands enough to homogenize the gene pools of these malaria parasites. Furthermore, the distribution of lizard malaria throughout the chain of islands as a whole is spotty, although many of these islands have habitat that is presumably suitable for the parasites and vectors (Staats & Schall, 1996a). This provides additional evidence that these parasite species have fragmented populations as a result of local extinctions.

A proposed colonization history of lizard malaria

Here I present hypotheses on the history of the two lizard malaria species based on inferences that can be derived from the outgroup-rooted networks. Figure 5 presents a scenario for the colonization history of *P. azurophilum* Red. The ancestor of this group was potentially a lizard malaria parasite in South America, as this region is rich in both *Anolis* and *Plasmodium* species (Ayala, 1977). Haplotype A, a product of this ancestor, gave rise to haplotype B on the island of Puerto Rico. Haplotype B later gave rise to haplotype D, which has colonized the northern Lesser Antilles and remains on St Kitts. The two missing haplotypes between B and D (Fig. 1) suggest that this lineage was once more prevalent in the northern islands. A second lineage colonized the islands northward, perhaps first landing on St Vincent as has been suggested for the anole hosts (Yang *et al.*, 1974). This first haplotype of the lineage (E) spread north to give rise to haplotype C in the anoles of the *bimaculatus* group, and south to Grenada to give rise to haplotype F. The move into the northern islands is interesting because it must represent either a failed colonization of a *roquet* anole into Dominica or the successful dispersal of the parasite in vectors.

The corresponding colonization hypothesis for *P. azurophilum* White is presented in Fig. 6. The root for this species appears to be either haplotype I or J, both now present only on St Kitts, although they might have originated on another island. *Plasmodium azurophilum*

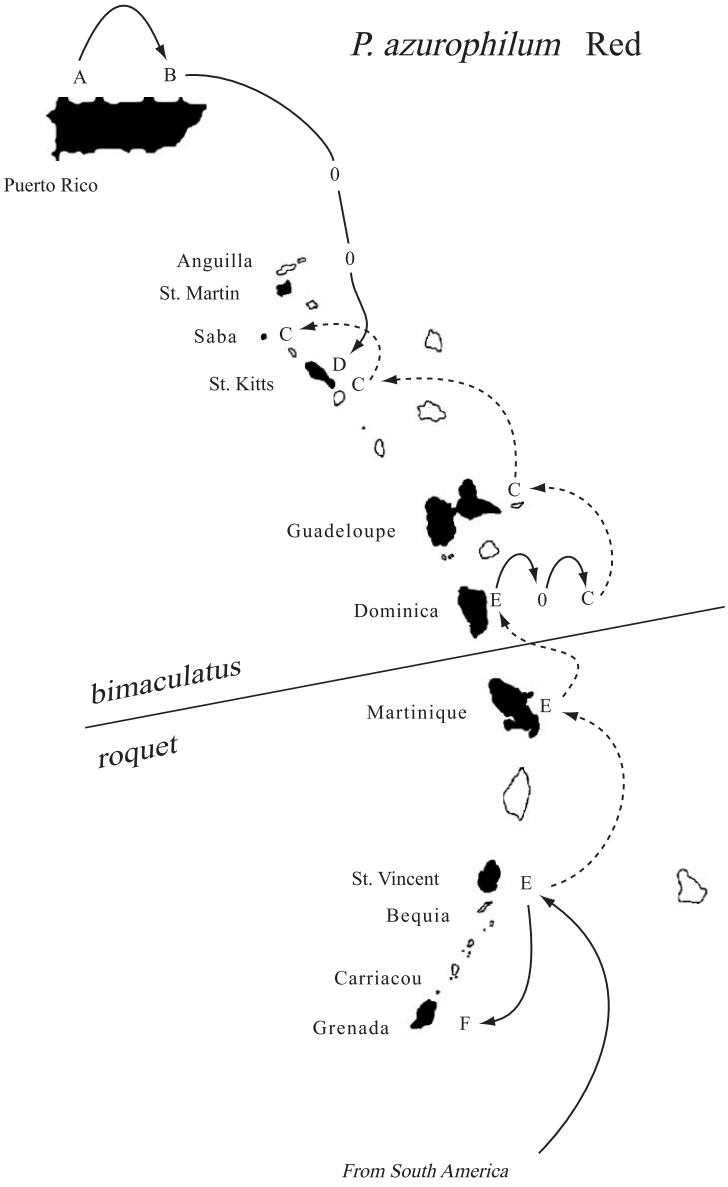

Fig. 5 Proposed scenario of the colonization history of *P. azurophilum* Red in the Lesser Antilles and Puerto Rico. Solid lines represent mutations to form new haplotypes; dashed lines represent interisland colonizations of haplotypes. The heavy line between Dominica and Martinique shows the break in distribution between the *bimaculatus* and *roquet* anoles. Scale is only approximate.

White then dispersed both north as far as Puerto Rico and south as far as St Vincent. Haplotype H has the broadest distribution, as it is found in *Anolis* from Saba, Guadeloupe and Dominica. The position of the root in the middle of the network as well as the middle of the island chain strongly argues that *P. azurophilum* White originated in the Lesser Antilles themselves and not in any mainland populations. *Plasmodium azurophilum* Red and White are monophyletic taxa, but are also sister taxa (S.L. Perkins, unpublished data). The use of erythrocytes, the typical strategy for *Plasmodium* parasites, is most probably the ancestral condition; thus, *P. azurophilum* White may have arisen from a population of *P. azurophilum* Red in the northern Lesser Antilles and then dispersed throughout the Caribbean islands. The very different colonization histories proposed here for the two taxa of *P. azurophilum* again illustrate that they are separate, independent species.

The malaria parasites of the Lesser Antillean *Anolis* lizards, share many biogeographical features with their vertebrate hosts, yet appear to have also moved independently throughout the islands, perhaps in windblown insect vectors (see also Charleston *et al.*, 2000). Nested clade analysis revealed that past fragmentation of parasite populations was probably responsible for the current association of geography and cytochrome *b* haplotypes, which is consistent with the susceptibility of vector-borne parasites to environmental or stochastic population processes, resulting in frequent local extinctions on islands.

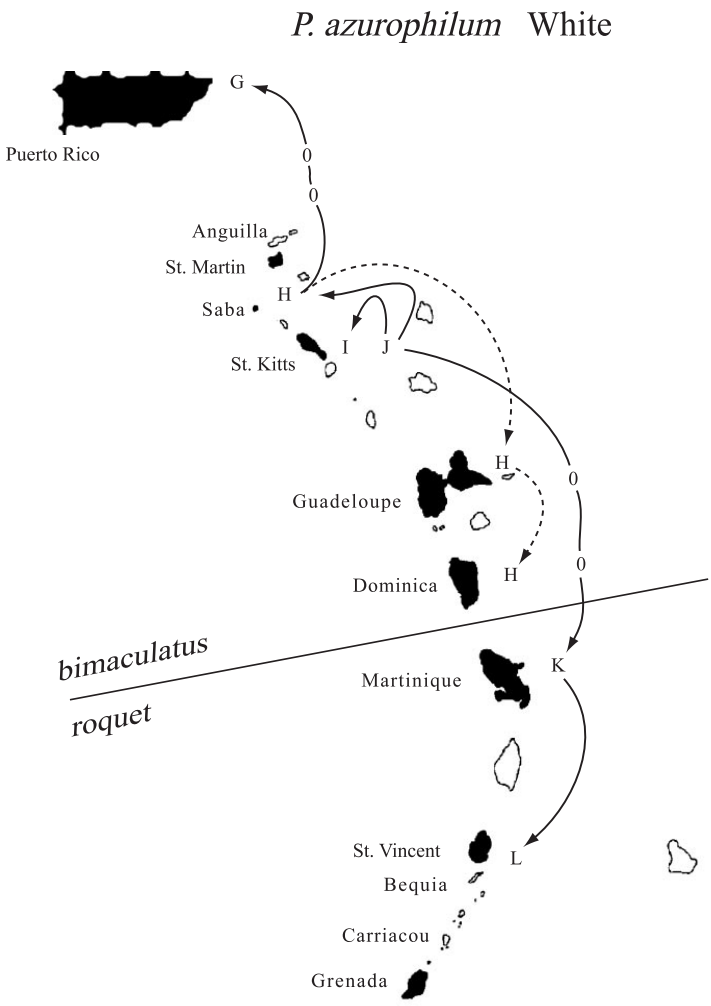

Fig. 6 Proposed scenario of the colonization history of *P. azurophilum* White in the Lesser Antilles and Puerto Rico. Solid lines represent mutations to form new haplotypes; dashed lines represent interisland colonizations of haplotypes. The heavy line between Dominica and Martinique shows the break in distribution between the *bimaculatus* and *roquet* anoles. Scale is only approximate.

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