DIVERSIFICATION OF MALARIA AND PINWORM PARASITES IN CARIBBEAN *ANOLIS* LIZARDS

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ABSTRACT

Parasites are ubiquitous, comprising a significant portion of biodiversity and occurring in host species across the tree of life. Nonetheless, both parasites and the processes contributing to parasite diversification are, in general, poorly studied. I attempt to shed light on parasite diversity and diversification by characterizing the malaria and pinworm parasite diversity in a model host group, the Caribbean *Anolis* lizards.

I began with a study of malaria parasites (genus *Plasmodium*) in Hispaniolan *Anolis* lizards. In the Caribbean, malaria parasite diversity is highest on this island, where six species were previously described using subtle and overlapping differences in morphology. Fifty-five infections were identified in 677 *Anolis* lizards collected from across the island, but only 24 of these infections could be assigned to species using morphological criteria. I tested these taxonomic hypotheses using a phylogenetic approach and both mitochondrial and nuclear loci. Four reciprocally monophyletic clades that generally contradict the morphological hypotheses were recovered, and consequently several taxonomic changes were made. Additionally, low average prevalence of these parasites among hosts was observed, as well as low genetic diversity in each of the parasite species.

I next attempted to explain the low intraspecific diversity of the malaria parasites in a study of the most common Caribbean species, *Plasmodium floridense*. This is among the most widespread of the lizard malaria parasites, and is distributed throughout the Caribbean and in parts of North and Central America. I predicted that low prevalence, in combination with the malaria parasite life cycle, shapes diversification in *P. floridense* through inbreeding. Sixty-three samples were collected from across the parasite's range, and were sequenced at seven independent loci. I employed Bayesian species delimitation to identify 11 independently evolving lineages within *P. floridense*, each of which is characterized by very low within-lineage variation. A molecular clock rate was used to infer very recent divergence among lineages, with some estimated to have diverged ~0.11 MYA. These patterns are consistent with inbreeding – a condition favored by the malaria parasite life cycle and transmission dynamics – and may be common to malaria parasites generally.

Lastly, I expanded on the effects of parasite transmission, asking whether differences in transmission among host species affect diversification. Specifically, I examined the effect of host specificity on the diversification of two multi-host pinworm parasites on the Puerto Rican Bank and St. Croix, testing the hypothesis that higher host specificity is associated with greater differentiation among populations. The pinworm parasites *Spauligodon anolis* and *Parapharyngodon cubensis* differ in host specificity; *S. anolis* infects *Anolis* lizards, whereas *P. cubensis* infects *Anolis* lizards and several other species of lizards and snakes. I collected 651 lizards from across the Puerto Rican Bank and St. Croix, and dissected them for parasites. A total of 233 pinworms were sequenced at both mitochondrial and nuclear loci, and, using a variety of phylogeographic

approaches, I showed that *S. anolis* exhibits greater differentiation among populations than does *P. cubensis*, particularly between populations permanently separated by ocean waters. This suggests that transmission among host species affects parasite diversification. I also provide evidence that *P. cubensis* may be a complex of several species.

Two main conclusions can be drawn from this research. First, parasite diversity in Caribbean *Anolis* lizards is largely underestimated, and molecular data are necessary to effectively delimit both malaria and pinworm parasites. Second, parasite transmission – between host individuals and host species – affects parasite diversification, and differences in transmission may be among the most important factors shaping the diversity of parasites alive today.

For Mom and Dad

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CHAPTER I

INTRODUCTION TO *ANOLIS* LIZARDS, THEIR PARASITES, AND PARASITE DIVERSIFICATION

Parasites form a large diversity of life on earth.

- Peter Price, 1980

Parasites are ubiquitous, and parasitologists are wont to say so (Bush *et al*. 2001; Roberts & Janovy 2010; Schmid-Hempel 2011). This ubiquity is apparent in the prominent role that parasites play in natural systems, where, for example, they contribute ~75% of food web connectivity (Dobson *et al*. 2008). Parasites pose obvious and major threats to the health of both human and wildlife populations (McCallum & Dobson 1995; Daszak *et al*. 1999; Daszak *et al*. 2000; Cunningham & Daszak 2001; Altizer *et al*. 2003; Skerratt *et al*. 2007), but can also be useful in guiding conservation efforts (Criscione & Blouin 2006; Whiteman & Parker 2006). In some cases, the parasites are themselves threatened with extinction (Gompper & Williams 1998; Dunn *et al*. 2009). Still, despite their importance and apparent omnipresence, we have a generally poor understanding of extant parasite diversity and which factors have contributed to this diversity (Poulin & Morand 2000).

Early efforts to explain parasite diversity were formulated into several "rules" (Brooks 1979, 1985; Brooks & McLennan 1991; Hoberg *et al*. 1997), which established a theoretical framework that provided parasitologists decades of hypothesis testing. Among these is Fahrenholz's Rule (Eichler 1948), which predicts that a parasite phylogeny will mirror the host phylogeny (i.e., strict co-speciation). A number of methods were

developed to test this hypothesis, and a number of studies sought to detect host and parasite co-speciation (Hafner & Nadler 1988; Page 2003; Huyse & Volckaert 2005). Nonetheless, it eventually became apparent that strict co-speciation occurs only in some cases (Huyse *et al*. 2005), and parasitologists began to look elsewhere for explanations for the great diversity of parasites.

Population genetics and phylogeographic studies of parasite diversification are becoming more common (Nadler 1995; Criscione *et al*. 2005; Huyse *et al*. 2005; Barrett *et al*. 2008), and complement the aforementioned systematic approaches. Studies using this approach have shown that parasite dispersal depends on host dispersal (McCoy *et al*. 2003), but also that parasite dispersal ability is negatively correlated with parasite population differentiation (Whiteman *et al*. 2007). The population-level scale of these studies has facilitated hypothesis testing that would not have otherwise been possible in systematic datasets, and these are promising for untangling the many potential factors affecting parasite diversification.

Potential Challenges

There are several potential challenges in research on parasite diversity and diversification. Some are not exclusive to parasitology, including a general a lack of funding and training (Brooks & Hoberg 2000, 2001, 2006). But, parasitologists do encounter unique obstacles that are not shared among their non-parasitologist colleagues. Primary among these is that the parasites themselves are difficult to obtain. Studies of parasite population genetics and systematics typically include just one parasite from each host individual (i.e., they include one parasite from each host infrapopulation), but parasite prevalence rarely reaches 100%. This means that a parasitologist must collect

more host individuals for the same-sized dataset of parasites than a biologist asking similar questions in a study of the host taxon. And, considering that adequate host sampling may be difficult or impossible to obtain (due to permitting, insufficient population sizes, or other practical reasons), acquiring even modestly sized datasets may not be possible for many parasites.

Museum collections of host taxa may provide an additional resource for the parasitologist, but parasite specimens and tissues are often not appropriately preserved. For example, macroparasite specimens (e.g., helminths) may be present in preserved host specimens, but the necessary fixation protocols for hosts and parasites are different, and the morphological features necessary for parasite delimitation and identification may not be present. Alternatively, some parasites simply cannot be detected after routine processing that is appropriate for the host. Blood parasites, for example, require the preparation of blood films from a still-living host. In many cases, traditional parasite recovery methods are destructive, meaning that host specimens are destroyed during dissection. Finally, and perhaps most importantly, commonly employed preservation techniques for the host specimens (e.g., formalin-fixation) preclude DNA amplification of the parasites.

Another major obstacle is the lack of genetic resources available many parasite taxa. Genomic data are becoming cheaper and easier to generate, but they are not yet available for many taxa. Nematodes, for example, are an ancient and diverse group, with an estimated one million species living today (Hugot et al. 2001). Still, only 11 nematode genomes have been sequenced to-date (http://www.nematodes.org, accessed 12/14/12), and these are biased towards either laboratory models (e.g., three of the 11 are

Caenorhabditis sp.) or root-knot nematodes (e.g. two are *Meloidogyne* sp.). Only three are animal parasites, and these all belong to the same clade (Clade III; *Ascaris suum*, *Brugia malayi*, and *Dirofilaria immitis*; Blaxter *et al*. 1998). This phylogenetically biased genome availability precludes novel primer design for many parasite taxa. And, molecular data are important in an integrative taxonomy (Hoberg 2002; Ferri *et al*. 2009), particularly as morphological crypsis may be more common in parasitic than non-parasitic taxa (de León & Nadler 2010; Perkins *et al*. 2011).

Anolis lizards in the Caribbean

Common and conspicuous, *Anolis* lizards are the dominant vertebrate fauna in the Caribbean, and have attracted a long history of attention by ecologists and evolutionary biologists. Early workers focused on *Anolis* taxonomy and systematics (Barbour 1930; Etheridge 1959; Williams 1976), and as phylogenetic theory and methodology expanded, so did knowledge of *Anolis* relationships (Gorman 1980; Guyer & Savage 1986; Nicholson *et al*. 2005). Likewise, Caribbean anoles have been the focus of studies across multiple sub-disciplines, including thermal ecology, invasion biology, developmental biology, island biogeography, and genome evolution (see Losos 2009, and references therein). Perhaps most notably, anoles on the large islands of the Greater Antilles have undergone a repeated pattern of adaptive radiation. These lizards have morphological, behavioral, and ecological adaptations to their microhabitat structure, and can usually be assigned into one of six "ecomorph" categories (Williams 1983; Losos 2009). These categories are not monophyletic clades, as ecomorphs evolved independently on each island (Losos 1998). Moreover, up to 12 anole species can co-occur at a single site. The *Anolis* communities on the smaller, satellite islands of the Greater Antilles and those on

the Lesser Antilles are less diverse. Many of these islands are populated by just one or two species, with species distributions often spanning several neighboring islands. Indeed, our understanding of Caribbean *Anolis* is considerable, and these lizards continue to be an excellent model system for studies in evolution and ecology.

Parasites in Caribbean *Anolis* lizards

Relatively little is known about the diversity and diversification of parasites in Caribbean anoles. The preponderance of taxonomic work has been completed by just a few individuals and has focused on parasite species discovery and host associations. Stephen Goldberg and Charles Bursey have characterized the geographic and host ranges of many helmith parasites (Goldberg *et al*. 1997, 1998; Bursey *et al*. 1998, 2012). Likewise, Sam Telford Jr. has described several malaria parasite species in *Anolis* lizards and conducted many of the first blood parasite surveys in Caribbean anoles (Telford 1975; Telford et al. 1989, Telford 2008).

The ecologist Jos Schall and his students have conducted a large number of ecological studies on the malaria parasites of anoles in the Lesser Antilles and in El Yunque Forest in Puerto Rico (Schall & Vogt 1993; Staats & Schall 1996a, 1996b; Schall & Staats 1997; Schall *et al*. 2000). In a classic study, he showed that on St. Maarten the competitively inferior *Anolis wattsi* co-occurs with the dominant *Anolis gingivinus* only when the latter is infected with the malaria parasite *Plasmodium azurophilum* (Schall 1992). A later study did not find *P. azurophilum* in these populations, however (Perkins, 2001), and host individuals on other islands exhibit little-to-no observable effects of malaria parasite infection (Schall & Pearson 2000; Schall *et al*. 2002), suggesting that the virulence of malaria parasites in anoles is minimal.

Of the few studies that address host/parasite ecology and evolution, most have been concerned with community composition and patterns of distribution. Both helminth community richness (Dobson *et al*. 1992) and trombiculid mite prevalence (Zippel *et al*. 1996) were found to be negatively correlated with increasing aridity in the Lesser Antilles and on Hispaniola, respectively. Likewise, similarities among helminth communities in Jamaican anoles were correlated with habitat type, not host phylogeny or ecomorph (Bundy 1987). Prevalence of malaria parasites (*Plasmodium*) showed no association with habitat in the Lesser Antilles, however (Staats & Schall 1996b). Only two studies have made comparisons of host and parasite evolutionary history. The phylogeography of two lizard malaria-parasite species were each compared to the biogeography of their *Anolis* hosts, and are only weakly correlated (Perkins 2001; Charleston & Perkins 2003).

My dissertation is centered on understanding the diversity and diversification of two parasite groups in Caribbean anoles: malaria parasites and pinworm parasites. I begin in Chapter II with a taxonomic revision of malaria parasite species on Hispaniola, and ask whether there are differences in parasite prevalence among anole ecomorphs. In Chapter III, I make predictions about how the malaria parasite life cycle and transmission dynamics may shape malaria parasite diversification, and I test these predictions in the widespread species *Plasmodium floridense*. Finally, in Chapter IV, I test the hypothesis that increased host specificity is associated in increased population structure in the multi-host pinworm parasites *Spauligodon anolis* and *Parapharyngodon cubensis*.

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CHAPTER II

TREE-BASED DELIMITATION OF MORPHOLOGICALLY AMBIGUOUS TAXA: A STUDY OF THE LIZARD MALARIA PARASITES ON HISPANIOLA

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Abstract

Malaria parasites in the genus *Plasmodium* have been classified primarily on the basis of differences in morphology. These single-celled organisms often lack distinguishing morphological features, which can encumber both species delimitation and identification. Six saurian malaria parasites have been described from the Caribbean island of Hispaniola. All six infect lizards in the genus *Anolis*, but only two of these parasites can be distinguished using morphology. The remaining four species overlap in morphology and geography, and cannot be consistently identified using traditional methods. We compared a morphological approach to a molecular, phylogenetic tree-based approach in assessing the taxonomy of these parasites. We surveyed for blood parasites 677 *Anolis* lizards, representing 26 *Anolis* species from a total of 52 sites across Hispaniola. Fifty-five of these lizards were infected with *Plasmodium* spp., representing several new host records, but only 24 of these infections could be matched to five of the six previously described species using traditional morphological criteria. We then estimated the phylogeny of these parasites using both mitochondrial (*cytb* and *coxI*) and nuclear (*EF2*) genes, and included carefully selected GenBank sequences to confirm identities for certain species. Our molecular results unambiguously corroborated our

morphology-based species identifications only for the two species previously judged to be morphologically distinctive. The remaining infections fell into two well-supported and reciprocally monophyletic clades, which contained the morphological variation previously reported for all four of the morphologically ambiguous species. One of these clades was identified as *Plasmodium floridense*, and the other as *Plasmodium fairchildi hispaniolae*. We elevate the latter to *Plasmodium hispaniolae* comb. nov. because it is polyphyletic with the mainland species *Plasmodium fairchildi fairchildi*, and we contribute additional morphological and molecular characters for future species delimitation. Our phylogenetic hypotheses indicate that two currently recognized taxa, *Plasmodium minasense anolisi* and *Plasmodium tropiduri caribbense*, are not valid on Hispaniola. These results illustrate that molecular data can improve taxonomic hypotheses in *Plasmodium* when reliable morphological characters are lacking.

Introduction

The taxonomy of the malaria parasites in the genus *Plasmodium* has for the most part followed the morphological species concept. As single-celled organisms, *Plasmodium* morphology is simple, and many species descriptions of these parasites rely on a handful of physical characteristics and measurements. In general, these characters are continuous (e.g., length measurements or ratios), and overlapping character variation among parasite species can encumber species delimitation. Reliable morphological characters are undoubtedly useful, but distinguishing traits may be rare or absent in many *Plasmodium* species, such that even the identification of previously described taxa is occasionally problematic. Among the reptile parasites, which account for approximately half of the roughly 200 species in the genus *Plasmodium*, the problem is particularly

acute as most species assignments have been made based only on the morphologies of the stages found in the circulating blood of the vertebrate host, which is just one part of the parasite's life cycle. The lack of diagnostic characters further confounds species delimitation and identification when the parasites also exhibit overlap in host preference and geographic distribution.

The saurian malaria parasites of Hispaniola, the Greater Antillean island comprised of the countries Haiti and the Dominican Republic, are exemplars of *Plasmodium* taxonomic uncertainty. Six species have been reported from this Caribbean island, all in *Anolis* lizard hosts. The first five are: *Plasmodium azurophilum* Telford, 1975; *Plasmodium fairchildi hispaniolae* Telford et al., 1989; *Plasmodium floridense* Thompson and Huff, 1944; *Plasmodium minasense anolisi* Telford et al., 1989; and *Plasmodium tropiduri caribbense* Telford et al., 1989 (Telford et al., 1989; Telford, 2009). *Plasmodium azurophilum* was originally described as a single species capable of infecting both erythrocytes (red blood cells) and leucocytes (white blood cells) of its hosts (Telford, 1975). A subsequent analysis showed that these two forms are reciprocally monophyletic lineages, one infecting red blood cells and other infecting white blood cells (Perkins, 2000). A new name was given to the form infecting white blood cells, *Plasmodium leucocytica* Telford, 2009; this is the sixth saurian malaria parasite on Hispaniola. *Plasmodium azurophilum* and *P. leucocytica* are widely distributed in the Caribbean, and *P. fairchildi hispaniolae, P. minasense anolisi,* and *P. tropiduri caribbense* are endemic Hispaniolan subspecies of species otherwise found in Central and South America. *Plasmodium floridense* has a broad distribution that includes Florida, the Greater and Lesser Antilles in the Caribbean, and mainland Central America.

Each of these parasites is assumed to have a life cycle that is typical for any *Plasmodium* species. The parasite first infects a lizard in the form of a sporozoite, which is transmitted from the saliva of an infected, blood-feeding fly, usually a mosquito. It undergoes schizogony (i.e., merogony), a form of asexual reproduction, in the liver, and these stages eventually reach the blood stream. Asexual reproduction continues within the blood cells, and male and female gametocytes develop. A blood-feeding fly then takes up these gametocytes during a meal, the parasites undergo sexual reproduction, and the cycle begins anew. While the mosquito *Culex erraticus* was demonstrated to transmit *P. floridense* in Florida (Klein et al., 1987), it is unknown whether the parasite retains this vector in the Greater Antilles. The identities of the vectors of the remaining parasite species on Hispaniola are unknown. Species identification and delimitation in these parasites has relied exclusively on the stages found circulating in the blood of their *Anolis* hosts.

Both *P. azurophilum* and *P. leucocytica* can be readily distinguished from the other Hispaniolan lizard malaria species, because both lack hemozoin pigment but each are found in different host cells. Discriminating between the remaining four co-occurring species based on fixed morphological differences is not possible. These species were described based upon minor morphological dissimilarities, and all four overlap in their physical appearance, host preference, and cell preference (Telford et al., 1989). Nonetheless, distinctions can be made in three instances. First, the schizonts (i.e., meronts) of *P. tropiduri caribbense* sometimes exhibit an elongate cytoplasmic projection not observed in the other species. Second, both the schizonts and gametocytes of *P. minasense anolisi* are sometimes smaller in size than the other parasite species.

Third, and similarly, the schizonts and gametocytes of *P. floridense* are sometimes larger than the other species (Telford et al., 1989). These size features for *P. minasense anolisi* and *P. floridense* are almost completely eclipsed by the variation observed in the other species, however, diminishing their use in identification (Figure 2.1). Moreover, any species identification that employs these features must assume that the size distributions of each of the four species are well characterized. Generally speaking, a gametocyte that is moderate in size, oval in shape, containing hemozoin, and found in an *Anolis* lizard erythrocyte may belong to any one of these four species. This ambiguity is additionally confounded by the low prevalence that characterizes this host-parasite system. *Plasmodium fairchildi hispaniolae* was described from a single blood smear, for example. Furthermore, typical rates of parasitemia in *Anolis* are low enough that it is not uncommon to observe only a few parasites in an entire blood smear (Telford, 1975; Staats & Schall, 1996; Vardo et al., 2005), forcing taxonomic inferences to be grounded on only a few observations. Even if many parasites are observed, representatives of trophozoite, gametocyte, and schizont stages may not be present. In chronic infections, for example, schizonts are rare and might not be sampled. Clearly, a morphological approach to species delimitation in this group is problematic.

When delimiting species that are poor in reliable morphological characters, molecular data can be used to inform species limits. This approach been used for the malaria parasites of birds (e.g., Bensch et al., 2004; Sehgal et al., 2006; Bensch et al., 2007; Martinsen et al., 2007; Valkiūnas et al., 2010) and mammals (e.g., Perkins et al., 2007; Singh and Divis, 2009; Duval et al., 2010), but only rarely has it been applied to the alpha taxonomy of malaria parasites in reptiles. Perkins (2000) implemented a tree-

based approach – with a phylogeny estimated using the mitochondrial gene cytochrome *b* – to reveal the aforementioned cryptic species diversity in *P. azurophilum*. Perkins and Austin (2009) used fixed molecular characters as part of their species descriptions of several lizard malaria parasites on New Guinea, explaining that such characters are particularly valuable when the sample sizes are small or not all life stages have yet been observed. These studies reveal the potential value of employing molecular data to inform species limits in saurian *Plasmodium* species.

When molecular data are analyzed in a phylogenetic framework, it allows a taxonomic assessment in concordance with the general lineage concept of species (GLC; de Queiroz, 1998; de Queiroz 2007). Under the GLC, species are regarded as independently evolving metapopulation lineages, and any of the recognition criteria of other concepts (e.g., the potential interbreeding criterion, the niche criterion, etc.) can be used to delimit species boundaries (de Queiroz, 1998; de Queiroz 2007). Taxonomic classifications using phylogenetic methods are consistent with the GLC. In this study, we use the recognition criterion of reciprocal monophyly in gene tree hypotheses. Because reciprocal monophyly for many phylogenetic markers occurs late in the speciation process (Knowles and Carstens, 2007), this is a conservative approach to species delimitation.

Tree-based inferences of species limits have frequently been made using only mitochondrial DNA (e.g., Sperling and Harrison, 1994; Morando et al., 2003; Pons et al., 2006; Monaghan et al., 2009). This maternally inherited, haploid locus typically exhibits greater variation than that found in the nuclear genome, and thus offers greater power for resolving relationships among closely related species (Brown et al., 1979; Avise, 2000;

Wiens and Penkrot, 2002). An approach that uses just one locus can be problematic, however. Gene trees may not provide accurate estimates of the true species tree due to incomplete lineage sorting, horizontal gene flow, gene duplication, or incorrect gene tree estimation (Maddison, 1997; Funk and Omland, 2003; Wiens and Penkrot, 2002). Confidence in gene tree estimation can be inferred using character-resampling techniques such as the bootstrap (Felsenstein, 1985). Estimating the phylogeny using additional loci can ameliorate potential problems arising from the remaining factors. Except under certain conditions that cause statistical inconsistency (e.g., long branch attraction for parsimony analyses, and trees in the "anomaly zone" for maximum likelihood analyses), the addition of more loci can improve estimation of the species tree (Pamilo and Nei, 1988; Wiens, 1998; Leaché and Rannala, 2010).

The purpose of this study was to evaluate the utility of a molecular-based, phylogenetic approach in assessing the phylogenetics and taxonomy of saurian malaria parasites on Hispaniola. We employed a broad geographic and host species sampling in order to allow an adequate estimation of parasite diversity. Morphology-based parasite identifications were made on the basis of previously reported differences, and we compared these results to those based on molecular data. We estimated phylogenetic trees using nucleotide data from the mitochondrial genes cytochrome *b* (*cytb*) and cytochrome oxidase I (*coxI*), and the nuclear gene elongation factor-2 (*EF2*), with both maximum parsimony and maximum likelihood as optimality criteria. We used the mitochondrial dataset to generate a taxonomic hypothesis, tested for congruence of the nuclear data, and then combined these loci. We recommend several taxonomic changes based on our findings, and we also use molecular data to update the description of one of

these species. The increased taxonomic resolution afforded by molecular data demonstrates its utility when assessing the taxonomy of this morphologically ambiguous group.

Materials and Methods

Sampling

We captured a total of 677 *Anolis* lizards, representing 26 of 39 Hispaniolan species, by noose or hand across 35 sites in the Dominican Republic (August 2008 & January 2010) and 17 sites in Haiti (August 2009; Figure 2.2 and Table 2.1). These included at least one locality where each of the six parasite species had been previously sampled (see Telford et al., 1989; Telford, 2009). From each lizard, we clipped a toe from the hind leg to obtain blood samples. We made blood smears for morphological analysis, first air-drying and then fixing the slides in 95% methanol. For molecular analysis, we applied 3-6 drops of blood to filter or FTA paper, and let them air-dry. We humanely euthanized most captured lizards and preserved them as voucher specimens using 95% ethanol or 10% buffered formalin, and we also preserved a sample of liver tissue from each lizard in 95% ethanol. Voucher host specimens and tissues are permanently stored at the Museum of Comparative Zoology at Harvard University. A subset of lizards, roughly 20% of all those captured, were not retained as vouchers and after sampling were released at the site of capture.

Morphological characterization

To determine whether a lizard was infected with malaria parasites, we stained the thin blood smears with Giemsa and searched for parasites using light microscopy under oil immersion at 1000x magnification for 3 – 6 minutes. When possible, we identified

malaria parasites to species, following Telford et al. (1989) and Telford (2009). Specifically, we identified species as either *P. azurophilum* or *P. leucocytica* if hemozoin was absent (based on host cell type; Figure 2.3A-D), as *P. tropiduri caribbense* if a schizont with a cytoplasmic projection was observed (Figure 2.3E-F), as *P. minasense anolisi* if parasite size was smaller than reported for the other three species (Figure 2.1 and Figure 2.3G), or as *P. floridense* if larger than reported for the others (Figure 2.1 and Figure 2.3H). Parasites lacking any of these distinguishing characters were left unidentified (Figure 2.3I-P).

Molecular characterization

Sequence data were amplified from all parasitized samples to confirm parasite identity. We extracted DNA using the QIAGEN DNeasy Animal Tissue Extraction kit (Valencia, California) following the manufacture's instructions, except with two final DNA elutions each using just 50µl AE buffer. A partial fragment of the mitochondrial gene *cytb* was amplified using the primer pair DW2 (5' – TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG – 3') and 3932R (5' – GAC CCC AAG GTA ATA CAT AAC CC – 3'). At least one representative of each unique haplotype at this locus was selected for further amplification and sequencing of additional gene regions. For these samples, the remainder of *cytb* was amplified using the primer pair 3932F (5' – GGG TTA TGT ATT ACC TTG GGG TC – 3') and DW4 (5' – TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG – 3'). The mitochondrial gene *coxI* was amplified using a nested reaction following Perkins et al. (2007). First, an initial outer reaction was performed using the primers coxIF (5' – CTA TTT ATG GTT TTC ATT TTT ATT TGG TA – 3') and coxIR (5' – GTA TTT TCT CGT AAT GTT TTA CCA AAG AA – 3').

Product from this reaction was used as template for two nested reactions, the first using the primer pair coIinF (5' – ATG ATA TTT ACA RTT CAY GGW ATT ATT ATG – 3') and coImidR (5' – CTG GAT GAC CAA AAA ACC AGA ATA A – 3'), and the second with coImidF (5' – TTA TTC TGG TTT TTT GGT CAT CCA G – 3') and coIinR (5' – GTA TTT TCT CGT AAT GTT TTA CCA AAG AA – 3'). A fragment of the nuclear gene *EF2* was also amplified using a nested reaction, first using the primer pair EF2F (5' – CAR GTT CGT GAR ATC ATG AAC A – 3') and EF2R (5' – AAT GCC CAD CCT TGT AA CCW GAA CC – 3'), and followed by a second with LizMalEF2F (5' – CAT GGA AAA TCA ACA TTA ACA GAT TCT – 3') and LizMalEF2R (5' – CAG GAT ATA CTT GAA TAT CAC CCA T – 3'). PCR products were cleaned with AMPure (Agencourt, Beverly, Massachusetts) and sequenced in both directions using BigDye v.3.0 (Applied Biosystems, Foster City, California). Sequences were edited in GENEIOUS v.4.8.3 (Biomatters, Auckland, New Zealand).

We obtained *cytb* and *coxI* sequences from GenBank for several potential ingroup taxa to corroborate parasite species identity. Each of these was previously identified, sequenced, and submitted to GenBank by one of the authors (SLP), and were collected in regions outside Hispaniola, minimizing confusion about parasite identity. These sequences were: *P. azurophilum* (Dominica: AY099055, EU254575), *P. leucocytica* (Dominica: AY099058, EU254576), *P. fairchildi fairchildi* (*cytb* only; Costa Rica: AY099056), and *P. floridense* (Florida: NC_009961). Sequences of the mammal parasites *Plasmodium berghei* (AF014115) and *Plasmodium knowlesi* (AY598141) were also downloaded from GenBank and included as outgroup taxa. These were also used as outgroups for the locus *EF2* (*P. berghei*: XM_673005; *P. knowlesi*: XM_002260326).

Multiple sequence alignments were generated using MUSCLE (Edgar, 2004), with 1000 iterations and default gap opening cost of -1. Leading and lagging ends were trimmed to remove any missing data at the alignment edges. Phylogenetic analyses were conducted under both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria. MP analysis was done in PAUP* v. 4.0 (Swofford, 2003), using random addition sequence and tree-bisection-reconnection (TBR). Gaps were treated as missing data. ML was implemented in RAxML (Stamatakis et al., 2005), using default settings and partitioning by gene in concatenated analyses. The best-fit model of nucleotide substitution was selected using the Akaike Information Criterion in FINDMODEL (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html), a web-based implementation of MODELTEST (Posada and Crandall, 1998). For both mitochondrial genes *cytb* and *coxI* the model GTR was selected, and GTR + Γ was selected for the nuclear gene *EF2*. For all analyses, bootstrap proportions (BP; Felsenstein 1985) were calculated to provide relative measures of nodal support, using 1000 replicates in each analysis.

We first inferred phylogenetic trees of the mitochondrial and nuclear datasets separately, then combined. We used the topology generated from the mitochondrial dataset to infer species boundaries of the parasites. We then assessed corroboration of those taxonomic hypotheses with the nuclear dataset. Finally, we combined the two loci and tested for conflicting signals using the incongruence length difference (ILD) test (Farris, 1994; see Dolphin et al., 2000, and Darlu & Lecointre, 2002, for a discussion on the utility of this test). This was implemented in PAUP* ("partition heterogeneity test") using 1000 ILD replicates, random addition sequences and TBR.

Species identifications from the morphological analysis were compared to results from the phylogenetic analyses. Because one clade recovered in the molecular analyses could not be identified using morphology or the GenBank sequence data, the blood smears were reexamined to further assess species identity. We quantified several morphological characters: length (as determined by the longest axis of the parasite cell); width (maximum width, measured perpendicular to the length axis); parasite area; host cell area; and host nucleus area (taken from the nearest uninfected host cell). All measurements were taken with a SPOT InTouch® digital camera and software. Area calculations were made by tracing the area of interest. We calculated three additional metrics: parasite length x width (LW), the ratio of parasite area to host cell area, and the ratio of parasite area to host nucleus area. For each metric, we present the whole range of observations as well as sample means and standard deviations.

We identified molecular characters to supplement these morphological characters (Desalle et al., 2005; and following Perkins & Austin, 2009). We aligned *cytb* and *coxI* sequences of this clade with homologous sequences of other *Plasmodium* species parasitizing reptile and birds, and used the protein coding sequences for *Plasmodium falciparum* (NC_002375) as reference for nucleotide position. We included the following species and GenBank accession numbers: *Plasmodium mexicanum* (NC_009960), *P. floridense* (NC_009961), *Plasmodium gallinaceum* (EU254535 and EU254578), *Plasmodium relictum* (AY733090), *Plasmodium azurophilum* (EU254532 and EU254575), *Plasmodium leucocytica* (EU254533 and EU254576), *Plasmodium giganteum* (EU254534 and EU254577), *Plasmodium chiricahuae* (*cytb* only, AY099061), *Plasmodium elongatum* (*cytb* only, AF069611), *Plasmodium fairchildi*

fairchildi (*cytb* only, AY099056), *Plasmodium agamae* (*cytb* only, AY099048), *Haemoproteus kopki* (*cytb* only, AY099062) and *Haemoproteus ptyodactylii* (*cytb* only, AY099057).

Results

Sampling and Morphology

Microscopic scans revealed that 55 of the 677 lizards were infected with malaria parasites. Of these, we identified 12 as *P. azurophilum* and three as *P. leucocytica* using morphological criteria (including the absence of hemozoin). Among the remaining 40 infections with parasite species containing hemozoin, we were able to distinguish nine of the infections based on previously reported physical differences. Two infections were identified as *P. tropiduri caribbense*, due to the observation in each of a single schizont with a cytoplasmic projection. Another six infections were characterized by the small sizes reported for *P. minasense anolisi*, and one infection had a single large gametocyte as reported for *P. floridense*; these were identified as such. We were unable to identify the remaining 31 infections using morphology.

Phylogenetic Analyses

Initial sequencing of parasite *cytb* (347 bp) from the blood samples of the 55 infected lizards showed that 11 unique mitochondrial haplotypes were present. Four samples had multi-allelic sequence chromatograms (i.e., clean sequences except at the segregating sites observed in the other samples), indicating infection by more than one parasite species. These mixed samples were excluded from subsequent phylogenetic analysis, though the identity of the component haplotypes was determined by comparison with single-infection sequences.

The topological hypotheses generated by MP and ML phylogenetic analyses of the concatenated *cytb* and *coxI* genes (924 bp and 848 bp, respectively, and 1772 bp combined) are similar, with the MP hypothesis generally showing greater resolution and support (Figure 2.4). Each of the sequences from parasites containing hemozoin fall into one of two well-supported clades. One of these clades (30 infections and two haplotypes; Groups A & B in Figure 2.4) contains all six samples that had been identified as *P. minasense anolisi*, the single *P. floridense* sample with large gametocytes, and one of the *P. tropiduri caribbense* samples. In fact, these three morphological species all share an identical mitochondrial haplotype (Group A). We identified all members of this clade as *P. floridense* because the clade is monophyletic with the GenBank sequence of *P. floridense*, because it contains the one sample we identified as *P. floridense*, and because the morphological identifications of *P. minasense anolisi* and *P. tropiduri caribbense* are not monophyletic. Members of the other clade (seven infections and three haplotypes; Groups C, D, & E in Figure 2.4) are not monophyletic with any GenBank sequences. This clade does include the other sample identified as *P. tropiduri caribbense*, which shares a haplotype with four other infections that we were unable to identify using morphological characters (Group C). We refer to all members of this clade as "*Plasmodium* sp." The clades containing *P. floridense* and *Plasmodium* sp. are reciprocally monophyletic. The GenBank sequences for each of *P. azurophilum* and *P. leucocytica* are monophyletic with the samples we had identified as such in the MP analysis. In the ML analysis, however, *P. azurophilum* is paraphyletic to *P. leucocytica*. The relative position of *P. fairchildi fairchildi* to *P. leucocytica* and *P. azurophilum* is also poorly resolved, possibly because we did not have *coxI* sequence data for *P.*

fairchildi fairchildi. When *P. fairchildi fairchildi* is removed, *P. azurophilum* and *P. leucocytica* are reciprocally monophyletic in the MP analysis, but their relationship remains poorly resolved in the ML analysis (not shown). Thus, all of our Hispaniolan samples fell into one of four reciprocally monophyletic groups in our MP analysis (*P. azurophilum*, *P. floridense*, *P. leucocytica*, and *Plasmodium* sp.), and two reciprocally monophyletic groups in our ML analysis (*P. floridense* and *Plasmodium* sp.) Hereafter we use these species identifications for our samples, unless we state otherwise.

The phylogenetic hypotheses generated from the nuclear *EF2* gene (410 bp; Figure 2.5) are similar to the mitochondrial hypotheses. The same clades are recovered in these analyses, again with strong support for the reciprocal monophyly of *P. floridense* and *Plasmodium* sp. *Plasmodium azurophilum* and *P. leucocytica* are reciprocally monophyletic in the nuclear MP tree (Figure 2.5A), though *P. azurophilum* is rendered paraphyletic in the ML analysis (Figure 2.5B). The ILD test further corroborated the congruence between the mitochondrial and nuclear loci ($P = 1.0$).

The phylogenetic hypotheses of the concatenated sequences of *cytb*, *coxI*, and *EF2* (2182 bp) are topologically similar to the mitochondrial and nuclear datasets, but with increased support (Figure 2.6). All four taxa described above are reciprocally monophyletic with strong support in the MP analysis, and with moderate support in the ML analysis. The reciprocal monophyly of these groups is consistent with our species delimitation criterion, particularly for the reciprocal monophyly observed for both *P. floridense* and *Plasmodium* sp. in the smaller, separate analyses of the nuclear and mitochondrial datasets.

Multiple lines of evidence suggest that the parasites forming the clade that belongs to *Plasmodium* sp. in our phylogenetic analyses are of the same that Telford et al. (1989) named *P. fairchildi hispaniolae*. *Plasmodium fairchildi hispaniolae* was described from a single *Anolis distichus* in Pedro Sanchez, in El Seibo Province of the Dominican Republic. Six of the 10 parasites that form the *Plasmodium* sp. clade (including the three from mixed infections; see below) were from anoles collected at Pedro Sanchez, and three of these were found in the host *A. distichus*. In addition, the morphological variation we observed in *Plasmodium* sp. overlaps significantly with the morphological variation reported for *P. fairchildi hispaniolae*. Broadly speaking, both *Plasmodium* sp. and *P. fairchildi hispaniolae* are characterized by elongated gametocytes, slightly larger in size than the host nucleus. Schizonts of these species are variable in shape, but are typically fan-shaped, and are also larger in size than host nuclei. We did not observe any distinguishing morphological characters that belong to previously described species, except for the one sample that contained a schizont with a cytoplasmic projection that we previously noted. We had identified this sample as *P. tropiduri caribbense* in our morphological analysis, but both its mitochondrial and nuclear sequences are identical to those belonging to several other members of the *Plasmodium* sp. clade, and this sample is polyphyletic with the other sample identified morphologically as *P. tropiduri caribbense*. This suggests that a cytoplasmic projection is not a useful morphological character to distinguish between schizonts belonging to the lineages of *P. floridense* and *Plasmodium* sp., and we do not consider the presence of a cytoplasmic projection on a schizont to be sufficient evidence to justify identifying all members of the *Plasmodium* sp. clade as *P. tropiduri caribbense*.

Plasmodium fairchildi hispaniolae was originally described as a subspecies of the mainland form, *P. fairchildi fairchildi*, on account of morphological resemblances. In the phylogenetic analysis, the Costa Rican form is polyphyletic with *P. fairchildi hispaniolae*. Accordingly, we elevate *P. fairchildi hispaniolae* to *P. hispaniolae* comb. nov. to retain monophyly and consistency with the GLC.

Taxonomic Summary

Plasmodium hispaniolae (Telford et al., 1989) comb. nov.

(Figure 2.3K-L,O-P and Figure 2.7A-P)

Main Diagnostic Characters: A *Plasmodium* (*Lacertamoeba*) species with ovoid-to-elongate gametocytes and fan-shaped schizonts. Vacuoles are present in fully-grown trophozoites and mature gametocytes, particularly macrogametocytes. Mature gametocytes often have irregular and ragged cell margins. The largest gametocytes and schizonts are positioned laterally in the host cell, whereas others are positioned terminally. Pigment is greenish-yellow-to-black. In schizonts, pigment granules form one or more clusters. Pigment granules are uniform in size and dispersed in microgametocytes, and irregular in size and clustered at cell margins in macrogametocytes.

Trophozoites (Figure 2.7A-D) are irregular in shape, though lacking outgrowths. Pigment is present in fully-grown forms, and is marginally distributed. Vacuoles are also sometimes present in fully-grown forms (Figure 2.7D, upper left).

Schizonts (Figure 2.3O-P; Figure 2.7E-J) are found in mature erythrocytes. Merozoite number ranges from 4 – 8 per schizont, with an average of 5 (±1.67). These are usually arranged in a fan shape, though they sometimes exhibit a rosette pattern

(Figure 2.7H) or are randomly distributed (Figure 2.7J). Rarely, the schizont exhibits a cytoplasmic projection (Figure 2.3E). Schizonts occupy a terminal position in the host cell, with no distortion of host cell or displacement of nucleus (Figure 2.3O-P; Figure 2.7E-J). They do not usually possess vacuoles. Pigment is clustered. Morphometric measurements are as follows (n = 20): length: 5.00 – 12.3 μm ($\overline{x}$ = 7.28 ± 1.51); width: 2.27 – 5.60 μm ($\overline{x}$ = 4.16 ± 0.73); LW: 14.0 – 50.4 μm^2 ($\overline{x}$ = 30.4 ± 8.53); parasite area: 14.4 – 65.4 μm^2 ($\overline{x}$ = 30.4 ± 10.0); parasite to host cell ratio: 0.12 – 0.47 ($\overline{x}$ = 0.216 ± 0.071); and parasite to host nucleus ratio: 0.70 – 2.37 ($\overline{x}$ = 1.29 ± 0.367).

Gametocytes (Figure 2.3K-L; Figure 2.7K-P) are typically elongate or ovoid, and immature forms are often wedge-shaped or with tapered ends (Figure 2.7K). These usually have a polar position in the host cell (Figure 2.3K-L; Figure 2.7M,O), though elongate forms more often have a lateropolar or lateral position (Figure 2.7N,P). The host cell is distorted only when gametocyte is very large (Figure 2.7P), and the nucleus is not displaced. Pigment is clustered at cell margins in macrogametocytes, and is diffuse in microgametocytes. Cell margins are often ragged or irregular in mature gametocytes (Figure 2.3L; Figure 2.7M,P), particularly macrogametocytes. Microgametocytes and macrogametocytes are otherwise similar in size and shape. Vacuoles are present in both immature (Figure 2.7L) and mature gametocytes (Figure 2.3L; Figure 2.7M-P), and tend to be smaller, numerous, and diffuse in the latter. Some gametocytes do not have vacuoles (Figure 2.3K). Morphometric measurements are as follows (n = 30): length: 3.56 – 15.2 μm ($\overline{x}$ = 8.14 ± 2.40); width: 2.19 – 5.76 μm ($\overline{x}$ = 3.70 ± 1.11); LW: 12.39 – 73.0 μm^2 ($\overline{x}$ = 31.6 ± 17.2); parasite area: 9.66 – 72.7 μm^2 ($\overline{x}$ = 27.15 ± 15.3); parasite

to host cell ratio: 0.08 – 0.41 ($\overline{x}$ = 0.194 ± 0.096); and parasite to host nucleus ratio: 0.51 – 2.69 ($\overline{x}$ = 1.11 ± 0.583).

Molecular Characters: These nucleotide characters in the cytochrome *b* gene are unique for *P. hispaniolae* comb. nov.: "C" at 121, "T" at 123, and "C" at 510. These nucleotide characters in the cytochrome oxidase I gene are unique: "C" at 508, "C" at 811, "C" at 981, and "C" at 1049. Positions refer to those of the coding regions for either *cytb* or *coxI* as annotated in the complete mitochondrial genome of *Plasmodium falciparum* (NC_002375).

Type host: Anolis distichus ignigularis Mertens, 1939 (Sauria: Polychrotidae).

Other hosts: Anolis cybotes Cope, 1862; *Anolis distichus ravitergum* Schwartz, 1968; *Anolis etheridgei* Williams, 1962.

Type locality: Pedro Sánchez, El Seibo Province, Dominican Republic (18.87967°N, 69.11958°W).

Additional localities: Constanza, La Vega Province, Dominican Republic (18.84137°N, 70.70745°W), Jarabacoa, La Vega Province, Dominican Republic (19.14088°N, 70.63128°W), and Matadero Village, Peravia Province, Dominican Republic (18.38963°N, 70.43035°W).

Type material: Hapantotype slide deposited in the United States National Parasite Collections, Beltsville (no. 80470; Telford et al., 1989).

Site of infection: Erythrocytes, rarely erythroblasts.

Prevalence: Telford et al. (1989) observed *P. hispaniolae* in 1/19 *A. distichus* in the Dominican Republic. We observed the following prevalence data within the Dominican Republic: 3/15 of *A. distichus ignigularis* and 3/12 of *A. cybotes* collected at

Pedro Sanchez; 1/2 of *A. etheridgei* at Constanza; 1/3 of *A. distichus ignigularis* at Jarabacoa; and 1/2 of *A. distichus ravitergum* and 1/2 of *A. cybotes* at Matadero Village.

Synonyms: Plasmodium fairchildi hispaniolae Telford et al., 1989; *Plasmodium tropiduri caribbense* Telford et al., 1989.

Remarks: Plasmodium azurophilum and *P. floridense* are also found in lizard erythrocytes in Hispaniola, and it is possible to distinguish *P. hispaniolae* from each of these if sufficient material is available (the fourth syntopic species on Hispaniola, *P. leucocytica*, is easily distinguishable because it is found only in white blood cells). *Plasmodium azurophilum* does not typically contain pigment, and the host nuclei are frequently displaced by its ovoid gametocytes (Figure 2.3A). *Plasmodium floridense* has a very similar morphology, but its size variation exceeds that observed for *P. hispaniolae*. Very small and very large forms can be assigned to *P. floridense* (e.g., mature schizonts with an area of less than 14.4 μm^2 or more than 65.4 μm^2, and mature gametocytes less than 9.66 μm^2 or more than 72.7 μm^2; Figure 2.3G-H). Additional sampling and characterization of *P. hispaniolae* might diminish the size differences between these species, however. Additionally, the gametocytes of *P. floridense* often contain more pigment granules and fewer vacuoles than those of *P. hispaniolae*. This vacuolization is perhaps the most useful morphological feature for discriminating *P. hispaniolae* from the other species, but note that this is not a fixed character and some gametocytes in this species lack vacuoles (Figure 2.3K).

Prevalence, Host Records, and Mixed Infections

Of the 677 lizards sampled, 55, or 8.1%, were infected with one or more *Plasmodium* parasites, as revealed by microscopic scans of the blood films and confirmed

with sequence data. Thirty-seven of these hosts, or 67%, were *A. cybotes*. This was the most frequently sampled host, and it had a relatively high overall prevalence of 37/220, or 17%. In addition to *A. cybotes*, another eight species were infected: *Anolis caudalis*, *Anolis chlorocyanus*, *Anolis christophei*, *Anolis coelestinus*, *Anolis distichus*, *Anolis etheridgei*, *Anolis insolitus*, and *Anolis marcanoi* (Table 2.2). This is the first record of any malaria parasite from *A. caudalis*, *A. christophei*, *A. etheridgei*, *A. insolitus*, and *A. marcanoi*. We also report new host-parasite associations for several *Anolis* and *Plasmodium* species pairs (Table 2.2).

Relative prevalence varied between parasite species and over the host species sampled. Of the four species, *P. floridense* was the most abundant, comprising 33/55, or 60%, of all infections. Not surprisingly, most *P. floridense* infections were found in the common host species *A. cybotes* (25/33, or 76%), as were the majority of *P. azurophilum* infections (8/12, or 67%), and all four of the *P. leucocytica* infections. In contrast, only 4/10, or 40%, of *P. hispaniolae* was found in *A. cybotes*. One was observed in *A. etheridgei* (10%), and the remaining 5/10 were found in *A. distichus*. Additional prevalence data are shown in Table 2.3.

As stated earlier, four of the 55 infected lizards were parasitized by more than one parasite species. Two of these were mixed *P. floridense* and *P. hispaniolae* infections. The third lizard was infected with *P. azurophilum* and *P. leucocytica*, and the fourth was infected with three parasite species: *P. floridense* and *P. hispaniolae*, and *P. leucocytica*. All four of these mixed infections occurred in *A. cybotes*, two of which occurred at the same locality (Pedro Sánchez, Dominican Republic; Table 2.3). All of these mixed infections were detected using sequence data, and were confirmed by repeated PCR and

sequencing. We re-examined the entire blood smears for the third and fourth infections, which were the only two that contained a species that could be unambiguously identified by morphology (*P. leucocytica*). In scanning each of these two blood films in their entirety, we were only able to visually identify *P. leucocytica* in one of them.

Discussion

The taxonomy of saurian Plasmodium *spp. on Hispaniola*

We observed significant discrepancies in the taxonomic hypotheses generated from our morphological and molecular phylogenetic data, and most discordance concerned the previously described species *P. minasense anolisi* and *P. tropiduri caribbense*. The phylogenetic analyses indicated that the morphology attributed to *P. minasense anolisi* on Hispaniola is contained wholly within the variation in *P. floridense*, and we consider the former subspecies to be a junior synonym of the latter species on Hispaniola. Outside of Hispaniola, *P. minasense anolisi* has been reported in *Anolis* at two localities in Panama (including its type locality) where *P. floridense* also occurs, with other subspecies of *P. minasense* occurring throughout Central America and northern South America in other host genera (Telford, 1979; Telford et al., 1989; Telford, 2009). Further study is needed to determine whether *P. floridense* and *P. minasense* are synonymous across their range. Similarly, the molecular phylogenetic data suggest that the distinguishing character reported for *P. tropiduri caribbense* on Hispaniola – a schizont with a cytoplasmic projection – is not useful, as this morphology was observed in both *P. floridense* and *P. hispaniolae*. Like *P. minasense anolisi*, *P. tropiduri caribbense* was described as one of many subspecies of a form originally described from Panama, although the cytoplasmic feature was described as unique to *P. tropiduri*

caribbense. We consider *P. tropiduri caribbense*, originally described from Hispaniola, to be a junior synonym of both *P. floridense* and *P. hispaniolae*. Further work is required to assess the validity of *P. tropiduri* across its range, above all because *P. floridense* and *P. tropiduri* are morphologically similar, and their assignment as separate species stems merely from their distinct geographic distributions (Garnham, 1966).

It is certainly possible that despite our broad geographic and host sampling, we did not fully sample the malaria parasite diversity of Hispaniola. Prevalence of *Plasmodium* species is variable between years in other populations of lizards (Schall & Marghoob, 1995; Staats and Schall, 1996; Schall et al., 2000) and birds (Bensch et al., 2007). Few data exist in regards to how this variability might be affected by the relatively high host and parasite diversity on Hispaniola; in some cases of fluctuating prevalence in more than one parasite species, the relative proportions of each species changes (Schall et al., 2000), whereas as in other cases it remains constant (Bensch et al., 2007). It is conceivable that *P. minasense anolisi* and *P. tropiduri caribbense* are valid species and have gone extinct on Hispaniola or that they have become so rare that we did not observe them. Nonetheless, neither low (i.e., unobserved) prevalence nor extinction of these parasites on Hispaniola would reject the hypotheses we presented for *P. floridense* and *P. hispaniolae.* The morphological variation attributable to *P. floridense* and *P. hispaniolae* has been expanded here to include that described for *P. minasense anolisi* and *P. tropiduri caribbense*, and if these taxa are indeed valid and extant on Hispaniola, a molecular-based approach will be required to confirm this.

Prevalence and Host Records

The prevalence of *Plasmodium* infection, among all anole species sampled as well as in the most commonly infected host *A. cybotes* was low (8.1% and 17%, respectively) compared to other studies of *Plasmodium* in Caribbean *Anolis* (e.g., 22 – 32% in *Anolis gundlachi* on Puerto Rico (Schall et al., 2000) and 47% in *Anolis sabanus* on Saba (Staats & Schall, 1996)). Although we consider it unlikely, it is possible that this difference is attributed to our microscopy protocol. We scanned each slide for 3 – 6 minutes, whereas each of the above studies scanned each slide for six minutes. Very light infections with 0.01-0.02% of host cells infected might not have been be detected by our protocol, but could possibly have been observed with longer scanning times. In their study of parasite prevalence in *A. sabanus*, Staats and Schall (1996) measured an average parasitemia of single infections for *P. azurophilum*, *P. floridense*, and *P. leucocytica* at 0.8%, and 0.4% for mixed infections. Thus, our protocol may be expected to yield false negatives only when parasitemia falls below 2.5% of the average parasitemia rates found in that system for single infections, and below 5% for mixed infections. This suggests that the effect of our microscopy protocol on our prevalence estimates is unlikely to account for the observed differences from other studies in Caribbean *Anolis* lizards.

Studies of mixed species infections of malaria parasites in birds show that PCR can preferentially amplify one species over others (Valkiûnas et al., 2006), which is another potential source error in our prevalence data. We detected four mixed infections using sequence data. Two of these samples were infected with an unambiguous species – *P. leucocytica* – but we observed this species in only one of two blood films. This suggests that in some cases, PCR does identify mixed species infections when

microscopy does not, though amplifications that are not confirmed with microscopy could be derived from circulating sporozoites, which are not true infections (Valkiūnas et al., 2009). Additionally, the total scanning time was ultimately longer than 3 – 6 minutes for many smears that we initially identified as infected. When we characterized the morphology of *P. hispaniolae* (after both the initial scans and phylogenic analyses), we reexamined all the blood films belonging to *P. floridense* and *P. hispaniolae*. We did not observe any other co-infections in those smears, further suggesting that we did not underestimate the number of mixed infections.

Another possible explanation for the low prevalence that we observed is our sampling scheme. We sampled several new localities – sometimes very heavily – where *Plasmodium* spp. were absent or present in very low numbers (e.g., 1/41 or 2.4% in Las Galeras, and 7/287 or 2.4% in La Cienaga). Other localities had relatively high prevalence (e.g., 13/28 or 46% in Pedro Sanchez). The large differences in prevalence among these localities suggest that the distributions of these parasites are patchy, which is consistent with other malaria parasite systems (Garnham, 1963; Greiner et al., 1975; Schall & Marghoob, 1995; Staats & Schall, 1996; Fallon et al., 2005).

Several anole species were consistently and conspicuously uninfected, and this could be because of host lineage effects (i.e., a genetically based resistance to malaria has evolved in some clades) or because of host ecology (e.g., the lizards perch in areas that are inaccessible to the parasite's vector). Oftentimes the uninfected *Anolis* species belonged to the same "ecomorph" category. Most Caribbean *Anolis* may be categorized into one of six ecomorph classes on the basis of their ecology, morphology, and behavior. Among islands, ecomorph classes do not form monophyletic groups, but

within islands, members of a particular ecomorph class sometimes do form a clade (reviewed in Losos, 2009). In our Hispaniolan sample, none of the species belonging to the crown-giant (n = 11) or grass-bush (n = 123) ecomorphs were infected, despite the presence of other infected anoles at the same localities. For example, eight uninfected crown-giant anoles (*Anolis baleatus*) were collected at Matadero Village, whereas three of the four other *Anolis* individuals from other species and ecomorphs at the same site were infected (we confirmed this by exhaustively examining the blood smears and with PCR). All the crown-giant anoles on Hispaniola form a clade, however, so we cannot distinguish between common ancestry versus host ecology as potential causes for the apparent absence of parasite infection (of course, additional sampling of Hispaniolan giant anoles will be required to test whether this is simply due to sampling artifact). Members of the grass-bush ecomorph on Hispaniola fall into two separate clades, and though we did not observe malaria parasites in the clade containing *Anolis olssoni* and *Anolis semilineatus* (n = 110), or the clade containing *Anolis bahorucoensis*, *Anolis dolichocephalus*, and *Anolis hendersoni* (n = 13), these lizards were largely sampled from localities with low overall prevalence of *Plasmodium* species. These data are insufficient to resolve whether the observed absence of infection in Hispaniolan grass-bush anoles is due to host ecology or to insufficient sampling. Moreover, malaria parasites have been reported in grass-bush anoles on other islands, for example *P. floridense* in *Anolis pulchellus* of Puerto Rico (Telford, 1975). Other studies have found generally poor evidence of cospeciation between malaria parasites and their vertebrate hosts (e.g., Bensch et al., 2000; Charleston & Perkins, 2003; Ricklefs et al., 2004), suggesting that there is minimal correlation between vertebrate host lineages and

parasitism among the malaria parasites. In any case, our data suggest patterns of malaria parasitism in Caribbean *Anolis* may be partially attributed to host ecology or correlated with host phylogeny, two possibilities that deserve further study.

Many of the host/parasite associations observed were new, largely because this was the first time that most of these host taxa had been sampled for malaria parasites. Some new associations were not unexpected, as infection had been previously reported in their ecomorph category and/or in a closely related species. For example, *A. marcanoi* is not surprising as a new host record for *P. floridense*; it is closely related to *A. cybotes*, and both are members of the trunk-ground ecomorph. Likewise, the trunk-crown ecomorph *A. chlorocyanus*, although exhibiting low prevalence (1/19 or 5%), is a new host record for *P. floridense*; this parasite had previously been reported in the closely related trunk-crown species *A. coelestinus* (Telford, 2009). In contrast, other novel associations were unexpected. Some new records (e.g., *A. christophei* for *P. azurophilum* and *P. floridense*, and *A. etheridgei* for *P. azurophilum* and *P. hispaniolae*) belong to lizards that are typically considered "unique" anoles, meaning that these species do not fit into any of the six traditional Greater Antillean ecomorph categories (Losos, 2009). These are the first records of any *Plasmodium* infections for any unique anoles. Perhaps most interesting is the presence of *P. floridense* in the twig anole *A. insolitus*. These diminutive lizards perch cryptically on twigs and thin branches, and can be found from just above the forest floor to the uppermost regions of the canopy. This is the first record of malaria parasite infection for any of 15 twig anole species found in the Caribbean.

Molecules and morphology in the malaria parasites

A number of the malaria parasites have a rich history of scientific research, and we have a good understanding of the morphological variation in both their vector and vertebrate hosts for many of these (Garnham, 1966; Valkiūnas, 2005; Telford, 2008). Molecular studies have corroborated the taxonomic hypothesis for several of these species (e.g., Hellgren et al., 2007; Palinauskas et al., 2007; Perkins et al., 2007). Many other *Plasmodium* spp., particularly those parasitizing reptiles, were described using only morphological features of the forms found in the circulating blood of the vertebrate host. Some, like *P. fairchildi hispaniolae*, are known only from a single blood film (e.g., Ball & Pringle, 1965; Telford & Landau, 1987), with a few species descriptions even originating from mixed infections on a single blood film (e.g., Telford, 1988).

For the taxonomist, these rare observations are problematic because accurate descriptions and identifications are confounded by a scarcity of informative characters. This should not be unexpected for malaria parasites, considering that these parasites are single-celled organisms, smaller in size than their hosts' erythrocytes, and that morphological observations are made using smeared – and potentially distorted – blood films. Morphological traits observed over a few blood smears may not always be translated into a truly diagnostic species description, and mixed infections can be misleading. The morphological description presented here for *P. hispaniolae*, for instance, does not clearly distinguish it from other *Plasmodium* species. It includes the modifiers "usually" and "sometimes," and the measurements overlap with the syntopic parasite *P. floridense*. These nebulous descriptions are the norm, unfortunately, and are impossible to avoid in organisms for which observable morphological features are very

few. We identify variation unique to *P. hispaniolae* in our taxonomic summary, but some infections remain in morphological crypsis. For example, the gametocytes in Figure 2.3, I-J fall within the variation reported for both *P. hispaniolae* and *P. floridense*.

An approach that incorporates molecular characters, like the one employed here, represents an improvement for species delimitation and identification in the malaria parasites. Placing molecular data in a phylogenetic framework allows taxonomic inference based on estimates of the evolutionary history of the organisms. Fixed differences – in the form of molecular characters – can be included in the species description (but see Fujita and Leachè (2011) for a discussion on why fixed characters might not be necessary for species descriptions). While a phylogeny from any single locus represents but one hypothetical estimate of the history of divergence for a group of organisms, the confidence that may be placed in such an estimate increases to the extent that it is corroborated by independent estimates from additional loci. To summarize, incorporating molecular data in a phylogenetic framework facilitates unambiguous species descriptions based on the evolutionary history of these parasites.

Nonetheless, we strongly advocate the continued collection of morphological and other types of data in these organisms. Vector information may be particularly valuable, as vector identities and vector switches have been associated with malaria parasite diversification (Martinsen et al., 2008). Taxonomic hypotheses based on parasite morphology may be tested with molecular data, and we suspect these will be corroborated in many cases. When not taxonomically informative, these data can be used to test other hypotheses, such as those related to convergence (e.g., Pérez-Losada et al., 2009) and phenotypic plasticity (e.g., Steinauer et al., 2007). Perhaps most importantly,

the description of morphological data in molecular taxonomic studies allows such studies to be smoothly integrated into more than 100 years of taxonomic research on saurian malaria parasites that was based primarily on morphology.

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Table 2.1. Locality information for sites where *Plasmodium* spp. were observed. Numbers correspond with areas in Figure 2.2 and prevalence data in Tables 2.2 and 2.3. Exact GPS coordinates are given in decimal degrees and are associated with nearby place names (e.g., town, province, country) provided for geographical reference; these names are shared when more than one site is nearest the same place.

Number	Name	Latitude	Longitude	Dates
1	Duchity, Sud, Haiti	18.36653°N	73.87885°W	Aug. 2009
2	Deloge, Artibonite, Haiti	18.96772°N	72.72537°W	Aug. 2009
3	Marotte, Oest, Haiti	18.82718°N	72.57038°W	Aug. 2009
4	Aubry, Oest, Haiti	18.72392°N	72.37355°W	Aug. 2009
5	San Juan, San Juan, Dominican Republic	18.77687°N	71.19923°W	Aug. 2008
6	La Cienaga, Barahona, Dominican Republic	18.05758°N	71.11297°W	Jan. 2010
7	Luperon, Puerto Plata, Dominican Republic	19.86266°N	70.96433°W	Aug. 2008
8a	Constanza, La Vega, Dominican Republic	18.91442°N	70.72942°W	Aug. 2008
8b	Constanza, La Vega, Dominican Republic	18.84137°N	70.70745°W	Aug. 2008
9a	Constanza, La Vega, Dominican Republic	18.86048°N	70.68315°W	Aug. 2008
9b	Constanza, La Vega, Dominican Republic	18.83973°N	70.69693°W	Aug. 2008
10	Jarabacoa, La Vega, Dominican Republic	19.14088°N	70.63128°W	Aug. 2008
11	Sabana Quéliz, La Vega, Dominican Republic	18.69602°N	70.59167°W	Aug. 2008
12	La Palma, La Vega, Dominican Republic	19.03310°N	70.54298°W	Aug. 2008
13	Matadero Village, Peravia Province, Dominican Republic	18.38963°N	70.43035°W	Aug. 2008
14	Recodo Road, Azua, Dominican Republic	18.38190°N	70.32977°W	Aug. 2008
15	Las Galeras, Samaná, Dominican Republic	19.30035°N	69.17233°W	Aug. 2008
16	Pedro Sanchez, El Seibo, Dominican Republic	18.87967°N	69.11958°W	Aug. 2008
17	Bayahibe, La Altagracia, Dominican Republic	18.37087°N	68.83145°W	Aug. 2008

Table 2.2. Observed prevalence of *Plasmodium* spp. in *Anolis* lizards sampled on Hispaniola. The total number of host individuals sampled is noted parenthetically after the host name, and the number of these individuals that were infected by each parasite species is listed in each column.

	Parasite species			
Host species	*Plasmodium azurophilum*	*Plasmodium floridense*	*Plasmodium hispaniolae* comb. nov.	*Plasmodium leucocytica*
Anolis aliniger (1)	0	0	0	0
Anolis armouri (4)	0	0	0	0
Anolis bahorucoensis (8)	0	0	0	0
Anolis baleatus (8)	0	0	0	0
Anolis barahonae (2)	0	0	0	0
Anolis caudalis (15)	0	1[a]	0	0
Anolis chlorocyanus (19)	0	1[a]	0	0
Anolis christophei (16)	1[a]	1[a]	0	0
Anolis coelestinus (93)	0	1	0	0
Anolis cybotes (220)	8	25	4[a]	5
Anolis distichus (130)	2	2	5	0
Anolis dolichocephalus (1)	0	0	0	0
Anolis etheridgei (17)	1[a]	0	1[a]	0
Anolis fowleri (1)	0	0	0	0
Anolis hendersoni (4)	0	0	0	0
Anolis insolitus (8)	0	1[a]	0	0
Anolis marcanoi (4)	0	1[a]	0	0
Anolis marron (1)	0	0	0	0
Anolis olsoni (95)	0	0	0	0
Anolis ricordi (1)	0	0	0	0
Anolis rimarum (2)	0	0	0	0
Anolis shrevei (2)	0	0	0	0
Anolis semilineatus (15)	0	0	0	0
Anolis sheplani (5)	0	0	0	0
Anolis singularus (1)	0	0	0	0
Anolis websteri (4)	0	0	0	0
Total (677)	12	33	10	5

[a] New parasite/host association.

Table 2.3. Relative prevalence among localities where *Plasmodium* spp. were observed. Locality numbers refer to areas and localities in Figure 2.2 and Table 2.1, respectively. The number of individuals sampled is given in parentheses after the host names.

Table 2.3.

Locality	Host Species	*Plasmodium* species			
		Plasmodium azurophilum	*Plasmodium floridense*	*Plasmodium hispaniolae* comb. nov.	*Plasmodium leucocytica*
1	*Anolis cybotes* (1)	0	0	0	100%
2	*Anolis chlorocyanus* (1)	0	0	0	0
	Anolis cybotes (2)	0	50%	0	0
	Anolis websteri (4)	0	0	0	0
3	*Anolis caudalis* (5)	0	0	0	0
	Anolis chlorocyanus (2)	0	50%	0	0
	Anolis cybotes (2)	0	0	0	0
	Anolis olssoni (1)	0	0	0	0
4	*Anolis caudalis* (6)	0	17%	0	0
	Anolis chlorocyanus (2)	0	0	0	0
	Anoli cybotes (3)	0	67%	0	0
	Anoli olssoni (1)	0	0	0	0
5	*Anolis chlorocyanus* (1)	0	0	0	0
	Anolis cybotes (7)	0	86%	0	0
	Anolis distichus (12)	0	0	0	0
6	*Anolis coelestinus* (93)	0	1%	0	0
	Anolis cybotes[a] (99)	5%	1%	0	1%
	Anolis olssoni (91)	0	0	0	0
7	*Anolis cybotes* (1)	0	100%	0	0
	Anolis distichus (5)	0	20%	0	0
8a	*Anolis cybotes* (10)	30%	10%	0	10%
	Anolis distichus (8)	0	0	0	0
8b	*Anolis cybotes* (1)	0	0	0	0
	Anolis distichus (8)	13%	0	0	0
	Anolis etheridgei (4)	0	0	25%	0
9a	*Anolis etheridgei* (4)	25%	0	0	0
	Anolis insolitus (2)	0	0	0	0
9b	*Anolis aliniger* (1)	0	0	0	0
	Anolis christophei (1)	0	100%	0	0
	Anolis fowleri (1)	0	0	0	0
10	*Anolis chlorocyanus (2)*	0	0	0	0
	Anolis cybotes (3)	0	33%	0	0
	Anolis distichus (4)	0	0	25%	0
11	*Anolis insolitus* (1)	0	100%	0	0
12	*Anolis christophei* (5)	20%	0	0	0
	Anolis etheridgei (1)	0	0	0	0
13	*Anolis baleatus* (8)	0	0	0	0
	Anolis cybotes[a] (2)	0	50%	50%	50%
	Anolis distichus (2)	0	0	50%	0
14	*Anolis cybotes* (2)	0	0	0	50%
	Anolis distichus (1)	0	0	0	0
	Anolis marcanoi (4)	0	25%	0	0
15	*Anolis cybotes* (22)	0	5%	0	0
	Anolis distichus (15)	0	0	0	0
	Anolis chlorocyanus (4)	0	0	0	0
16	*Anolis chlorocyanus* (1)	0	0	0	0
	Anolis cybotes[a] (12)	0	67%	25%	0
	Anolis distichus (15)	7%	0	20%	0
17	*Anolis chlorocyanus* (1)	0	0	0	0
	Anolis cybotes (3)	0	33%	0	0
	Anolis distichus (8)	0	13%	0	0

[a] One or more of these host individuals was infected with more than one parasite species.

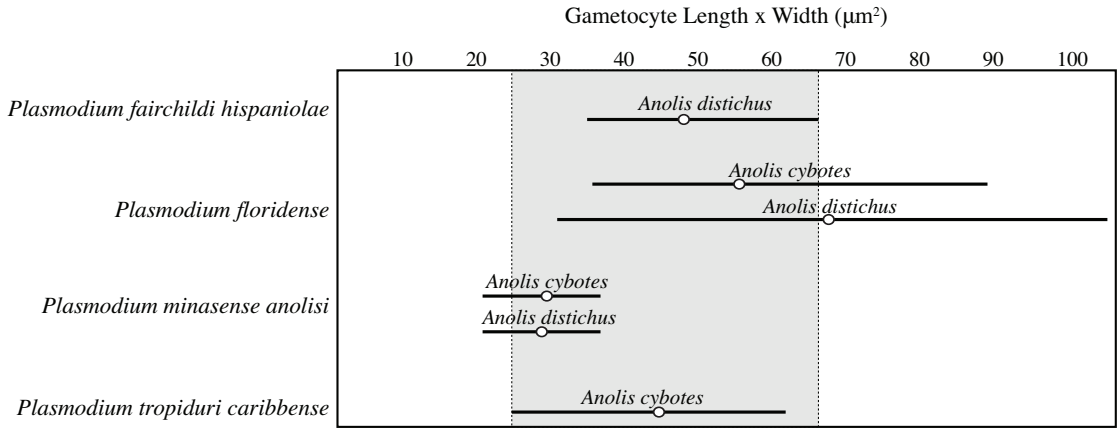

Figure 2.1. Previously reported gametocyte size variation among the four pigmented *Plasmodium* species (according to Telford et al., 1989) in the Hispaniolan anoles *Anolis cybotes* and *Anolis distichus*. Bars represent total size variation, with the mean represented by a hollow circle. Grey shading indicates where two or more parasite species overlap in gametocyte length x width, precluding unambiguous identification of these species by this metric over much of its observed range.

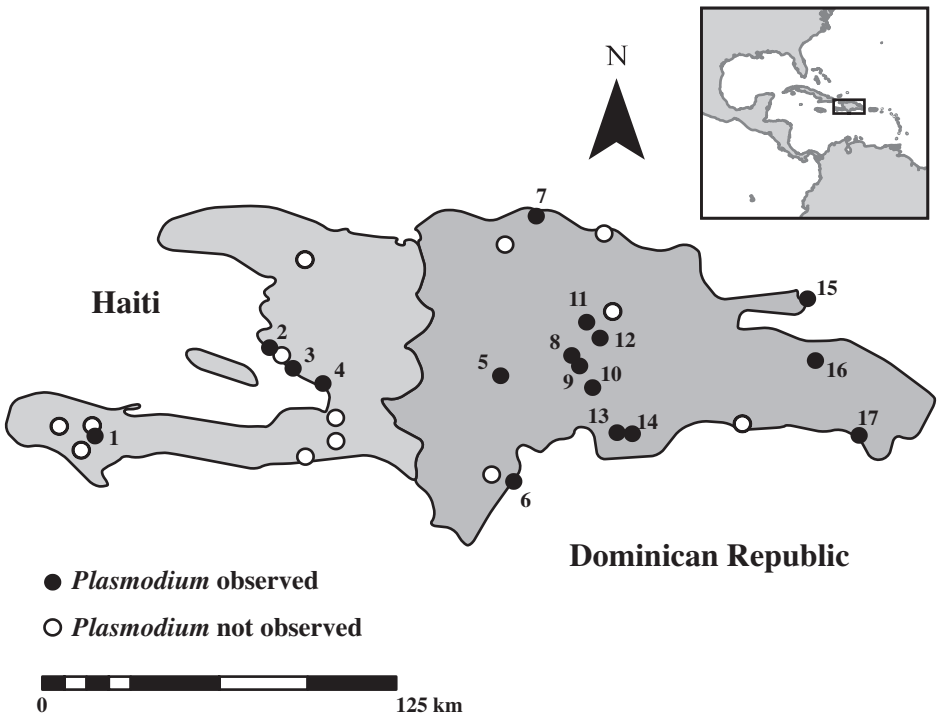

Figure 2.2. Sampling sites on Hispaniola. Localities where *Plasmodium* spp. were observed have numbers corresponding to GPS coordinates given in Table 2.1.

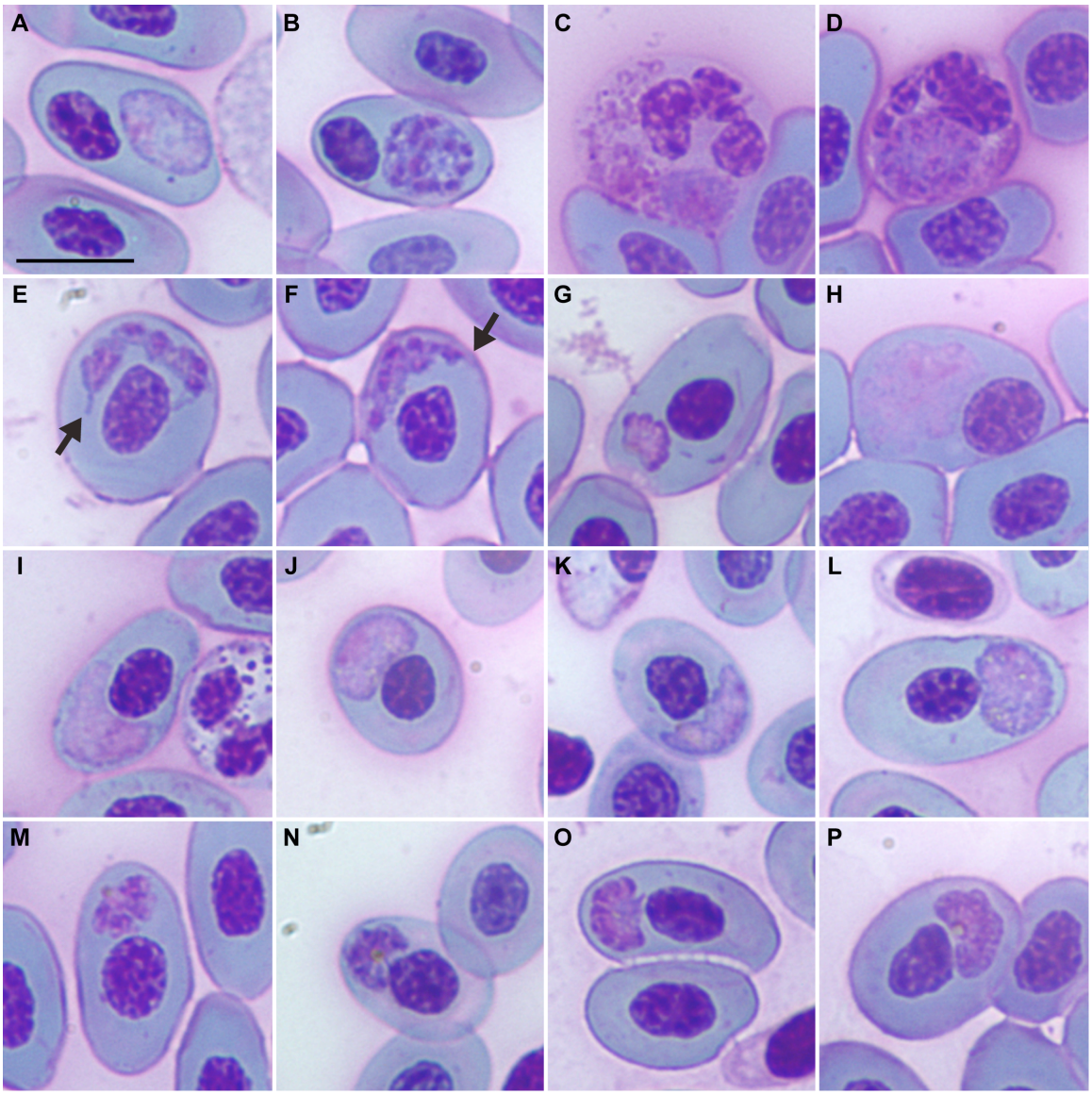

Figure 2.3. Morphological variation previously described for lizard malaria parasites from Hispaniola. Images A-H show parasites with features that allow confident identification (see text for explanation). These are: *Plasmodium azurophilum* gametocyte (A) and schizont (B), *Plasmodium leucocytica* gametocyte (C) and schizont (D), *Plasmodium tropiduri caribbense* schizonts (arrow indicates cytoplasmic projection; E-F), *Plasmodium minasense anolisi* schizont (G) and *Plasmodium floridense* gametocyte (H). Images I-P show parasites that cannot be confidently assigned to any species using previously reported morphological variation. These gametocytes (I-L) and schizonts (M-P) could belong to any of the pigmented species (i.e., *Plasmodium fairchildi hispaniolae*, *P. floridense*, *P. minasense anolisi*, or *P. tropiduri caribbense*). Molecular analyses (see text and Figures 2.4-6) identified all forms as either *P. floridense* (F-J, M-N) or *Plasmodium hispaniolae* comb. nov. (K-L, O-P; "*Plasmodium* sp." in Figures 2.4-6). Scale bar = 10 μm.

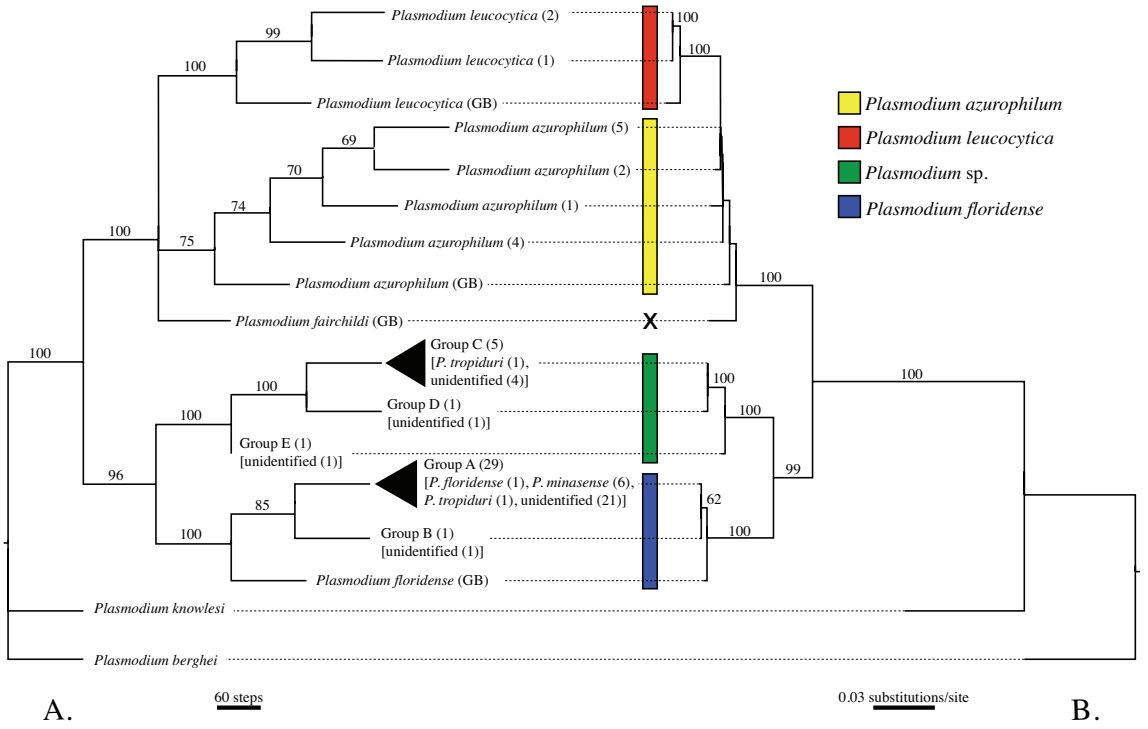

Figure 2.4. Phylogenetic hypotheses of Hispaniolan *Plasmodium* spp. inferred by the analysis of concatenated mitochondrial genes *cytB* and *coxI*. Tree tip labels reflect identifications made on the basis of previously described morphological features, or, where indicated, pre-existing GenBank accession identifications (GB). For haplotypes for which we were unable to identify species using morphological means, tree tips are given provisional labels indicating shared hapotype (e.g., "Group A"). When morphological identifications were made to some members one of these haplotype groups, these are indicated in brackets. The number of individuals that share a haplotype is noted parenthetically after the species or group name. Vertical bars show the species hypotheses inferred from this study (*Plasmodium* sp. is identified and described as *Plasmodium hispaniolae* comb. nov.; see text). (A). Strict consensus of 2 most parsimonious trees with a length of 598 steps and with 288 parsimony-informative characters. (B). ML tree (lnL = -5268.9) generated under a GTR model and partitioned by gene.

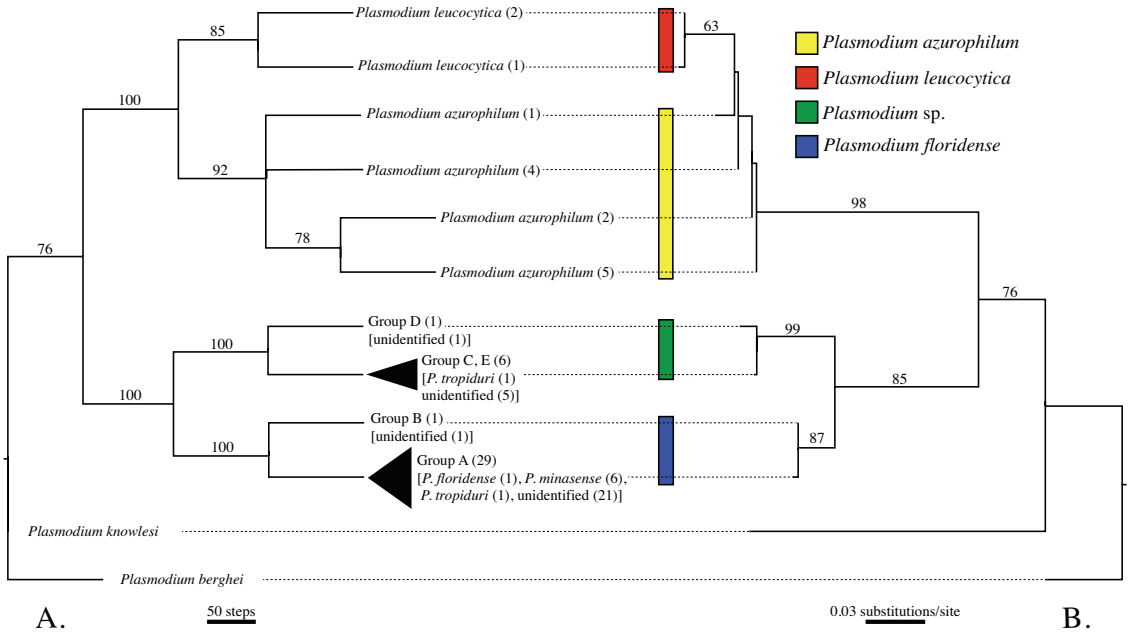

Figure 2.5. Phylogenetic hypotheses of the nuclear gene *EF2*. Tree tip labels and vertical bars representing taxonomic hypotheses correspond to those assigned in the mitochondrial DNA analysis (Figure 2.4). Numbers above branches indicate bootstrap support. (A). Strict consensus of 3 most parsimonious trees from a maximum parsimony analysis with a length of 152 steps and with 72 parsimony-informative characters. (B). ML tree (lnL = -1306.9) generated under a GTR + Γ model.

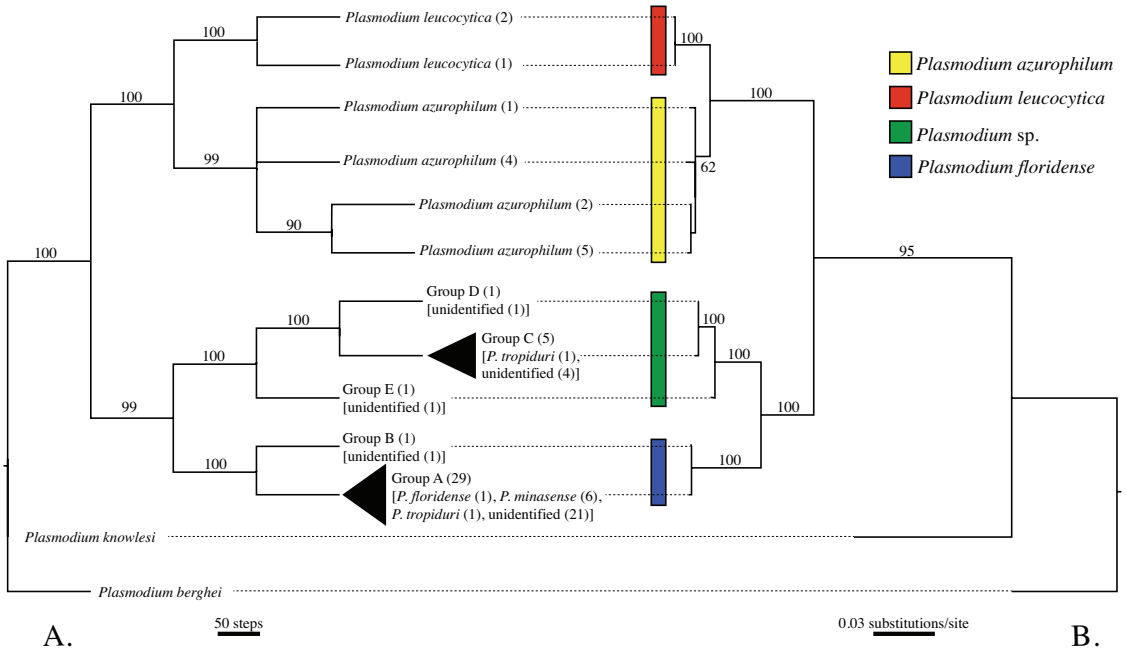

Figure 2.6. Phylogenetic hypotheses of the concatenated *cytB*, *coxI*, and *EF2* genes. Tree tip labels and vertical bars representing taxonomic hypotheses correspond to those assigned in the mitochondrial DNA analysis (Figure 2.4). Numbers above branches indicate bootstrap support. (A). Strict consensus of 3 most parsimonious trees from a maximum parsimony analysis with a length of 724 steps and with 359 parsimony-informative characters. (B). ML tree (lnL = -6453.7) partitioned by gene and generated under a GTR+ Γ model.

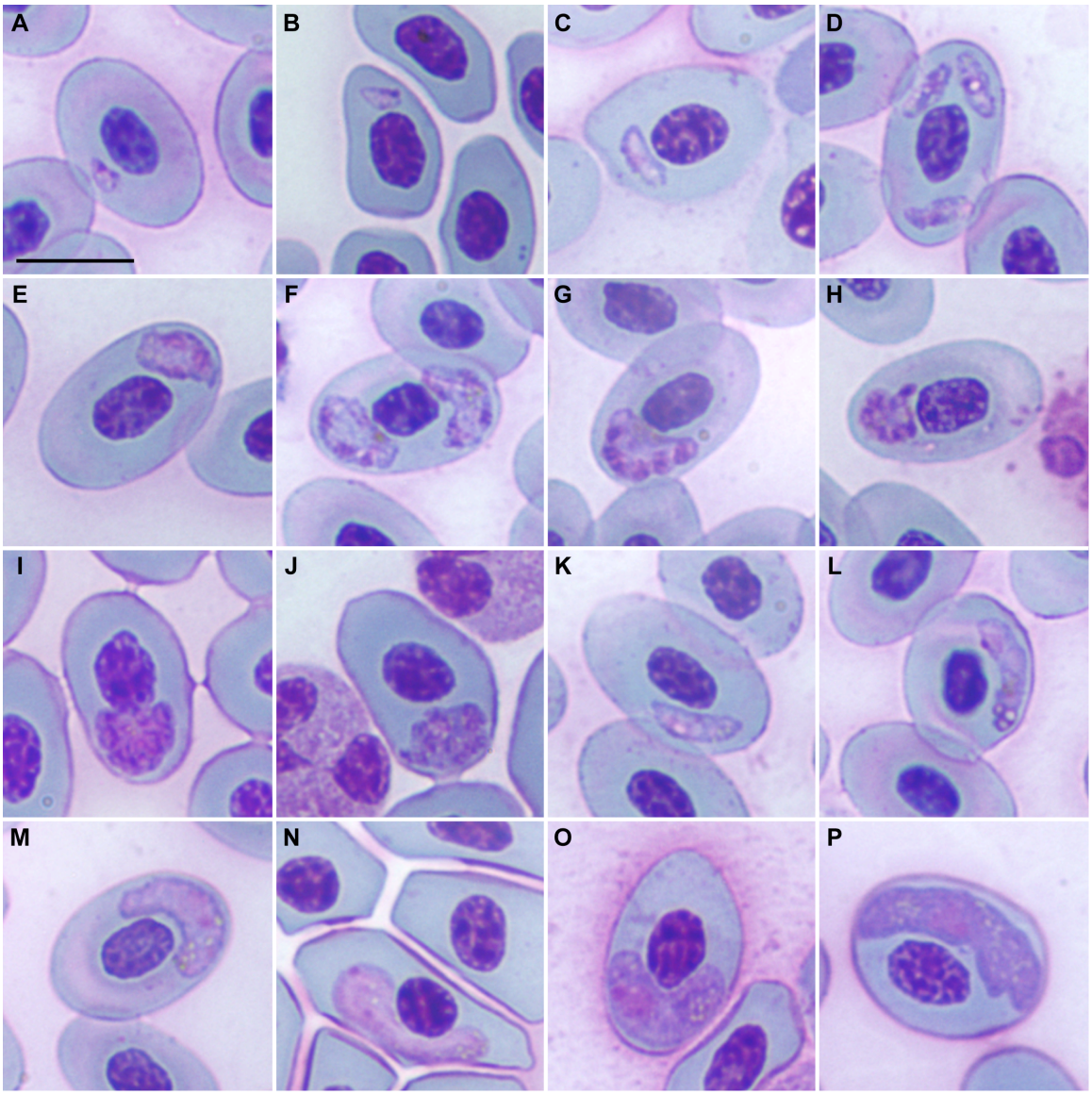

Figure 2.7. Morphology of *Plasmodium hispaniolae* comb. nov. Variation in trophozoites (A-D), schizonts (E-J), immature gametocytes (K-L), microgametocytes (M-O), and macrogametocytes (O-P) of *Plasmodium hispaniolae* comb. nov. Scale bar = 10 μm.

CHAPTER III

LIFE CYCLE AND TRANSMISSION SHAPE DIVERSIFICATION IN THE LIZARD MALARIA PARASITE *PLASMODIUM FLORIDENSE*

ABSTRACT

Malaria parasites in the genus *Plasmodium* all share the same life cycle wherein they alternate between invertebrate and vertebrate hosts. This life cycle, in combination with potentially common patterns of moderate-to-low prevalence, may shape the parasite populations through inbreeding. Inbreeding brings about a decrease in the effective population size (N_e), which in turn causes rapid divergence among populations and minimal within-population variation. We test these predictions in the lizard malaria parasite *Plasmodium floridense*. This is among the most widespread of the lizard malaria parasites, ranging from southeastern North America, throughout the Caribbean, and in parts of mainland Middle America. We collected, identified, and sequenced 63 single-infection samples from across the parasite's range for two mitochondrial, one apicoplast, and five nuclear genes. We employed Bayesian species delimitation to identify 11 independently evolving lineages within *P. floridense*. As predicted, both N_e and within-lineage variation are low, and the majority of polymorphisms are fixed between lineages. We observed very recent divergence estimates; some lineage pairs are estimated to have diverged ~110,000 years ago. These results are consistent with the predictions given the parasite life cycle and transmission patterns, and we suggest that these patterns may be common to malaria parasites generally.

Introduction

The first malaria parasite in the genus *Plasmodium* was described in 1885, and the genus has grown to contain nearly 200 species at present (Levine 1988; Valkiūnas 2004; Telford 2008). These parasites can be found on every continent except Antarctica, and in hundreds – possibly thousands – of reptile, bird, and mammal host species. The phylogenetic relationships are reasonably well characterized for many of these parasite species (Perkins & Schall 2002; Martinsen *et al*. 2008), but the patterns of diversification within species remain unknown for most.

Plasmodium falciparum – the causative agent of malignant malaria – is the exception. It has been the focus of numerous population genetics studies from which we can make several generalizations. Many mitochondrial and nuclear loci contain minimal variation that result in estimates of small population size (N_e) and recent diversification (Rich & Ayala 2000; Volkman *et al*. 2001; Joy *et al*. 2003; Hartl 2004). Reproduction is clonal (i.e., highly inbred) under conditions of low-moderate transmission (Razakandrainibe *et al*. 2005; Nkhoma *et al*. 2013), although this is not always the case (Mzilahowa *et al*. 2007; Pumpaibool *et al*. 2009). Similarly, genetic variation is limited in the other human parasites *P. malariae* and *P. vivax* (Lecerc *et al*. 2004; Tazi & Ayala 2011; Neafsey *et al*. 2012). Nonetheless, it is unclear whether these patterns (e.g., low variation and recent divergence) uniquely result from the exceptional population history of their vertebrate host and associated selection regimes, or whether they are common to all malaria parasites.

All *Plasmodium* species share a life cycle that may engender common patterns of within-species diversification. The life cycle can be divided in two ways that are largely

congruent: it can be split by host class or by ploidy. The invertebrate host (i.e., vector), which is often a mosquito but is always a dipteran, takes a blood meal from a vertebrate host, and injects haploid sporozoites that make their way into the new host's bloodstream. The parasite undergoes asexual reproduction first in the host's organs (e.g., the liver) and later in circulating blood cells, and eventually produces haploid gametocytes. A vector takes a blood meal from the vertebrate host and ingests blood cells containing the gametocytes. These blood cells burst in the vector's gut, releasing parasite gametes, which exflagellate and fuse to form a diploid zygote. The zygote develops into an ookinete – this is when meiosis and recombination occurs – and this in turn develops into an oocyst in the gut wall. These diploid oocysts produce haploid sporozoites that invade to the vector's salivary glands, and, if the vector survives, are ready infect another vertebrate host.

This life cycle may have the capacity to profoundly affect parasite population genetics: when parasite prevalence is moderate or low, selfing rates will be high. This is because as prevalence decreases, the proportion of host individuals infected with more than one clone nears zero, as does the probability of a vector taking an infected blood meal from more than one host. The N_e of diploid loci is reduced by half in populations that are 100% inbred, reducing variation within populations while increasing variation among populations (Hartl & Clark 2007; Wakeley 2009). It also reduces the incidence of incomplete lineage sorting (Funk & Omland 2003), leading to greater congruence between mitochondrial and nuclear gene trees. Inbreeding also brings about observable changes in homozygosity. While this cannot be measured from the haploid samples collected from the vertebrate hosts, allelic diversity among parasites in the same host

population will be low. So, given the malaria parasite life cycle, we predict that all malaria populations subsisting at moderate or low prevalence will exhibit the same characteristics as the primate parasites: low N_e, low variation within populations but high variation among populations, and recent divergence among populations.

There is some evidence that the populations of non-primate parasites are shaped by their life cycle. Bensch *et al*. (2004) sequenced a multitude of avian parasite samples at both the mitochondrial gene *cytb* and the nuclear gene *DHFR-TS*, and found that most mitochondrial lineages also had unique nuclear sequences. Similarly, Falk *et al*. (2011) observed strict congruence between the mitochondrial genes *cytb* and *coxI* with the nuclear gene *EF2* in their phylogeny containing multiple parasite species. Both of these studies meet the expectations of rapid coalescent times of nuclear loci and complete lineage sorting. Nonetheless, the scope of both of these studies extended across several parasite species – rather than within species – and each included just two independent loci.

In order to better understand the population genetics of malaria parasites in wildlife, we studied the population genetics of the lizard parasite *Plasmodium floridense* Thompson & Huff, 1944. *Plasmodium floridense* is among the most widely distributed of the lizard malaria parasites. It is reported from southeastern North America, from the Caribbean throughout the Greater Antilles and parts of the northern Lesser Antilles, and from parts of mainland Middle America from Panama northwards to Mexico (Figure 3.1; Telford, 2008). The mosquito *Culex erraticus* (Diptera: Culicidae) is a competent vector of *P. floridense* in Florida (Klein *et al*. 1987, 1988). This mosquito is distributed throughout the Americas (Mendenhall *et al*. 2012), and though its distribution exceeds

the distribution of *P. floridense*, it is unknown whether the parasite retains this same vector throughout its range. The vertebrate hosts of *P. floridense* are primarily anole lizards; of its 34 reported host species, 31 are *Anolis* spp. (Squamata: Dactyloidae) whereas three are *Sceloporus* spp. (Squamata: Phrynosomatidae) (Telford 2008; Falk *et al*. 2011).

Generation time in *P. floridense* depends on the average time spent in each its vertebrate and invertebrate hosts. In the mosquito, the time from blood meal to sporogony is determined by the infection intensity (i.e., how many parasites it consumes with its blood meal). If the blood meal is heavily infected, sporogony occurrs 11-14 days later (Klein et al. 1987). If the infection is light, sporogony may take longer than 20 days, if it happens at all (Klein et al. 1987). The time spent in the vertebrate host is also variable. In experimental infections of *Anolis carolinensis*, gametocytes were present on blood films as early as two weeks following inoculation, and parasitmemia peaked in 4-6 weeks following inoculation (Klein *et al*. 1987). Each of these benchmarks was prolonged when the animals experienced cooler temperatures (Thompson & Winder 1947; Klein *et al*. 1987). Still, it is unknown for how long lizards maintain their infections in the wild, or whether the infections are fatal. Infected lizards do not typically live longer than 1-3 months in the laboratory (Thompson & Huff 1944; Thompson & Winder 1947; Klein *et al*. 1987), but this may be an artifact of frequent blood sampling and/or poor husbandry. Wild-caught *Anolis* lizards with natural infections typically exhibit low parasitemia of primarily gametocytes (Falk, pers. obs.), suggesting long-term infections. And, there are no observable differences in body condition, tail breakage, and male-male competitive success between infected and uninfected wild-caught *Anolis* spp.

(Schall & Pearson 2000; Schall & Staats 2002), providing further evidence that virulence of *P. floridense* is low many of its hosts, and that lizards may maintain low-level infections for a long time under natural conditions.

Average prevalence of *P. floridense* in its lizard hosts is generally low, and varies over space and time. In a survey of Puerto Rico, for example, *P. floridense* was observed in El Yunque National Forest, but was absent from eight other localities throughout the island (Guerrero & Pickering 1984). Within El Yunque, prevalence in the most-commonly infected host *Anolis gundlachi* ranged 10-30% over a nine-year period (Schall *et al*. 2000). Similarly, 92 of 554 (17%) *Anolis sagrei* from 28 localities in Florida were infected, but 89 of these infections were reported from 270 lizards at two localities (33%), with prevalence at 1% for the remaining 284 lizards from 26 localities (Perkins *et al*. 2007). In a recent sample of 677 anoles from 19 localities on Hispaniola, just 4.8% were infected with *P. floridense*, with the parasite absent or in very low numbers in many host populations (Falk *et al*. 2011). These rates contrast with those observed on Saba in the Lesser Antilles; approximately 21% of *Anolis sabanus* were infected with *P. floridense* (Staats & Schall 1996a). Observed prevalence of *P. floridense* in subsequent years has dropped to ~5%, however (Falk & Perkins unpublished). Overall, these patterns are consistent with the conditions that may favor inbreeding in *P. floridense*.

Our aim was to answer two general questions relating the effect of the malaria parasite life cycle and transmission to diversification in *P. floridense*. First, what is the lineage diversity in *P. floridense*? Second, do the lineages exhibit population genetics parameters consistent with the life cycle / transmission predictions? Specifically, do these populations exhibit low N_e, low within-lineage variation, and recent divergence? In

order to answer these questions, we collected samples of *P. floridense* from across its range, developed several new nuclear markers, sequenced our samples using these markers, and analyzed these data using a variety of population genetic and phylogeographic methods.

Methods

Parasite Sampling, Identification, and Sequencing

We collected lizards by noose or hand along roads and trails in Cuba (July-August, 2002), Florida (March, 2002; December, 2002; April, 2006), Hispaniola (June, 2006), Jamaica (May, 2012), the Puerto Rican Bank (Puerto Rico and the Virgin Islands: August, 2011; October, 2011), Saba (May, 2005; May, 2009), and Las Tuxtlas in Mexico (January, 2011). From each of these lizards (except those collected in Cuba – see below), we clipped the distal portion of one toe to obtain blood samples. One drop was used to make a thin blood smear, and these were fixed in absolute methanol immediately after drying. We applied 3-6 additional drops to Whatman filter paper for molecular analysis. Once dry, each paper was individually placed in a coin envelope and these were stored together in a sealed plastic bag along with silica beads. The blood-dot papers were kept at room temperature for up to four weeks while in the field and at -20°C thereafter. In preparation for microscopic analysis, blood smears were fixed in methanol a second time, stained with phosphate-buffered Geimsa stain for 50-60 minutes, rinsed with tap water, and let air-dry. We scanned each smear under oil immersion at 1000x magnification for 3-6 minutes to identify positive infections.

We extracted DNA of each positive sample from a single, dried blood dot that was cut from the filter paper. We used Qiagen DNeasy Animal Tissue Extract kits

(Valencia, CA, USA), following the manufacturer's instructions except with two modifications: (1) we did not transfer the piece of cut filter paper to the spin column after the digestion step, and (2) we used two elutions of 50µl each (as opposed to 200µl each) in the final step so that the parasite DNA would not be too diluted. We had liver tissues of 42 lizards from Cuba, and because we did not have blood smears to identify positive samples, we extracted DNA from all of these using the Qiagen kits – this time following the manufacture's instructions – and screened them for infections using PCR.

Plasmodium floridense and *P. hispaniolae* are co-occurring, morphologically cryptic species that cannot be distinguished using blood smears alone (Falk *et al*. 2011). We used a phylogenetic approach to confirm the identity of the positive infections detected through microscopy and to identify *P. floridense* infections in our Cuban samples. We sequenced the mitochondrial gene cytochrome b (*cytb*) because this is the most commonly sequenced locus in studies of haemosporidian parasites in wildlife (Escalante *et al*. 1998; Bensch *et al*. 2000; Perkins & Schall 2002; Ricklefs & Fallon 2002; Valkiūnas *et al*. 2010), and because it allows discrimination between *P. floridense* and *P. hispaniolae*. We amplified this gene (along with a small portion of the gene cytochrome C oxidase subunit I [*coxI*]) in two reactions using the primers DW2 / 3932R and 3932F / DW4 (Perkins & Austin 2009; primer information in Supplementary Table S3.1,) and Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Pittsburg, PA, USA). We cleaned PCR products with AMPure (Agencourt, Beverly, MA, USA), sequenced them in both directions using BigDye v.3.0 (Applied Biosystems, Foster City, CA, USA), and edited them in GENEIOUS v.5.4.6 (Biomatters, Auckland, New Zealand). We combined these with sequences of previously identified samples of *P.*

floridense and *P. hispaniolae*, along with the lizard parasites *P. azurophilum*, *P. fairchildi*, *P. leucocytica*, and *P. mexicanum*, and the mammal parasites *P. berghei* and *P. knowlesi* as outgroup taxa (GenBank accession numbers in Supplementary Table S3.2). Multiple sequence alignments were generated using the MUSCLE plugin (Edgar 2004) in GENEIOUS using default parameters. Mixed infections of more than one parasite species (i.e., those with clean chromatograms except with double peaks at segregating sites between species) were identified and discarded from this and subsequent analyses. We estimated a phylogeny using Bayesian inference in MrBayes v.3.2.1 (Ronquist *et al*. 2012). We ran two analyses simultaneously for 10 million generations, sampling every 1000 generations, and discarding the first 25% as burn-in. In each analysis we used three hot chains, one cold chain, and employed a GTR + Γ substitution model. We assessed performance and convergence of the MCMC chains by checking that the average standard deviation of split frequencies was < 0.01 and that the effective samples sizes (ESS) were > 200 in TRACER v1.5 (Rambaut & Drummond 2007). We also assessed convergence in AWTY (Nylander *et al*. 2008), paying attention to the "split frequency of run 1 vs. run 2" and the "cumulative split frequency." We used this phylogeny, along with the molecular characters provided in the species description of *P. hispaniolae* (Falk *et al*. 2011), to distinguish *P. floridense* from *P. hispaniolae*.

We sequenced all positive, single-infection samples of *P. floridense*, including four Hispaniolan samples that we previously identified and included in the above analysis as GenBank data (Falk *et al*. 2011), at an additional seven loci using new primers that we designed for this study. We also sequenced one *P. hispaniolae* sample at these loci for use as an outgroup taxon. These additional loci consisted of one mitochondrial gene:

cytochrome C oxidase subunit I (*coxI*); one apicoplast gene: caseinolytic protease C (*clpC*); and five nuclear genes: adenylosuccinate lyase (*Adsl*), alpha-tubulin I (*Atub*), elongation factor 2 (*EF2*), histone H3 (*HisH3*), and heat shock protein (*HSP70*). We employed nested PCR for all of these loci, using degenerate outer primers and specific internal primers to avoid amplifying non-target DNA and also to increase the amount of starting template. We attempted PCR using the internal, specific primers with the DNA extractions as template (instead of the PCR product resulting from the outer primers), but were unsuccessful for all except *coxI* (where greater success was observed using the nested protocol), suggesting that the quantity of starting non-mitochondrial parasite DNA in these extractions is too low (i.e., parasitemia in these lizards is too low). Primer information and thermocycler protocols are given in Supplementary Table S3.1, and sequencing methods were the same as those for *cytb*.

We evaluated each locus for evidence of selection, evidence of clock-like evolution, and established the best-fit model of sequence evolution for use in downstream analyses. We tested for evidence selection using both the McDonald-Kreitman test (McDonald & Kreitman 1991) and Tajima's D (Tajima 1989) in DnaSP v5.10.01 (Librado & Rozas 2009). The McDonald-Kreitman test compares the proportion of synonymous to non-synonymous fixed substitutions, relative to the number of polymorphic sites, between species (i.e., *P. floridense* and *P. hispaniolae*). A significant difference in the fixation rate between synonymous and non-synonymous substitutions is interpreted as evidence of selection. Tajima's D infers deviations from neutrality under a standard coalescent model by comparing the estimate of the population mutation rate (θ) generated using the number of segregating sites versus the estimate generated using the

average number of pairwise mismatches. These deviations result from relatively high or low numbers of polymorphisms, and are interpreted to be the result of demographic changes and/or selection pressures. We estimated Tajima's D at each locus using all *P. floridense* samples. We tested for non-clocklike evolution of each locus of our ingroup taxon using likelihood ratio tests in MEGA v.5.05 (Tamura *et al*. 2011). Substitution models were chosen for each locus using Bayesian information criterion (BIC) scores in jModelTest v0.1.1 (Posada 2008).

Lineage Identification

We employed a three-step procedure to delimit lineages within *P. floridense* that centers on the species-delimitation program BPP (Rannala & Yang 2003; Yang & Rannala 2010). BPP employs a reversible-jump MCMC to estimate the probability of alternative species delimitation models, conditioned on the probabilities of population size and time since divergence among species in the various possible delimitations. It accommodates the species phylogeny and incomplete lineage sorting via a coalescent model. The BPP algorithm samples a user-defined, strictly-bifurcating guide tree of putative lineages, so we first identified putative lineages and then inferred the relationships among those lineages. Following that, we used BPP to infer which of the lineages are independently evolving.

We initially inferred putative lineages using Discriminant Analysis of Principal Components (DAPC; Jombart *et al*. 2010). DAPC is a multivariate analysis that uses sequential *K*-means clustering of principal components to identify groups of individuals, and then employs discriminant analysis to maximize variation between groups. DAPC was run using the ADEGENET package (Jombart 2008) in R (R Development Core

Team 2010). We first extracted SNP data from the concatenated multiple sequence alignments of *P. floridense* samples. We attempted to choose the optimal cluster number (K) of this dataset using each of the five available criteria (e.g., "diffNgroup" and "goodfit") and BIC scores in ADEGENET. The optimal K was variable over several runs with every criterion, however, with ≥ 10 clusters identified in every run. For example, the "goodfit" criterion was the most stable, but inferred 11-18 clusters over 10 runs. Six clusters were reliably inferred over all analyses that generally correspond to samples collected in each area, so we chose to infer additional clusters within those six clusters using an interactive approach. We first divided the dataset into the six sets. Next, we grouped the samples from each of these sets into 1-4 clusters, used discriminant analysis of just one principal component (to avoid over-fitting), and chose the greatest cluster number that maximized the membership of each sample to just a single cluster.

We also identified putative lineages using *cytb* sequences sensu Bensch *et al*. (2004). We trimmed the *cytb* alignment to be homologous with the region that would have amplified with the avian malaria primers HAEMF and HAEMR2 (Bensch *et al*. 2000). This resulted in 459-bp fragments, which is slightly shorter than the 479-bp fragment that these primers amplify because we extracted 20-bp of our primer sequences from the samples. We assigned samples to lineages based on shared haplotype identities at this locus.

We employed a species tree approach in *BEAST v1.6.2 (Drummond & Rambaut 2007; Heled & Drummond 2010) to infer the relationships among putative lineages to use as a guide tree in the BPP analyses. *BEAST is a Bayesian MCMC method that uses a multi-locus coalescent model to estimate the species tree, with a species defined as an

interbreeding, metapopulation lineage (i.e., the general lineage concept of species, or the GLC; de Queiroz 1998; 2007). We chose substitution models based on the results of JModelTest. We used a relaxed clock with a lognormal prior for *Adsl* (Drummond *et al*. 2006) and a strict clock for all other loci (based on the tests for clock-like evolution – see Results). We chose a birth-death tree prior and a piecewise-linear-and-constant-root population size prior. We ran the analysis several times and adjusted the prior distributions for several parameters until we observed appropriate sampling for all parameters (i.e., unimodal distributions that pull away from the prior). The final analysis was set to run for 4.0×10^8 generations, sampled every 3.0×10^4 generations, with the first 10% discarded as burn-in. We assessed convergence in TRACER, checking that the ESS values for every parameter were ≥ 200. Anticipating the assumptions of the BPP analyses, we made a final modification once the *BEAST analysis finished. The BPP algorithm can collapse and resolve previously collapsed nodes on the guide tree, but it cannot move branches on the tree or incorporate phylogenetic uncertainty into its estimations. Accordingly, we took a conservative approach and collapsed any terminal nodes on the species tree with < 95% posterior probability. We gave each putative lineage an arbitrary name according to where the samples were collected (e.g., "Hispaniola 1").

We estimated the probability that putative lineages are reproductively isolated using BPP v2.1. We employed algorithm 0 with multiple values of the fine-tuning parameter ε (5, 10, 20) to ensure adequate performance of the rjMCMC (following Burbrink *et al*. 2011). Prior distributions for ancestral population mutation rate (θ) and root age (τ_0) may affect the posterior probabilities of the species models (Yang &

Rannala 2010). Consequently, and similar to Leaché & Fujita (2010), we used three different – but still diffuse – prior combinations to evaluate the sensitivity of our dataset to these priors. These priors are assigned a gamma (Γ) distribution (α, β), with a mean $m = \alpha/\beta$ and standard deviation $s = (\alpha/\beta^2)^{1/2}$. In order to inform our prior combinations, we estimated θ of each putative lineage and all lineages together using the per-site number of segregating sites (i.e., Watterson's estimator of θ; Watterson 1975) from the concatenated dataset and using DnaSP. The mean estimates ranged $1.3\times10^{-4} – 7.8\times10^{-3}$. The first prior combination assumes small ancestral population sizes and shallow divergences between species: $\theta \sim \Gamma(1, 5000)$ and $\tau_0 \sim \Gamma(1, 5000)$; $m = s = 2.0\times10^{-4}$ for both θ and τ_0. The second prior combination assumes much larger population sizes and deep divergences between species: $\theta \sim \Gamma(1, 100)$ and $\tau_0 \sim \Gamma(1, 100)$; $m = s = 0.01$ for both θ and τ_0. The third prior combination assumes large population sizes and shallow divergences between species: $\theta \sim \Gamma(1, 100)$ and $\tau_0 \sim \Gamma(1, 5000)$; $m = s = 0.01$ for θ; $m = \mathrm{s} = 2.0\times10^{-4}$ for τ_0. This latter prior combination of large populations and shallow divergences is conservative in that it is biased towards lumping species together (Leaché & Fujita 2010). We parameterized our model to accommodate rate variation among loci, imposing a Dirichlet prior distribution with vector $\alpha = 2$. We also used $\alpha = 10$, which corresponds to greater variation among loci, over all prior combinations for $\varepsilon = 10$ to check the sensitivity of our dataset to this prior. Each analysis was run for 150,000 generations and sampled every three generations, with the first 10% discarded as burn-in. We adjusted the step proposals of the fine-tune parameters and allowed the program to automatically adjust these during burn-in, and these were satisfactory for all analyses, remaining in the interval (0.2, 0.6). Each analysis was run twice with different starting seeds to confirm

consistency among runs, and the effective sample size (ESS) values of all parameters in all runs were ≥ 200. We considered lineages to be independently evolving when the posterior probabilities were ≥ 95% in all nine parameterizations (three values of ε under three prior combinations).

Lineage Characterization – Diversity, Dating, and Demography

We estimated several population genetics parameters of the BPP-identified lineages to assess whether these were consistent with our predictions from the parasite life cycle and transmission dynamics. For each lineage separately and combined, we used DnaSP to measure the nucleotide diversity (π), haplotype diversity (H_d), and the number of haplotypes in each locus (N_H).

We employed a species-tree approach in *BEAST to infer an ultrametric, dated phylogeny of the BPP-identified lineages in order to test the prediction that independently evolving lineages have recently diverged. We incorporated a molecular clock rate for malaria parasites as estimated by Ricklefs & Outlaw (2010). They compared a phylogeny of malaria parasites to a phylogeny of their avian hosts, and used the proportional differences between host sister taxa and parasite sister taxa, conditioned on the relative age of host-switching events and the molecular clock rate of birds, to estimate a mean parasite per-lineage *cytb* rate of 0.6% per million years. This clock-rate estimate places the most recent common ancestor of the human parasite *P. falciparum* with its sister taxon *P. reichenowi* at 2.49 million years ago (Mya) [95% CI: 1.93-3.79 Mya] (Ricklefs & Outlaw 2010), which is roughly congruous with an estimate using host-fossil and biogeographic calibrations (2.96 Mya [95% CI: 1.75-4.71] or 3.42 Mya [95% CI: 2.25-4.67], depending on methodology; Pacheco *et al*. 2011). We used this *cytb* rate, but

otherwise this analysis employed the same parameterizations and priors as in the abovementioned guide-tree inference. The ESS values for every parameter were ≥ 200.

We compared the extent of divergence among the most recently diverged lineage pairs. We used DnaSP to measure the number of polymorphic sites, the number of fixed sites, and the average number of nucleotide substitutions per site (D_{xy}). We employed an analysis of molecular variance (AMOVA) to estimate Φ_{st} among these closely related lineage pairs in ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010). Φ_{st} is an F_{st} analog, and measures the extent of variation that is partitioned among versus within populations (Excoffier *et al*. 1992). We utilized FaBox v.1.35 (Villesen 2007) to convert our files into the ARLEQUIN format.

We estimated N_e of each lineage using the species tree inferred in *BEAST and the Python script "starbeast_demog_log" available in the package BIOPY v.0.1.7 (Heled 2011) in Python v2.7 (http://www.python.org). This extracts from the phylogeny both ancestral and descendant N_e estimates for each branch, allowing for a crude assessment of both current and historical demography while also incorporating uncertainty from the phylogeny. Estimates are scaled to generation time, and we transformed these into N_e values (i.e., the number of "breeders") by assuming generation times of each 3- and 12-months, which we believe represent the lower and upper range, respectively, for generation times in *P. floridense*. Thus, for each extant lineage we obtained two alternative estimates – each using two different generation times – for both current and ancestral N_e. ESS values for N_e estimates of all extant lineages were ≥ 200.

Results

Parasite Sampling, Identification, and Sequencing

We initially identified 64 blood smears with parasite infections characterized by morphology consistent with *P. floridense*, *P. hispaniolae*, or other members of the *Lacertamoeba* subgenus. We used PCR to confirm that 62 of these were infected with a single parasite species. Another seven of the Cuban samples that we screened using PCR had single-species infections, resulting in a total of 69. We sequenced a 1189-bp fragment containing part of *coxI* and all of *cytb* for all of these.

We inferred a phylogeny using these data and several GenBank sequences to identify the 69 samples (Figure 3.2; Supplementary Table S3.3). All the samples are contained in one of three major clades. Both *P. floridense* GenBank sequences are monophyletic with 59 samples, and we identified these as *P. floridense*. Both *P. hispaniolae* GenBank sequences are monophyletic with nine samples. These nine samples – all collected on the Puerto Rican Bank – also share two of the three fixed nucleotide characters in *cytb* reported in Falk *et al*. (2001) for Hispaniolan *P. hispaniolae* infections: "C" at positions 121 and 510, but contained a "G" instead of a "T" at position 123, (where the position refers to the annotated *cytb* region of *P. falciparum* on GenBank [NC_022375]). We identified these nine samples as *P. hispaniolae*. The single sample from Mexico is monophyletic with *P. fairchildi*, and these belong to a larger clade that also contains *P. azurophilum* and *P. leucocytica*. We identified this mainland sample as *P. fairchildi*.

We successfully sequenced all eight genes for the 63 *P. floridense* samples – including the 59 newly sequenced samples and the four GenBank samples we previously

collected on Hispaniola – and one *P. hispaniolae* sample. All combined, this resulted in 4202-bp of data, and the alignments contained no missing data or gaps. We could not reject neutral evolution for any locus using the McDonald-Kreitman test or Tajima's D, and a molecular clock was not rejected for any locus except *Adsl*. Sequence lengths and best-fit substitution models are shown in Table 3.1.

Lineage Identification

We identified almost twice as many putative lineages using DAPC than *cytb* haplotypes. We inferred a total of 17 genetic clusters using DAPC (Figure 3.3A), and recovered nine unique haplotypes of 459-bp *cytb* (Figure 3.3B). In four instances the DAPC and *cytb* haplotype cluster inferences are congruent, and the remaining 13 DAPC clusters are contained within five *cytb* haplotypes.

We inferred a guide tree of 15 putative lineages using the DAPC results and *BEAST (Figure 3.3C). There were four DAPC-inferred lineages on the Puerto Rican Bank, and the relationships among three of these – corresponding to samples collected on Puerto Rico, St. Thomas / St. John, and Virgin Gorda – are unresolved. We collapsed these nodes for the guide tree. Except for two nodes near the base of the tree, all the remaining nodes are well supported with ≥ 95% posterior probability.

Of the 15 putative lineages, 11 were recovered as being reproductively isolated in each of the nine BPP analysis run under three different prior combinations and three different values for fine-tune parameter ε (Figure 3.4). In all analyses, "Hispaniola 2" and "Hispaniola 3" are collapsed into a single lineage, as are "Cuba/Florida 2" and "Florida 2." In six of the nine analyses, "Cuba 1" and "Cuba 2" are collapsed into a single lineage, as are "Jamaica 2" and "Jamaica 3." These results were consistent

between the two independent runs, and were unchanged in analyses using $\alpha = 10$ instead of $\alpha = 2$ for the Dirichlet prior on locus rate variation.

Lineage Characterization – Diversity, Dating, and Demography

Genetic variation within the BPP-inferred lineages is low. The number of haplotypes, haplotype diversity, and nucleotide diversity at each locus for each lineage are summarized in Table 3.2. We observed just 30 unique 4202-bp sequences among the 63 samples, and many lineages contained just one haplotype at each locus (e.g., all lineages except "Puerto Rican Bank 2" possess just one haplotype at *clpC*). Likewise, where a lineage contains more than one haplotype per locus, nucleotide diversity is very low.

Estimates of *Ne* are low for all lineages (Table 3.3). These range ~13,000-47,000 for extant populations (assuming a 1-year generation time; estimates that assume a 3-month generation time are 4x larger). For every lineage except "Jamaica 2,3" and "Saba," estimates for the extant population are higher than ancestral populations, suggesting a general pattern of population growth, although in every case the mean estimates for the extant populations are contained in the 95% CI for the ancestral populations.

We observed recent divergence between lineages using the species tree approach and the molecular clock rate in *BEAST (Figure 3.5). The crown age of our samples is estimated at 0.89 Mya [95% CI: 0.58-1.2 Mya]. Divergence dates and summary statistics of the four most recently diverged population pairs are shown in Table 3.4. These pairs diverged ~0.11-0.27 Mya [95% CI: 0.038-0.41 Mya], and differ by just ~0.2-1%. But, the majority of polymorphic sites are fixed between populations, and the Φ_{st} estimates

indicate that most variation is between – rather within – lineages. These are significant for all except those on the Puerto Rican Bank (P=0.0567), which may be because one of these lineages is comprised of a single sample.

Discussion

We hypothesized that populations of malaria parasites are shaped by both transmission rates and their life cycle. We predicted that lineages would be characterized by small N_e estimates, that most variation would be between lineages, and that lineages would achieve reproductive isolation over short timescales. We tested these predictions in the lizard parasite *P. floridense* using a multi-locus dataset of samples collected from throughout the parasite's range. We identified 11 evolutionary independent lineages with characteristics that are consistent with our predictions.

Understanding diversification in malaria parasites

The patterns of diversification among *P. floridense* lineages are remarkably similar to those observed in the human parasites: low N_e, minimal variation, and recent divergence. We contend that these shared patterns are a result of a common life cycle, particularly since the parasites have little else in common, and that research on human and wildlife hosts may be reciprocally informative. Information from human parasites can be used to guide studies of wildlife parasites, as we did here. Likewise, and remarkably, information gleaned from studies of malaria parasites in wildlife may be transferrable to the human parasites, and these may serve as models for the study of human disease.

We assert that untangling the factors that contribute to differences in parasite prevalence is fundamental to understanding malaria parasite diversification, because

prevalence rates determine transmission rates, which in turn may determine the extent of inbreeding within populations. Malaria parasite prevalence varies between species, and over time and space within species (Staats & Schall 1996b; Schall *et al*. 2000; Perkins *et al*. 2009). Already, we have information on some of the factors that contributed to these differences. For example, year-round transmission, as opposed to seasonal transmission, is associated with higher prevalence rates among avian parasites (Pérez-Tris & Bensch 2005). Similarly, generalist parasites reach higher prevalence rates in their avian hosts than parasites specializing on fewer species (Hellgren *et al*. 2009). Also, transmission success may depend on differences in virulence among clones in multi-clonal infections (Mackinnon & Read 1999), and sex ratio dynamics in monoclonal versus multi-clonal infections (Schall 2000). Still, many questions remain, including, for example, how differences between the vector's feeding preferences and the parasite's vertebrate host specificity affect prevalence.

We did not directly measure transmission rates in *P. floridense*, but our data are consistent with low transmission among hosts. None of our samples are mixed with more than one *P. floridense* lineage (although two samples were mixed with *P. floridense* and another species). But, this pattern may be artifactual if the single-infection sequences we observed are the result of PCR bias (Valkiūnas *et al*. 2006). We think this is unlikely, however, because: 1) each of the nine different PCRs was clean for every sample; and 2) most lineages share the same haplotype at each locus. Thus, we believe the apparent absence of multi-clonal infections to be real, and that there is limited opportunity for outcrossing between lineages.

Spatial and temporal diversification in **Plasmodium floridense**

Generally speaking, the distribution of lineages makes geographic sense, and suggests that allopatry is important in diversification of *P. floridense*. With two exceptions, all lineages contain samples collected from the same island or area. Additionally, the samples in "Puerto Rican Bank 2*"* form a polytomy of three clusters that were identified in DAPC, and correspond to three distinct haplotypes contained in the lineage. Each of these haplotypes is geographically associated with Puerto Rico, St. Thomas / St. John, or Virgin Gorda. These islands formed one continuous landmass during the glacial maxima of the Pleistocene, and rising sea levels contributed to the separation of the Virgin Islands from each other and from Puerto Rico most recently ~7000 years ago (Pregill & Olson 1991). Thus, the *P. floridense* populations on these islands may be at an early stage of speciation in response to recent allopatry. Another interesting pattern emerged on Jamaica. The lineage "Jamaica 1" is distributed in the extreme eastern end of Jamaica, and is non-overlapping with "Jamaica 2,3", which is distributed throughout the remainder of the island. This biogeographic boundary coincides with a pattern observed in birds (e.g., *Trochilis polytmus* and *Trochilis scitulus*) and reptiles (e.g., *Anolis grahami aquarum* and *Anolis grahami grahami*), and is hypothesized to be the effect of the northern Rio Grande Valley and the southern Morant River Valley acting in concert as a barrier to gene dispersal (Gill *et al*. 1973; Schwartz & Henderson 1991; McCormack *et al*. 2012). Barriers to dispersal for some lineages are less clear, for example those on Hispaniola. Many Hispaniolan taxa are distributed on either side of Mertens' Line (Schwartz & Henderson 1991; Glor & Warren 2010), but we

did not find evidence for this – or any other pattern – in the Hispaniolan *P. floridense* lineages. Incidentally, the divergence dates between these lineages are among the oldest of the within-island pairs (Table 3.4), making it possible that any causal evidence has been obscured by time. Biogeographic patterns at a larger scale (e.g., colonization to/from the mainland) remain unclear until more data are available.

We inferred very recent divergence dates among lineages, with those on Jamaica and Cuba having diverged ~0.11 Mya. These estimates are much more recent than the most recent divergence dates reported for the *Anolis* host spp. (e.g., *Anolis desechensis* ~1.3 Mya, Brandley & de Queiroz 2004; *Anolis fuscoauratus* ~3 Mya, Glor *et al*. 2002), and are more similar to the rapid divergence times observed in selfing organisms (e.g., 0.010-0.065 Mya in the plant *Clarkia xantiana*; Pettengill & Moeller 2012). While there are numerous assumptions in the clock rate and unincorporated uncertainty in our estimates (Pulquerio *et al*. 2007), we contend that diversification events in *P. floridense* were almost certainly "recent" for two reasons. First, the 0.6% per-lineage-per-million-years rate is less than the ~1.0% *cytb* rate commonly employed for vertebrates (Bromham 2002; Weir & Schluter 2008) and the 1.0-1.2% rate employed for invertebrates (Bower 1994). And, it is less than or similar to the 0.5-1.0% rate previously used for the parasite's *Anolis* lizard hosts (Glor *et al*. 2002; Thorpe *et al*. 2005). This is contrary to the assertion that parasites evolve faster than their hosts, owing to the parasites' faster generation times (Hafner *et al*. 1994). Rates that are more similar to or faster than these other rates would make the diversification date estimates in *P. floridense* more recent. Second, observed substitution rates often increase among closely related species because

of the coalescent process (Hickerson *et al*. 2003), providing further evidence that our estimates may be biased to be older than they actually are.

There are two possible biogeographic calibrations that we could have used to calibrate our phylogeny, but these were not useful. First, Jamaica may have completely submerged in the Eocene and re-emerged in the late Miocene (Graham 2003), providing a maximum calibration of 10 million years for Jamaican endemics (Burbrink *et al*. 2012). Alternatively, the Blue Mountain region of Jamaica may have been continuously emergent for the last 33-35 million years (Iturralde-Vinent & MacPhee 1999; Iturralde-Vinent 2006). Even if we ignore the Blue Mountain possibility, placing a 10 million-year maximum calibration on our Jamaican lineages provides a lower bound on the per-lineage *cytb* rate of ~ 0.0066%, which is too low to be informative. A second possible calibration is for the Lesser Antillean island of Saba. The age of oldest rocks on Saba are estimated to be ~ 0.4 million years old (Defant *et al*. 2001), suggesting that all extant fauna arrived after a volcanic eruption at that time. Unfortunately, we cannot confidently place this calibration anywhere in our phylogeny. All five Saba samples are genetically identical, and parent node is the hypothetical ancestor of every sample in our study except those from Hispaniola. It is a very strong assumption that this hypothetical ancestor existed on Saba, so we do not place our calibration on that node. Generally speaking, biogeographic calibrations such as these on Saba and Jamaica are problematic because both the timing and comprehensiveness of geologic events are largely equivocal, and they assume that the taxon did not survive on a nearby island and subsequently go extinct (Heads 2011). Again, since we are only interested in inferring whether divergence

events in *P. floridense* are recent, and not associating them with any particular causal event, we believe the clock rate to be sufficient.

Independently evolving lineages

We inferred 11 independently evolving lineages (i.e., species) contained within *P. floridense*. We are not able to formally revise *P. floridense* at this time, however, because we do not have morphological specimens for the samples collected on Cuba, and do not expect the availability of such material in the near future. The International Code of Zoological Nomenclature requires a designated type for each nominal taxon (Article 16.4; ICZN 1999), making invalid any descriptions of the two Cuban species without types. An alternative is to leave the Cuban samples in *P. floridense*, along with the Florida samples to which the name belongs, while also naming the remaining lineages as species. This would render *P. floridense* polyphyletic, however. For now, *P. floridense* remains a complex of several species.

The only Middle American sample in our study was one that we collected from a population where *P. floridense* was previously reported as the only malaria parasite species (21-41% prevalence in *Anolis* sp.; Lowichik *et al*. 1988). We identified the parasite as *P. fairchildi*, however. *Plasmodium hispaniolae* was originally described as a subspecies of *P. fairchildi*, and all of these species – *P. fairchildi*, *P. floridense*, and *P. hispaniolae* – belong to the subgenus *Lacertamoeba*. Members of this group are characterized as being average in size and average in shape (Telford 1988), making species delimitation and identification using morphological criteria particularly difficult (Rand *et al*. 1983). The systematics of the Middle American lizard malaria parasites will remain equivocal until assessed with molecular data.

We were moderately successful inferring lineages using DAPC, although we were forced to use an interactive, and potentially subjective, approach to determine the optimal cluster number. But, in concert with *BEAST and BPP, we were able to test these lineage hypotheses. Some previous studies of primate malaria parasites have used methods that optimize Hardy-Weinberg Equilibrium (HWE) to infer genetic clusters from samples collected from vertebrate hosts [e.g., STRUCTURE (Pritchard *et al*. 2000); Mu *et al*. 2005; Neafsey *et al*. 2008; Gupta *et al*. 2012]. Nonetheless, HWE is an inappropriate criterion for samples of malaria parasites from their vertebrate hosts. These parasite stages are always haploid at all loci, and any heterozygous samples must be the result of multiple sporozoite inoculations (i.e., a sample from a single host that contains multi-allelic loci is a population that may be of mixed ancestry). DAPC – or other methods that can handle haploid data and do not optimize HWE – are better suited for inferring clusters among samples of malaria parasites collected from their vertebrate hosts.

Bensch *et al*. (2004) showed that samples with unique *cytb* haplotypes are potentially cryptic species, suggesting that current numbers of malaria parasite diversity are gross underestimates. We inferred 11 evolutionarily independent lineages from samples that share nine *cytb* haplotypes in *P. floridense.* (Note that this relationship is not perfectly nested - the samples collected on Cuba belong to lineages that are incongruous with their *cytb* haplotypes.) This suggests that there may be more *Plasmodium* spp. than *Plasmodium cytb* haplotypes, and that the latter approach is a conservative one that may slightly underestimate species diversity. MalAvi is a database of avian malaria parasite *cytb* sequences (Bensch *et al*. 2009), and it currently contains

over 330 *Plasmodium* sequences (http://mbio-serv4.mbioekol.lu.se/avianmalaria/, accessed 11/2/12). Given that the avian parasites are perhaps the best studied malaria parasites in wildlife, and that at present, only about 40 *Plasmodium* species have been described from birds (Valkiūnas 2005), we can surmise that malaria parasite systematists have plenty of work ahead.

We believe that the methods employed here will be useful in malaria parasite systematics. Species delimitation in malaria parasites traditionally employs a three-pronged approach that makes use of differences in morphology, host preference, and geographic range to infer species limits (Garnham 1966). If we consider a species to be an independently evolving, metapopulation lineage (i.e., the GLC), then the traditional criteria may grossly underestimate species diversity. We show that reproductive isolation can evolve in just a short period of time, potentially outpacing morphological change. Moreover, neither host nor geographic information is consistently useful to identify lineages in our study. Approaches that combine molecular and traditional techniques in species delimitation are becoming more common (Perkins *et al*. 2009; Valkiūnas *et al*. 2010; Falk *et al*. 2011). But, notably, molecular characters alone are sufficient to satisfy The Code's requirements of a character-based description (Article 13.1.1), allowing for the description of truly cryptic species. While we advocate the continued use of the traditional criteria, we contend that molecular diagnostics are necessary to effectively describe any new malaria parasite species.

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Table 3.1. Summary information for each locus. The best-fit substitution models were selected using BIC scores, both with and without *P. hispaniolae* as an outgroup taxon.

Locus	Length (bp)	Polymorphic sites	Substitution model (with outgroup)	Substitution model (no outgroup)
Adsl	580	47	GTR + Γ	GTR + Γ
Atub	541	25	HKY + I	HKY
clpC	419	14	HKY + I	HKY + I
coxI	553	19	TrN + I	TrN + I
cytb	1187	32	HKY	HKY
EF2	351	8	F81	HKY
HisH3	304	5	TrN + I	HKY + I
HSP70	267	5	HKY + I	HKY
All	4202	155	n/a	n/a

Table 3.2. Locus-by-locus summary statistics for each lineage. Haplotype and nucleotide diversity is generally low for all lineages, with many sharing just one haplotype per locus. All 63 samples share 30 unique haplotypes, and these contain low nucleotide diversity.

Locus	Lineage	N	N_h	H_d	π	Locus	Lineage	N	N_h	H_d	π
Adsl	All	63	18	0.901	0.02393	*EF2*	All	63	10	0.856	0.00646
	Cuba 1, 2	4	1	0	0		Cuba 1, 2	4	3	0.833	0.00285
	Cuba/Florida 1	3	1	0	0		Cuba/Florida 1	3	1	0	0
	Cuba/Florida 2, Florida 2	6	4	0.867	0.00218		Cuba/Florida 2, Florida 2	6	1	0	0
	Florida 1	1	1	0	0		Florida 1	1	1	0	0
	Hispaniola 1	5	3	0.700	0.00138		Hispaniola 1	5	1	0	0
	Hispaniola 2, 3	8	2	0.250	0.00043		Hispaniola 2, 3	8	2	0.536	0.00153
	Jamaica 1	3	1	0	0		Jamaica 1	3	1	0	0
	Jamaica 2, 3	12	1	0	0		Jamaica 2, 3	12	1	0	0
	Puerto Rican Bank 1	1	1	0	0		Puerto Rican Bank 1	1	1	0	0
	Puerto Rican Bank 2	15	2	0.248	0.00043		Puerto Rican Bank 2	15	1	0	0
	Saba	5	1	0	0		Saba	5	1	0	0
Atub	All	63	13	0.855	0.01165	*HisH3*	All	63	6	0.640	0.00410
	Cuba 1, 2	4	2	0.500	0.00092		Cuba 1, 2	4	2	0.500	0.07031
	Cuba/Florida 1	3	2	0.667	0.00016		Cuba/Florida 1	3	1	0	0
	Cuba/Florida 2, Florida 2	6	3	0.600	0.00123		Cuba/Florida 2, Florida 2	6	2	0.333	0.00219
	Florida 1	1	1	0	0		Florida 1	1	1	0	0
	Hispaniola 1	5	2	0.400	0.00074		Hispaniola 1	5	1	0	0
	Hispaniola 2, 3	8	2	0.250	0.00046		Hispaniola 2, 3	8	1	0	0
	Jamaica 1	3	1	0	0		Jamaica 1	3	1	0	0
	Jamaica 2, 3	12	1	0	0		Jamaica 2, 3	12	1	0	0
	Puerto Rican Bank 1	1	1	0	0		Puerto Rican Bank 1	1	1	0	0
	Puerto Rican Bank 2	15	1	0	0		Puerto Rican Bank 2	15	1	0	0
	Saba	5	1	0	0		Saba	5	1	0	0
clpC	All	63	10	0.874	0.00703	*HSP70*	All	63	8	0.796	0.00089
	Cuba 1, 2	4	1	0	0		Cuba 1, 2	4	2	0.500	0.07031
	Cuba/Florida 1	3	1	0	0		Cuba/Florida 1	3	1	0	0
	Cuba/Florida 2, Florida 2	6	1	0	0		Cuba/Florida 2, Florida 2	6	1	0	0
	Florida 1	1	1	0	0		Florida 1	1	1	0	0
	Hispaniola 1	5	1	0	0		Hispaniola 1	5	1	0	0
	Hispaniola 2, 3	8	1	0	0		Hispaniola 2, 3	8	2	0.250	0.00094
	Jamaica 1	3	1	0	0		Jamaica 1	3	1	0	0
	Jamaica 2, 3	12	1	0	0		Jamaica 2, 3	12	1	0	0
	Puerto Rican Bank 1	1	1	0	0		Puerto Rican Bank 1	1	1	0	0
	Puerto Rican Bank 2	15	2	0.514	0.00245		Puerto Rican Bank 2	15	1	0	0
	Saba	5	1	0	0		Saba	5	1	0	0
mtDNA	All	63	16	0.901	0.00813	Complete	All	63	30	0.958	0.01005
(*coxI* &	Cuba 1, 2	4	1	0	0	dataset	Cuba 1, 2	4	4	1.0	0.00071
cytb)	Cuba/Florida 1	3	2	0.667	0.0038		Cuba/Florida 1	3	2	0.667	0.00016
	Cuba/Florida 2, Florida 2	6	3	0.600	0.00057		Cuba/Florida 2, Florida 2	6	6	1.0	0.00086
	Florida 1	1	1	0	0		Florida 1	1	1	0	0
	Hispaniola 1	5	1	0	0		Hispaniola 1	5	4	0.900	0.00029
	Hispaniola 2, 3	8	1	0	0		Hispaniola 2, 3	8	4	0.786	0.00031
	Jamaica 1	3	1	0	0		Jamaica 1	3	1	0	0
	Jamaica 2, 3	12	3	0.621	0.00040		Jamaica 2, 3	12	3	0.621	0.00017
	Puerto Rican Bank 1	1	1	0	0		Puerto Rican Bank 1	1	1	0	0
	Puerto Rican Bank 2	15	1	0	0		Puerto Rican Bank 2	15	3	0.648	0.00030
	Saba	5	1	0	0		Saba	5	1	0	0

N = number of samples
N_h = number of haplotypes
H_d = haplotype diversity
π = nucleotide diversity, or average number of differences per site

Table 3.3. Estimates of per-lineage N_e in *P. floridense* inferred using the *BEAST species tree and BIOPY. We transformed these estimates using two different generation times, 1 year and 3 months, which we believe are on the opposite ends of the range of possible generation times in *P. floridense*. Both mean estimates and 95% confidence intervals are provided.

Lineage	Population size (generation = 1 year)		Population size (generation = 3 months)	
	Extant	Ancestral	Extant	Ancestral
Cuba 1, 2	40,264 [10,809 – 76885]	23,130 [692 – 51,265]	161,056 [43,236 – 307,540]	92,520 [2528 – 205,060]
Cuba/Florida 1	24,951 [3298 – 51,111]	14,266 [571 – 35,473]	99,804 [13,190 – 204,444]	57,064 [2286 – 141,892]
Cuba/Florida 2, Florida 2	47,435 [17,195 – 84,060]	17,524 [571 – 41,141]	189,740 [68,780 – 336,240]	70,096 [2284 –164,564]
Florida 1	32,923 [3414 –70,483]	24,917 [716 –56,370]	131,692 [13,356 – 251,932]	99,668 [2863 – 225,480]
Hispaniola 1	25,145 [6870 – 47,171]	16,301 [199 – 38,395]	100,580 [27,480 – 188,684]	65,204 [797 – 153,580]
Hispaniola 2, 3	29,088 [7384 – 58,577]	15,161 [772 – 36,395]	116,352 [29,536 – 234,308]	60,644 [3091 – 145,580]
Jamaica 1	22,050 [2343 – 48,756]	15,784 [263 – 38,117]	88,200 [9374 – 195,024]	63,136 [1050 – 152,468]
Jamaica 2, 3	12,974 [118 – 30,744]	18,975 [4029 – 37,767]	51,896 [471 – 122,976]	75,900 [16,117 – 151,068]
Puerto Rican Bank 1	33,280 [4371 – 73,604]	17,650 [311 – 42,456]	133,120 [17,483 – 294,416]	70,600 [1244 – 169,824]
Puerto Rican Bank 2	21,770 [5162 – 42,830]	15,229 [314 – 35,705]	87,080 [20,648 – 171,320]	60,916 [1255 – 142,820]
Saba	15,640 [2318 – 33,133]	17,404 [1635 – 39,468]	62,560 [9270 – 132,532]	69,616 [6540 – 157,872]

Table 3.4. Differentiation among the four most closely related sister lineages. Lineages diverged in the last ~0.27 million years, exhibit low variation, and almost all variation is contained between lineages. Likewise, most polymorphic sites are fixed between lineages.

Lineage X	Lineage Y	Polymorphic sites	Fixed differences	d_{xy}	AMOVA		tMRCA
					Φ_{st}	significance	
Cuba 1, 2	Cuba/Florida 2, Florida 2	53	42	0.01111 (0.00365)	0.769	$p = 0.0342$	0.1101 [0.0382-0.2009]
Hispaniola 1	Hispaniola 2, 3	19	12	0.00357 (0.00102)	0.916	$p = 0.0000$	0.2080 [0.0951-0.3510]
Jamaica 1	Jamaica 2, 3	10	8	0.00203 (0.00087)	0.932	$p = 0.0196$	0.1117 [00447-0.1931]
Puerto Rico 1	Puerto Rico 2	18	15	0.00380 (0.00230)	0.912	$p = 0.0567$	0.2695 [0.1357-0.4055]

d_{xy} = pairwise nucleotide difference between lineages X and Y, reported as mean and standard deviation
Φ_{st} = proportion of variation portioned among (vs. within) populations, scaled 0-1.
tMRCA = time to most recent common ancestor, reported as mean per million years [95% CI]

Figure 3.1. Putative distribution for *Plasmodium floridense*. Areas from where the parasite is previously reported are labeled, and the hypothesized distribution is shown in green.

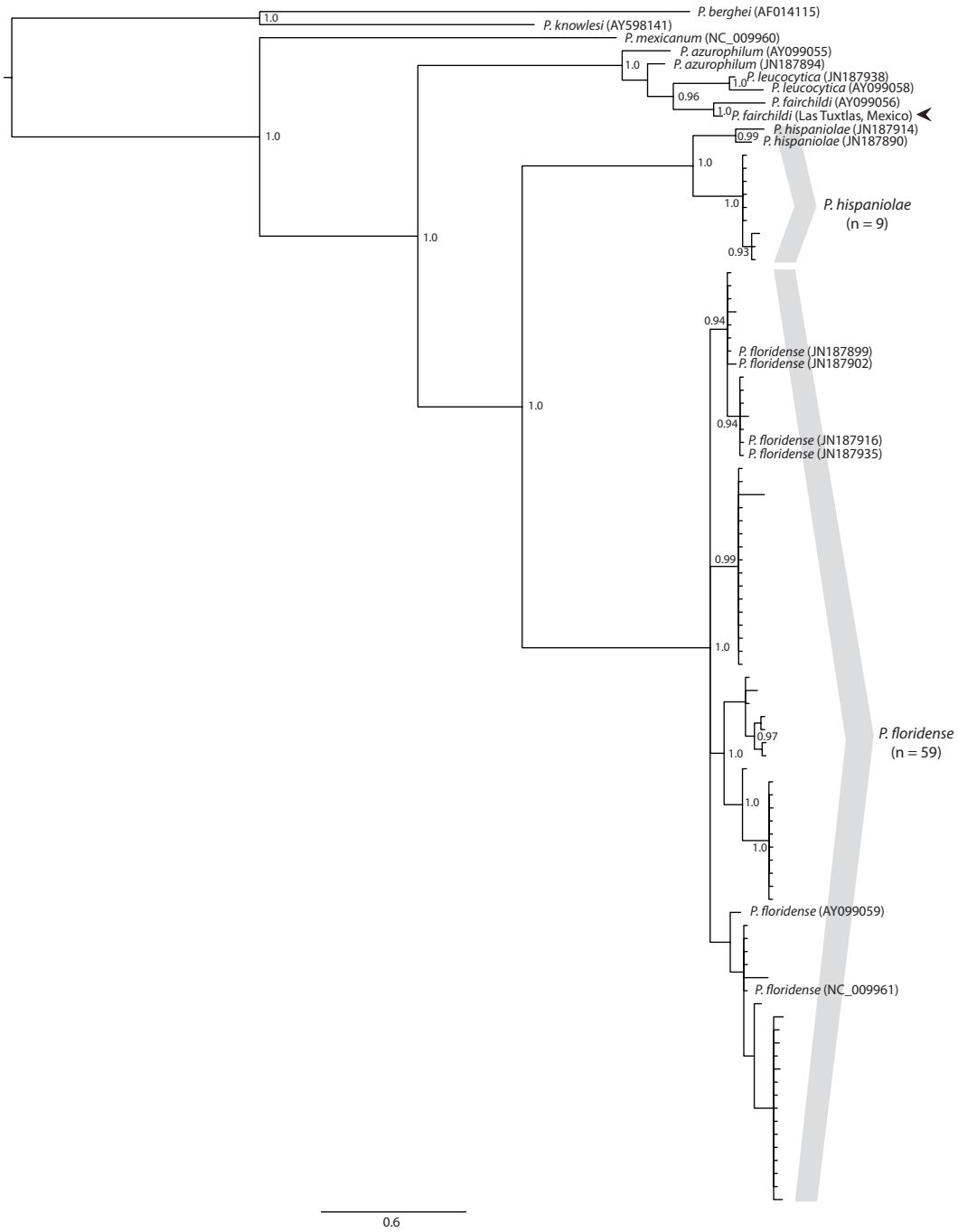

Figure 3.2. Phylogeny used for malaria parasite identification. The tree was inferred in MrBayes from 69 unidentifiable samples and 16 GenBank samples, sequenced at 1189-bp mtDNA (partial *coxI* and all of *cytb*). Based on their monophyly with GenBank samples, we identified 59 of the unidentified samples as *P. floridense*, nine samples as *P. hispaniolae*, and one sample as *P. fairchildi* (black arrow). Nodal support is shown for nodes with ≥ 0.90 posterior probability. The scale bar represents the average number of substitutions per site.

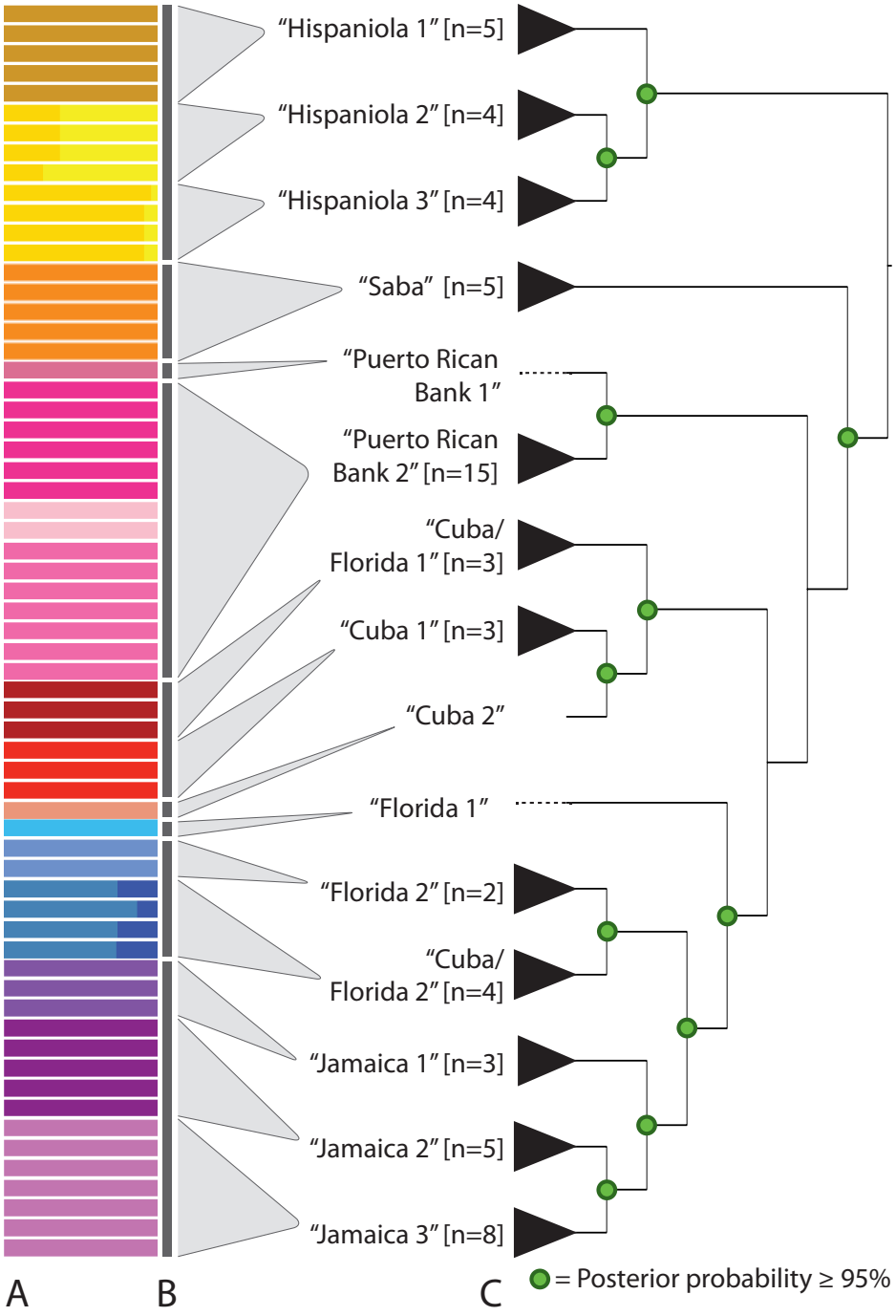

Figure 3.3. Putative lineage identification and guide tree. A) Results of DAPC showing the 17 identified clusters. Each bar represents a single sample and color represents inferred cluster identity. B) Nine shared haplotypes (459-bp cytb) among samples, as indicated by each continuous grey bar. C) Guide tree of 15 putative lineages for BPP analyses, inferred using a species tree approach in *BEAST. Each of the 17 DAPC clusters was initially included as a species, and any unsupported terminal nodes were collapsed until posterior probabilities reached 95%.

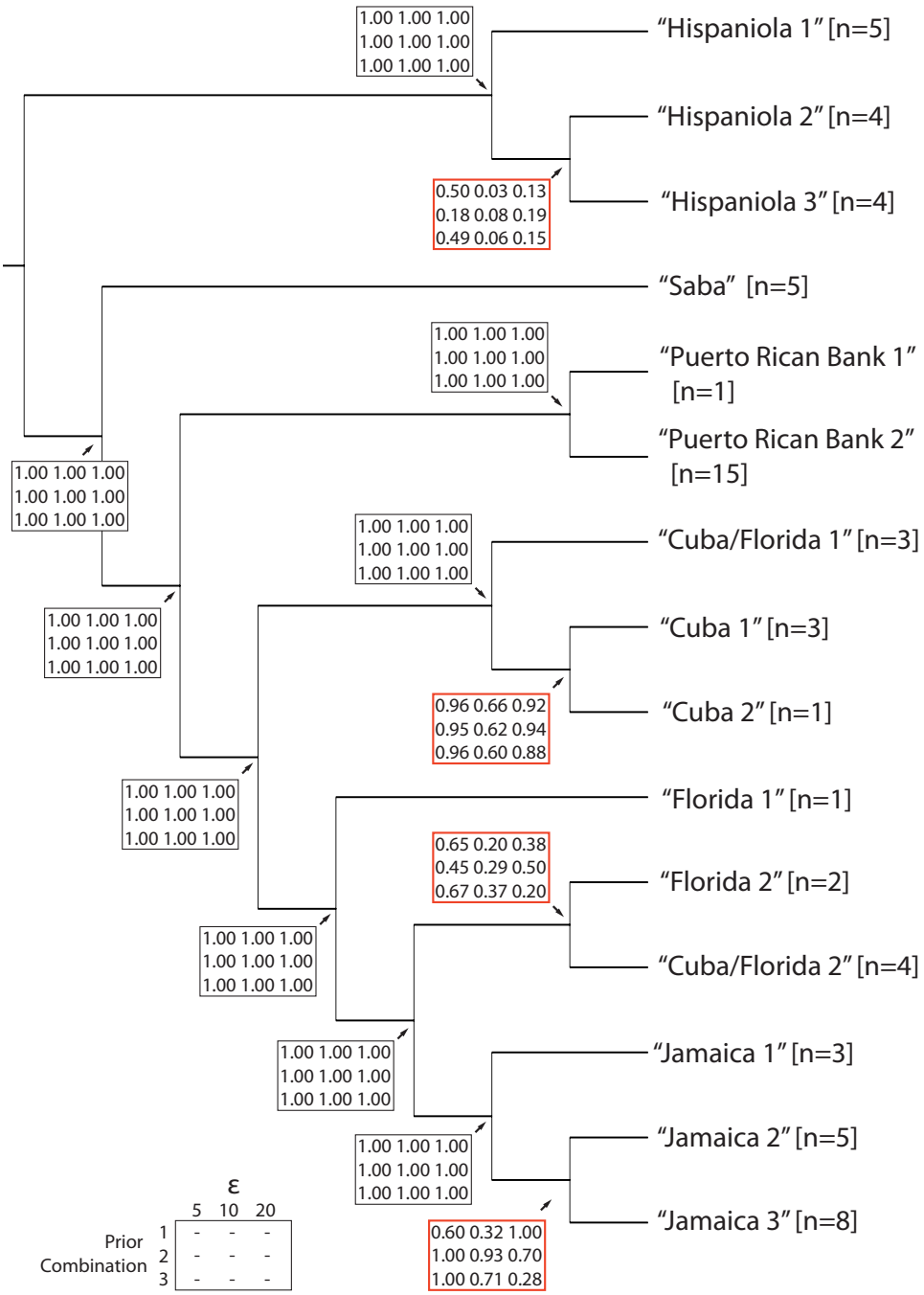

Figure 3.4. Posterior probabilities of reproductive isolation between lineages, as inferred from nine BPP analyses. Node labels represent the probability that descendent lineages are reproductively isolated, given the model parameters and priors. Of the 15 putative lineages, 11 are inferred to be reproductively isolated in every analysis.

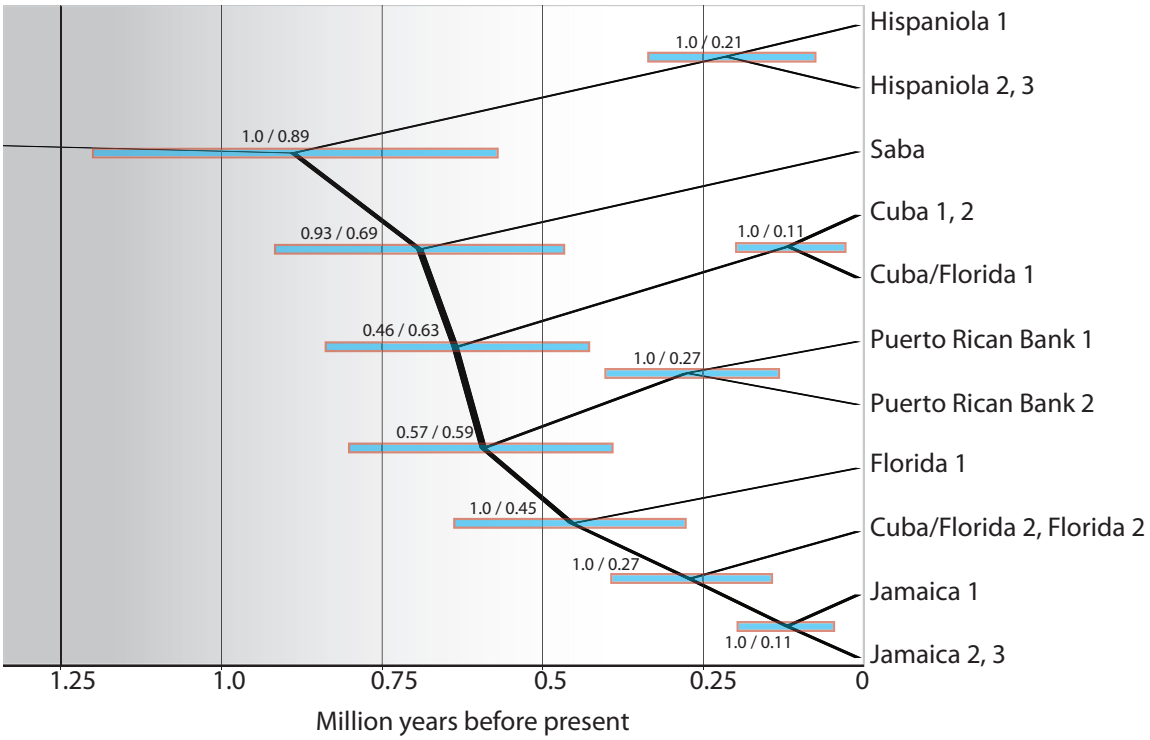

Figure 3.5. Chronogram of *P. floridense* lineages inferred using a species-tree approach in *BEAST and a molecular clock rate for malaria parasites. Nodes are labeled with posterior probability / mean divergence time, and node bars indicate the 95% CI for the age of that node.

CHAPTER IV

HOST SPECIFICITY SHAPES POPULATION STRUCTURE OF PINWORM PARASITES IN CARIBBEAN REPTILES

Abstract

Among the potential factors affecting parasite diversification is variation in host specificity, because gene flow may be facilitated or constrained by the number of host species that a parasite can exploit. We test this hypothesis in two co-distributed pinworm parasites – *Parapharyngodon cubensis* and *Spauligodon anolis* – on the Puerto Rican Bank and St. Croix in the Caribbean. Each of these parasites occurs in several host species and can be classified as a generalist, but each has a different host range. *Spauligodon anolis* specializes on *Anolis* lizards, whereas *P. cubensis* parasitizes *Anolis* lizards as well as many other species of lizards and snakes. We sampled 651 lizards from across the Puerto Rican Bank and St. Croix. We extracted DNA from 60 *S. anolis* and 195 *P. cubensis* individuals, and sequenced them at the mitochondrial gene *coxI* and the nuclear ribosomal gene *18s*. We used the *18s* dataset to detect any cryptic diversity, and show that *P. cubensis* is comprised of several operational taxonomic units (OTUs). We used a phylogeographic approach and the *coxI* dataset to demonstrate that – consistent with our predictions – *S. anolis* exhibits greater variation among populations than the *P. cubensis* OTUs. We also provide evidence that the distribution of *P. cubensis* OTUs is maintained by competitive exclusion, and, in contrast to previous theoretical work, these parasites with the greatest number of hosts reach the highest prevalence rates. Overall,

our results confirm that host specificity shapes parasite diversification, and that differences in host specificity are important even among multi-host parasites.

Introduction

Parasitism is a predominant life mode among metazoans, and it is estimated that 30-50% of all extant species are parasitic (Price 1977; de Meeûs & Renaud 2002). Still, the factors contributing to parasite diversification remain unclear (Poulin & Morand 2000). Metazoan parasites are taxonomically disparate, belonging to several phyla, and are united by just one feature: each relies on a host for at least part of its life cycle. Accordingly, most research on parasite diversification has focused on correlating patterns of host and parasite differentiation in a search for evidence of cospeciation. The notion that diversification in parasites should mirror that of their hosts is known as Fahrenholz's Rule (Eichler 1948), and for decades this was a dominant hypothesis of how parasites diversify (Brooks 1979; Hafner & Nadler 1988; Page & Charleston 1998). We now know that strict cospeciation of hosts and their parasites occurs in only some cases (see Huyse *et al*. 2005). A major exception is when host specificity extends beyond one host species (i.e. when the parasite is a generalist), and many parasites fall into this category (Woolhouse *et al*. 2001).

Nadler (1995) generated several hypotheses about which factors may influence parasite diversification, and among these is host specificity. He predicted that multi-host parasites would exhibit reduced population structure, because additional hosts allow for additional opportunities for parasite dispersal. While Nadler's hypothesis has been restated in the literature (Huyse *et al*. 2005; Barrett *et al*. 2008), and has been used to explain the minimal population structure in multi-host parasites (Hillburn & Sattler 1986;

Archie & Ezenwa 2011), empirical tests are few. Most support comes from studies showing that parasite dispersal depends on host dispersal (Blouin *et al*. 1995; McCoy *et al*. 2003; Criscione & Blouin 2006). Ideally, tests of the effects of host specificity on parasite diversification would compare diversification among co-distributed parasites that vary only in their host range.

Such an approach was used to evaluate the relationship between population structure and host specificity in the ectoparasites of birds. Johnson *et al*. (2002) used 379-bp of mitochondrial DNA and nested-clade analysis to compare the population genetics of two feather lice taxa – *Physconelloides* spp. and *Columbicola* spp. – that differ in host specificity. They showed that *Physconelloides* spp. are very host specific, exhibiting genetic differences among host species, and that these parasites also exhibit greater genetic differentiation among localities than the *Columbicola* spp., which exhibit little host specificity. These differences were extended to higher taxonomic levels; the host-specific *Physconelloides* spp. have more tightly coevolved with their hosts, consistent with Fahrenholz's Rule, than the generalist *Columbicola* spp. (Clayton & Johnson 2003). But, because these studies made comparisons between parasites that exhibit extreme differences in host specificity (i.e. those with a one-to-one host/parasite relationship *vs*. those parasitizing many hosts), it is unknown whether the same population differences may be observed among parasites with moderate differences in host specificity. Indeed, differences in host range among generalist parasites may be so minor that their population structure remains unaffected.

We compared the population structure of two generalist parasite species that differ in host range, in order to infer the effect of differences in host specificity on

diversification of multi-host parasites. These two parasites - *Parapharyngodon cubensis* and *Spauligodon anolis* – both belong to the family Pharyngodonidae, and their host ranges are well characterized (Table 4.1; Bursey *et al*. 2012). Both of these parasites live in the large intestine of their hosts. Both are assumed to share the common oxyurid patterns of direct transmission between hosts (via fecal-oral contact) and haplo-diploidy (Anderson 2000). Both are distributed throughout the Caribbean and in parts of Central America. Their differences lie primarily in their host specificity. *Spauligodon anolis* infects *Anolis* lizards, a group that is among the most abundant and conspicuous of the Caribbean vertebrate fauna (Losos 2009). *Parapharyngodon cubensis* infects *Anolis* lizards as well as other many species of non-herbivorous lizards and snakes.

We focused our study in Puerto Rico and the surrounding islands because these islands have a unique geography and host composition that makes them well suited for testing hypotheses about parasite dispersal. The Puerto Rican Bank (hereafter "PRB") islands – including those belonging to Puerto Rico, the U.S. Virgin Islands, and the British Virgin Islands – comprise what was once a continuously emergent landmass during the lowered sea levels of the Pleistocene glacial maxima (Pregill & Olson 1991; Siddall *et al*. 2003). The islands were separated by rising sea levels into their current physiography approximately 7,000 years ago (Pregill & Olson 1991). St. Croix is the exception. It is near these other islands, and politically part of the U.S. Virgin Islands, but has not been connected to another landmass since at least the early Oligocene (33–35 MYA), if ever (Iturralde-Vinent & MacPhee 1999). The pattern of reptile diversity in these areas reflects the islands' history. The PRB islands share many of the same reptile species (e.g. *Anolis cristatellus, Anolis pulchellus,* and *Anolis stratulus),* but the species

found on St. Croix are typically endemics with sister taxa on the PRB (e.g. *Anolis acutus*). Both *P. cubensis* and *S. anolis* are reported from the PRB and St. Croix, and if we assume that they can infect any extant species belonging to the same family as a previously reported host species, then *S. anolis* has 11 available hosts and *P. cubensis* has 41 available hosts on the PRB and St. Croix (Table 4.2).

We tested the hypothesis that higher host specificity would be associated with greater population structure in *S. anolis*, relative to *P. cubensis*. We collected lizards from throughout the PRB and St. Croix, and dissected them for pinworm parasites. We extracted DNA from specimens of *S. anolis* and *P. cubensis*, and sequenced from them both nuclear and mitochondrial loci. We tested our predictions using summary statistics, AMOVA, Mantel tests for isolation-by-distance, and topology-based tests.

Methods

Sampling, DNA sequencing, and identification of OTUs

We captured 641 *Anolis* lizards, comprising six of 11 species on the PRB and St. Croix, by noose or hand from 30 sites on Puerto Rico and Vieques (October 2011), from seven sites on St. John and St. Thomas in the U.S. Virgin Islands (August 2011), from six sites on Anegada, Jost van Dyke, Tortola, and Virgin Gorda in the British Virgin Islands (August 2011), and from three sites on St. Croix in the U.S. Virgin Islands (August 2011; Figure 4.1 and Table 4.3). We focused our sampling on *Anolis* lizards because these are among the most abundant host species on the islands, and are possible to collect in sufficient numbers. For example, populations of several potential hosts for *P. cubensis* (e.g. *Ameiva exsuul* and *Borikenophis portoricensis*) are nearly extinct on many islands due to predation from the introduced mongoose (Pimentel 1955; MacLean 1982). We

also collected three *Hemidactylus mabouia* individuals (Squamata; Gekkonidae) and seven *Sphaerodactylus macrolepis* individuals (Squamata; Sphaerodactylidae) to look for evidence of cryptic host specificity, because it is possible that the *P. cubensis* specimens reported from non-*Anolis* host taxa are morphologically cryptic species (i.e. that host specificity in *P. cubensis* and *S. anolis* is the same; Poulin & Keeney 2008). We humanely euthanized each lizard using tricaine methanesulfonate (i.e. MS-222; Conroy *et al*. 2009), and preserved the entire gastrointestinal (GI) tract, the heart, the lungs, the liver, and the gall bladder in a vial containing absolute ethanol. We later separated each of these tissues and sections of the GI tract (i.e. stomach, small intestine, large intestine), and dissected them under a Nikon SMZ800 stereomicroscope (Nikon Inc., Mellville, NY, USA). We preserved parasite specimens in absolute ethanol at -20°C, and set aside a subset of *P. cubensis* and *S. anolis* specimens in DNA buffer for immediate DNA extraction.

We extracted DNA from whole, individual nematodes using QIAGEN DNeasy Animal Tissue Extraction kits (Valencia, CA, USA), following the manufacture's instructions except using two final DNA elutions of just 50 µl AE buffer each (as opposed to 200 µl each) so that the DNA would not be too diluted. We used Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare, Pittsburg, PA, USA) to amplify the small subunit ribosomal gene *18s* using the primers MN18F (5'-CGC GAA TRG CTC ATT ACA AC AGC-3') and Nem_18sR (5'-GGG CGG TAT CTG ATC GCC-3'; Bhadury *et al*. 2006) and the mitochondrial gene cytochrome oxidase I (*coxI*) using the primers Ent_coxIF (5'-AGA GAA CAA GAC ATA AAG ATA TTG G-3') and Ent_coxIR (5'-TAA ACC TCA GGA TGA CCA AAA AAT CA-3'; this study). We re-

attempted any amplification failures for each sample for each locus at least twice. We cleaned PCR products with AMPure (Agencourt, Beverly, MA, USA), sequenced them in both directions using BigDye v.3.0 (Applied Biosystems, Foster City, CA, USA), and edited them in GENEIOUS v.5.4.6 (Biomatters, Auckland, New Zealand). Multiple sequence alignments were generated using the MUSCLE plugin (Edgar 2004) in GENEIOUS using default parameters. Gaps were treated as missing data in all analyses.

We used the *18s* sequences to detect any cryptic diversity. We chose this locus because of the availability of primers that amplify universally across nematodes, and also because it is commonly used in nematodes to identify molecular operational taxonomic units, or (M)OTUs (Floyd *et al*. 2002; Blaxter 2004; Blaxter *et al*. 2005). We visualized the relationships among groups of *18s* sequences, along with a GenBank sequence for the human pinworm *Enterobius vermicularis* (JF934731), using a haplotype network inferred via the Neighbor-Net algorithm (Bryant & Moulton 2004) in SplitsTree v.4.12.6 (Huson & Bryant 2005). We regarded each group of samples that share a single *18s* sequence to be an OTU.

Population structure

We characterized the structure of variation among populations using the *coxI* datasets of each OTU separately. We considered each sampling locality on Puerto Rico to be a population, but, owing to their close geographic proximity, we grouped together localities 9 and 12 as well as localities 10 and 11 (Figure 4.1; Table 4.3). We also grouped together all samples from each of the smaller Virgin Islands, for a total of 26 "populations." We acknowledge that these may or may not represent true populations (i.e. panmictic groups). Instead, we use this population assignment scheme to compare

the relative extent of geographic variation in each *P. cubensis* and *S. anolis* over the same landscape. When applicable and for OTUs that occur on both the PRB and St. Croix, we conducted analyses using all samples and also restricting the dataset to PRB samples only.

We characterized the genetic diversity of each OTU via several summary statistics inferred in DnaSP v.5 (Librado & Rozas 2009): the number of haplotypes, haplotype diversity, the average number of nucleotide differences per site (π), and the population mutation rate (θ) inferred using the number of segregating sites (i.e. θ_S; Watterson 1975). We also estimated Tajima's D, which uses differences between θ estimates that are derived using segregating sites (θ_S) and from those using the average number of nucleotide differences (θ_N) to infer selection or demographic changes (Tajima 1989).

We tested the null hypothesis that the *coxI* sequences of each OTU are panmictic using an exact test of sample differentiation (Raymond & Rousset 1995) in ARLEQUIN v.3.5.1.3 (Excoffier & Lischer 2010). This test uses a Markov chain method to explore contingency tables of haplotype frequencies among populations, and estimates the probability that haplotypes are not randomly distributed among populations (i.e. that populations are not panmictic). Following that, we employed an analysis of molecular variance (AMOVA) of the *coxI* datasets in ARLEQUIN to estimate the extent of variation that is partitioned within versus among populations (i.e. Φ_{st}, Excoffier *et al*. 1992). We converted our files into the ARLEQUIN format using FaBox v.1.35 (Villesen 2007).

In order to compare range-wide dispersal patterns among the parasites, we tested for isolation-by-distance (IBD) among populations using Mantel tests via ADEGENET (Jombart 2008) in R (R Development Core Team 2012). A Mantel test – in the context of IBD in ADEGENET – measures the correlation between a genetic and a geographic distance matrix, and significance is estimated using the proportion of 999 random permutations that infer a positive correlation coefficient (Mantel 1967; Jombart 2008). We used ARLEQUIN-derived pairwise F_{st} estimates for our distance estimates. We predicted that *P. cubensis* would exhibit less IBD due to greater number of hosts available to facilitate its dispersal across the landscape.

We visualized the relationship between the genetic and geographic distances using scatterplots generated in R. Significant IBD inferences can be made from a single population exhibiting clinal variation (i.e. classic IBD) or several discrete populations that differ along a gradient (i.e. an island model of differentiation; Handley *et al*. 2007). We used the pattern of relative densities in these plots to visually assess which of these patterns better explains our data (Jombart 2012), paying particular attention to the differences between datasets including the PRB and St. Croix and those including the PRB only. For example, plots showing a single high-density nucleus suggest clinal variation, whereas multiple high-density nuclei suggest the population is evolving under an island model of differentiation.

We visualized the relationships among haplotypes using both a phylogenetic and haplotype network approach. We inferred a phylogeny using maximum-likelihood in RAxML (Stamatakis *et al*. 2005; Stamatakis 2006) via the raxmlGUI v.0.9 (Silvestro & Michalak 2010). We employed a GTR + Γ substitution model and estimated nodal

support with 1000 bootstrap replicates (Felsenstein 1985). We inferred a median-joining haplotype network in SPLITSTREE. We initially attempted to use the Neighbor-Net algorithm because these better represent the different possible connections in each network, but these networks contained so many potential paths that visualization was not possible.

We wanted to know whether samples from the same population are similar because they share the same common ancestor, and so we applied an approximately unbiased (AU) test (Shimodaira 2002), a topology-based test, to infer whether sequences from samples collected from each island are monophyletic. The AU test employs a multiscale bootstrap (Zharkikh & Li 1995) of likelihood values from a set of trees to create a set of expected log-likelihood values for a given dataset (i.e. a null distribution). For each tree it tests whether the likelihood is larger or equal to these expected values (i.e. it tests whether some or all trees are not equally good explanations of the data). We tested whether a geographically constrained topology is not an equally good explanation of the data. We first inferred a ML phylogeny of each OTU in RAxML, and resampled using 1000 bootstrap replicates. We rooted each phylogeny using one sample from a different OTU. Next, we inferred a ML tree from the same dataset while imposing a somewhat liberal constraint so that samples from each of the small island populations are monophyletic. More specifically, groups of samples collected from each of the eight Virgin Islands (e.g. St. Croix, Vieques, Virgin Gorda, etc.) were constrained to be monophyletic, while those from Puerto Rico were allowed to fall anywhere in the tree. We inferred the per-site log-likelihoods for each of these 1002 trees (1000 bootstrap trees, the constrained ML tree, and the unconstrained ML tree), and used these data to

conduct the AU tests in CONSEL v.0.1k (Shimodaira & Hasegawa 2001), using 10,000 multiscale bootstrap replicates. We interpreted any *p*-values ≤ 0.05 as a rejection of geographically associated population structure, and that samples from the same island do not share a single common ancestor.

Results

Sampling, DNA sequencing, and identification of OTUs

Of the 641 *Anolis* lizards we collected, 100 were infected with *S. anolis* and 221 with *P. cubensis* (Table 4.4). Of the seven *Sphaerodactylus macrolepis* individuals, one – collected on St. Croix – was infected with a single *P. cubensis* pinworm. None of the three *Hemidactylus mabouia* individuals were infected with any pinworm parasite.

We extracted DNA from 65 *S. anolis* and 195 *P. cubensis*. Of the 65 *S. anolis*, we successfully sequenced 817-bp of *18s* from 61 individuals and 641-bp of *coxI* from 55 individuals. Of the 195 *P. cubensis*, we successfully sequenced 819-bp of *18s* from 172 individuals and 640-bp of *coxI* from 178 individuals. The *18s* alignment contained a single 2-bp indel separating *S. anolis* and *P. cubensis*, and, similarly, the *coxI* alignment contained a single 1-bp indel separating *S. anolis* and *P. cubensis*.

The Neighbor-Net haplotype network inferred from the *18s* data is shown in Figure 4.2. All *S. anolis* individuals share a single *18s* sequence. All *P. cubensis* individuals contain one of three unique sequences, and we arbitrarily named them *P. cubensis* A, *P. cubensis* B, and *P. cubensis* C. The *P. cubensis* sample collected from the gecko *S. macrolepis* contained a *P. cubensis* B haplotype, confirming that *P. cubensis* is a squamate generalist. We considered each of the three *P. cubensis* groups, as well as the group of *S. anolis* individuals sharing the single *18s* sequence, to be an OTU. Because *P.*

cubensis C was comprised of just two individuals collected on St. Croix, we removed it from all downstream analyses.

Population structure

Locality sampling for each *S. anolis*, *P. cubensis* A, and *P. cubensis* B is shown in Table 4.5. We observed at least one parasite species at each locality, and each parasite at a total of 16, 18, and 13 localities, respectively, where localities are grouped into 26 populations as described above. Notably, *P. cubensis* A was collected only on the PRB, while *P. cubensis* B and *S. anolis* were collected both on the PRB and St. Croix. Summary statistics for each of these parasites are shown in Table 4.6. The haplotype diversity for all OTUs is high, and is slightly higher for the *P. cubensis* OTUs. Nucleotide diversity (π) is highest in *S. anolis*, as are estimates of the population mutation rate θ_S. Estimates of Tajima's D are negative for each of the *P. cubensis* OTUs and positive for *S. anolis*, though none are significant.

Estimates of population divergence are also shown in Table 4.6. We rejected the null hypothesis of panmixia for each of the OTUs using the exact test of sample differentiation. *Parapharyngodon cubensis* A and *P. cubensis* B exhibit moderate population structure, with estimates of Φ_{st} (including all samples and also restricting to the PRB samples only) ranging 0.34-0.35. Estimates for *S. anolis* indicate greater relative population differentiation and are different when the St. Croix samples are included, with $\Phi_{st} = 0.76$ for all samples and $\Phi_{st} = 0.65$ when restricting to samples from the PRB.

Isolation-by-distance correlations are significant for both *S. anolis* and *P. cubensis* B when including all samples and also when restricting to the PRB, with greater

correlation coefficients in *S. anolis* (Table 4.7). There is no significant correlation between genetic and geographic distances in *P. cubensis* A. The density plot for the *S. anolis* dataset from both the PRB and St. Croix contains more high-density nuclei than the PRB-only dataset (Figure 4.3), suggesting that dispersal between St. Croix and the PRB is not clinal (i.e. the open ocean water between St. Croix and the PRB presents a greater barrier than an equal distance within the PRB). In contrast, the density plots for *P. cubensis* B are relatively uniform and unchanged between the two datasets. This pattern is consistent with the aforementioned pattern in Φ_{st} estimates (Table 4.6), which were similar in *P. cubensis* B whether or not we included the St. Croix samples in the dataset.

The patterns in the phylogeny and the haplotype networks show greater geographically associated population structure in *S. anolis* than the *P. cubensis* OTUs. *Spauligodon anolis* samples from each of the small islands are monophyletic, often with strong nodal support (Figure 4.4). In contrast, there is minimal monophyly of same-island samples and generally poor nodal support in *P. cubensis* A and *P. cubensis* B, particularly in the former. The haplotype networks (Figure 4.5) show that in *S. anolis*, samples from the same islands are clustered together, and when multiple individuals share the same haplotype, these are always from the same island. Once again, the pattern is different for both *P. cubensis* A and *P. cubensis* B; haplotypes collected from the same area are scattered throughout the network, and, in many cases, identical haplotypes are shared among samples collected from different islands. The AU tests confirmed these patterns. We could not reject geographically associated population structure in *S. anolis*

($p = 0.841$). But, geographically associated population structure is rejected for both *P. cubensis* A ($p = 3.0 \times 10^{-4}$) and *P. cubensis* B ($p = 6.0 \times 10^{-6}$).

Discussion

Consistent with Nadler's prediction (1995), we show that differences in host specificity are associated with differences in population structure in *S. anolis* and *P. cubensis*. We provide evidence that *P. cubensis* on the PRB and St. Croix is comprised of three OTUs, and two of these – *P. cubensis* A and *P. cubensis* B – are widely distributed, as is *S. anolis*. Compared to the *P. cubensis* OTUs, a greater proportion of genetic variation in *S. anolis* is contained between populations, rather than within populations. Populations of *S. anolis* exhibit greater isolation-by-distance than the *P. cubensis* OTUs, and the St. Croix and PRB populations are not clinally differentiated, in contrast to the *P. cubensis* B populations. Finally, we reject the monophyly of populations occurring in each of the smaller islands for the *P. cubensis* OTUs, but not *S. anolis*.

The differences in population structure between *S anolis* and the *P. cubensis* OTUs suggest that many more dispersal opportunities are available to the latter. *Anolis* lizards are remarkably good overwater dispersers (Williams 1969; Calsbeek & Smith 2003; Glor *et al*. 2005; Nicholson *et al*. 2005), but dispersal is apparently infrequent enough that *S. anolis* exhibits relatively strong population structure. Dispersal patterns and capabilities for other squamates – which may facilitate gene flow among populations of the *P. cubensis* complex – are not well known, though overwater dispersal is perhaps not uncommon in many animal taxa (de Queiroz 2005). For example, amphisbaenians are hypothesized to have colonized the Americas via transatlantic dispersal in the Eocene

(Vidal *et al*. 2008), suggesting that overwater dispersal in these hosts is possible. Additionally, floating rafts of vegetation (i.e. flotsam) that are generated during hurricanes facilitate inter-island dispersal in several Caribbean animal species (Heatwole & Levins 1972; Censky *et al*. 1998; Hedges 2006), and this is a potential source of host dispersal for the *P. cubensis* complex. In any case, movement of these parasites between the PRB and St. Croix cannot occur without overwater host dispersal, and our data suggest that this may not be infrequent among squamates.

The prevalence we observed in *S. anolis* and the *P. cubensis* OTUs are in contrast with theoretical work that suggests that there may be some trade-off between host specificity and prevalence (Holt *et al*. 2003; Dobson 2004; Keesing *et al*. 2006). This work uses the reasoning that because a specialist parasite is adapted to one or a few hosts, it is more efficient, and so will infect a greater proportion of those hosts than will a generalist parasite that is not so well adapted. But, even though we did not sequence some *P. cubensis* individuals in order to assign them to an OTU (i.e. our observed prevalence data for the *P. cubensis* OTUs is slightly low), we observed a similar number of host species infected with either *P. cubensis* A or *P. cubensis* B than *S. anolis* in their *Anolis* lizard hosts (Table 4.1). Moreover, if we include only the PRB samples (i.e. exclude *Anolis acutus*), prevalence of each of the *P. cubensis* OTUs is higher than the prevalence of *S. anolis*. One possible explanation for this discrepancy is that pinworm infections are generally not considered harmful to their hosts (Lane & Mader 2005; Jacobson 2007), potentially diminishing the pervasiveness of host-specific adaptations and making more important other factors that may determine prevalence among host species. Still, such a discrepancy was also found in malaria parasites of birds (Hellgren

et al. 2009), begging the question of whether host specificity plays a role in observed prevalence, and if yes, just what that role is.

Currently, *P. cubensis* is the only recognized species of *Parapharyngodon* reported from Caribbean squamates, though we provide evidence that it is a complex of several species. Only two other species – *Parapharyngodon garciae* and *Parapharyngodon osteopili* – are described from Caribbean herpetofauna, but their hosts are frogs, not squamates (Schmidt & Whittaker 1975; Adamson 1981). *Parapharyngodon cubensis* is distinguished from these and other species by differences in the male spicule length, the number of papillae, and the characteristics of the cloacal lip (smooth or echinate; Barus & Coy Otero 1969; Bursey *et al*. 2005; Jiménez *et al.* 2008). Samples from Hispaniola and Saba provide further evidence of cryptic species diversity within *P. cubensis* (data not shown), and we expect that the taxonomy will remain uncertain until more molecular data are collected from throughout its range.

We observed minimal co-occurrences of the *P. cubensis* OTUs within localities, suggesting the potential for competitive exclusion among these taxa. For example, *P. cubensis* B was abundant on both Jost van Dyke and Anegada but was not observed on Virgin Gorda. We observed the exact opposite in *P. cubensis* A; it occurs on Virgin Gorda but not Jost van Dyke or Anegada. Similarly, the two co-occur at only three of the 18 localities in Puerto Rico. In contrast, in the 12 localities on Puerto Rico where *S. anolis* occurs, we also observed either *P. cubensis* A or *P. cubensis* B at 10 of them, and sometimes they co-occurred within a single host individual. We suspect this pattern to be associated with another pattern – differences in infection intensity (i.e. the number of nematode individuals per host). Intensity in *P. cubensis* is low; for example, intensity in

Anolis acutus ranges 1-4, with an average of 1.6 worms per host individual (Goldberg *et al*. 1997). Intensity of *S. anolis* in the same host species at the same locality is much higher, ranging 1-130, with an average of 21.3 worms per host individual (Goldberg *et al*. 1997). Given that autoinfection via asexual reproduction is possible in pinworms (i.e. production of haploid males via thin-shelled, autoinfective eggs; Anderson 2000), these data suggest that the maximum number of *P. cubensis* individuals in a single host is limited. Thus, individuals from each *P. cubensis* OTU must compete for the same set of local host individuals, and these parasite populations may undergo periodic local extinctions and recolonizations.

An open and interesting question remains regarding the conditions necessary to prevent gene flow among populations of multi-host nematode parasites, since we show that the open ocean waters between the PRB and St. Croix do not prevent dispersal in *P. cubensis* B. Mayr (1963) hypothesized that allopatric speciation is predominant among nematodes, although it is unclear whether he was referring to either the parasitic or free-living taxa, or both. Inglis (1971) clarified Mayr's arguments in the context of parasitic nematodes, proposing that allopatric speciation is chiefly responsible for species diversification among these parasites. While there is some contention on the concept of "allopatry" in the context of parasites (e.g. among hosts, among localities, etc.), we consider allopatric speciation to be speciation in response to some extrinsic barrier to gene flow (McCoy 2003, 2004; Le Gac & Giraud 2004). This hypothesis can be tested using a DNA-based, phylogeographic approach (Criscione *et al*. 2005; Huyse *et al*. 2005; Perkins *et al*. 2011), and already some evidence exists that geographic barriers – not host differences – prevent gene flow among nematode populations (Nieberding *et al*. 2008;

Wu *et al*. 2009). Our study demonstrates that the strength of those barriers is labile according to a parasite's host specificity, and that host specificity has a profound affect on parasite diversification.

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Table 4.1. Summary of known host taxa for *Spauligodon anolis* and *Parapharyngodon cubensis*. *Spauligodon anolis* infects anole lizards, while *P. cubensis* infects anoles and many other squamate taxa. Host reports are taken from the summary provided in Bursey et al. (2012), and higher taxa are as listed in the Reptile Database (http://www.reptile-database.org, accessed 11/15/12).

Table 4.1.

Parasite species	Common name (host)	Higher taxa (host)	N	Species (host)
Spauligodon anolis	Anoles	Polychrotidae (Dactyloidae), Iguania, Sauria	17	*Anolis acutus, Anolis bimaculatus, Anolis conspersus, Anolis cristatellus, Anolis ferreus, Anolis gingivinus, Anolis leachi, Anolis lineatopis, Anolis lividus, Anolis marmoratus, Anolis oculatus, Anolis richardii, Anolis sabanus, Anolis scriptus, Anolis stratulus, Anolis valencienni, Anolis wattsi*
Parapharyngodon cubensis	Worm lizards	Amphisbaenidae, Amphisbaenia	1	*Amphisbaena cubana*
	Anoles	Polychrotidae (Dactyloidae), Iguania, Sauria	43	*Anolis acutus, Anolis aeneus, Anolis allisoni, Anolis allogus, Anolis bartschi, Anolis bimaculatus, Anolis bremeri, Anolis brevirostris, Anolis chlorocyanus, Anolis coelestinus, Anolis cristatellus, Anolis distichus, Anolis eugenegrahami, Anolis extremus, Anolis ferrus, Anolis gingivinus, Anolis grahami, Anolis griseus, Anolis homolechis, Anolis jubar, Anolis leachii, Anolis lineatopis, Anolis lividus, Anolis luciae, Anolis lucius, Anolis luteogularis, Anolis marmoratus, Anolis maynardi, Anolis monticola, Anolis oculatus, Anolis olssoni, Anolis pogus, Anolis porcatus, Anolis quadriocellifer, Anolis richardii, Anolis sabanus, Anolis sagrei, Anolis schwartzi, Anolis scriptus, Anolis stratulus, Anolis valencienni, Anolis vermiculatus, Anolis wattsi*
	Colubrid snakes	Colubridae, Dipsadinae, Alsophiini, Serpentes	1	*Cubophis cantherigerus*
	Curly-tailed lizards	Leiocephalidae, Iguania, Sauria	3	*Leiocephalus carinatus, Leiocephalus cubensis, Leiocephalus macropus*
	Dwarf boas	Tropidophiidae, Henophidia, Serpentes	2	*Tropidophis melanurus, Tropidophis semicinctus*
	Geckos	Gekkonidae, Sauria	2	*Cyrtopodion scabrum; Hemidactylus mabouia*
	Geckos	Sphaerodactylidae, Sauria	5	*Gonatodes albogularis, Sphaerodactylus cinereus, Sphaerodactylus fantasticus, Sphaerodactylus torrei, Sphaerodactylus vincenti*
	Whiptail lizards	Teiidae, Sauria	3	*Ameiva auberi, Ameiva exsul*, Ameiva pleei*

**Ameiva exsul* was reported to host *Pharyngodon anolis* Chitwood, 1934 (Acholonu 1976) in Puerto Rico, and the parasite's identity remains equivocal. We place it in *Parapharyngodon cubensis* here. Bursey and Goldberg (1998) examined the two available specimens from that study (USNPC73292), and found their morphology consistent with *Spauligodon anolis*. But, given that this was the only pinworm taxon collected from the large intestines of 246 lizards on Puerto Rico, and that *P. cubensis* is a common parasite of the large intestine in squamates on the Puerto Rican Bank (Goldberg et al. 1998; Dyer et al. 2001; this study) we believe that the parasites termed *"Pharyngodon anolis"* in that study include both *S. anolis* and *P. cubensis*. Following that, and in combination with the fact that *S. anolis* has been reported only in *Anolis* lizard hosts in every other study, we believe the parasites reported from *Ameiva exsul* in Acholonu (1976) are *P. cubensis*.

Table 4.2. Potential hosts available to *Spauligodon anolis* and *Parapharyngodon cubensis* on the Puerto Rican Bank and St. Croix. Potential host species belong to the same family as previously reported host species for each parasite (Table 4.1), are extant, and occur on the Puerto Rican Bank and/or St. Croix. Taxonomy and occurrence records are as listed in the Reptile Database (http://www.reptile-database.org, accessed 12/5/12), Rivero (1978), MacLean (1982), and Schwartz and Henderson (1991).

Parasite species	Common name (host)	Higher taxa (host)	N	Species (host)
Spauligodon anolis	Anoles	Polychrotidae (Dactyloidae), Iguania, Sauria	11	*Anolis acutus, Anolis cooki, Anolis cristatellus, Anolis cuvieri, Anolis evermanni, Anolis gundlachi, Anolis krugi, Anolis occultus, Anolis poncensis, Anolis pulchellus, Anolis stratulus*
Parapharyngodon cubensis	Worm lizards	Amphisbaenidae, Amphisbaenia	5	*Amphisbaena bakeri, Amphisbaena caeca, Amphisbaena fenestrata, Amphisbaena schmiditi, Amphisbaena xera*
	Anoles	Polychrotidae (Dactyloidae), Iguania, Sauria	11	*Anolis acutus, Anolis cooki, Anolis cristatellus, Anolis cuvieri, Anolis evermanni, Anolis gundlachi, Anolis krugi, Anolis occultus, Anolis poncensis, Anolis pulchellus, Anolis stratulus*
	Colubrid snakes	Colubridae, Dipsadinae, Alsophiini, Serpentes	4	*Borikenophis portoricensis, Borikenophis sanctaecrucis, Magliophis exiguum, Magliophis stahli*
	Dwarf boas	Tropidophiidae, Henophidia, Serpentes	6	*Typhlops catapontus, Typhlops granti, Typhlops hypomethes, Typhlops platycephalus, Typhlops richardi, Typhlops rostellatus,*
	Geckos	Gekkonidae, Sauria	2	*Hemidactylus brooki, Hemidactylus mabouia*
	Geckos	Sphaerodactylidae, Sauria	10	*Sphaerodactylus beattyi, Sphaerodactylus gaigeae, Sphaerodactylus klauberi, Sphaerodactylus levinsi, Sphaerodactylus macrolepis, Sphaerodactylus microlepis, Sphaerodactylus micropithecus, Sphaerodactylus nicholsi, Sphaerodactylus parthenopion, Sphaerodactylus roosevelti*
	Whiptail lizards	Teiidae, Sauria	3	*Ameiva exsul, Ameiva polops, Ameiva wetmorei*

Table 4.3. Locality information. Localities have numerical labels that correspond to the map in Figure 4.1 and prevalence data in Table 4.5. These are accompanied by both exact GPS coordinates in decimal degrees and nearby place or road names for geographical reference (names are shared when more than one site is nearest the same place).

#	Locality Name	Latitude	Longitude
1	Mayagüez, Mayagüez, Puerto Rico	18.21339	-67.13757
2	Isabela, Isabela, Puerto Rico	18.51195	-67.06575
3	Parguera, Lajas, Puerto Rico	17.98195	-67.03824
4	PR-119, Maricao, Puerto Rico	18.17595	-67.02090
5	Lago de Guajataca, San Sebastián, Puerto Rico	18.37535	-66.92910
6	Yauco, Guánica, Puerto Rico	18.00466	-66.85098
7	PR-123, Arecibo, Puerto Rico	18.36646	-66.68867
8	Ponce, Ponce, Puerto Rico	17.98140	-66.66600
9	PR-143, Orocovis, Puerto Rico	18.16853	-66.49990
10	Manatí, Manatí, Puerto Rico	18.38191	-66.49680
11	Manatí, Manatí, Puerto Rico	18.38034	-66.48800
12	PR-590, Orocovis, Puerto Rico	18.18846	-66.45745
13	Rabo del Buey, Salinas, Puerto Rico	18.03267	-66.24347
14	Represa de San Juan, Aguas Buenas, Puerto Rico	18.27778	-66.13858
15	PR-184, Cayey, Puerto Rico	18.13538	-66.08157
16	Loiza, Carolina, Puerto Rico	18.43764	-65.88540
17	Yabucoa, Yabucoa, Puerto Rico	18.04458	-65.85477
18	PR-186, Rio Grande, Puerto Rico	18.29482	-65.85041
19	Rio Mar, Rio Grande, Puerto Rico	18.38813	-65.75592
20	Naguabo, Naguabo, Puerto Rico	18.21836	-65.74065
21	PR-994, Vieques, Puerto Rico	18.11591	-65.54987
22	Laguna Playa Grande, Vieques, Puerto Rico	18.09369	-65.50729
23	PR-200, Vieques, Puerto Rico	18.13345	-65.50548
24	PR-201, Vieques, Puerto Rico	18.10911	-65.48299
25	Esperanza, Vieques, Puerto Rico	18.09492	-65.47132
26	PR-200, Vieques, Puerto Rico	18.14154	-65.46638
27	Calle 10, Vieques, Puerto Rico	18.12784	-65.45504
28	PR-997, Vieques, Puerto Rico	18.11494	-65.45106
29	Puerto Ferro, Vieques, Puerto Rico	18.10081	-65.42842
30	PR-200, Vieques, Puerto Rico	18.15762	-65.42194
31	Fortuna Hill, St. Thomas, USVI	18.35126	-65.00710
32	Bordeaux Rd., St. Thomas, USVI	18.36181	-65.00259
33	Dorothea, St. Thomas, USVI	18.36852	-64.96253
34	Hull Bay, St. Thomas, USVI	18.37065	-64.95063
35	St. Peter Mtn. Road, St. Thomas, USVI	18.35664	-64.95004
36	Creque Dam Rd, St. Croix, USVI	17.73820	-64.88882
37	Old Mill, St. Croix, USVI	17.73550	-64.88696
38	Creque Dam, St. Croix, USVI	17.74582	-64.87627
39	Fish Bay, St. John, USVI	18.32669	-64.76401
40	Great Harbour, Jost Van Dyke, BVI	18.44556	-64.74791
41	Coral Harbour, St. John, USVI	18.34926	-64.72855
42	Belmont Pond, Tortola, BVI	18.39614	-64.69241
43	Fresh Gut Pond, Tortola, BVI	18.38927	-64.66298
44	Sage Mountain, Tortola, BVI	18.41208	-64.65601
45	Nail Bay, Virgin Gorda, BVI	18.49719	-64.40387
46	Settlement Rd., Anegada, BVI	18.72728	-64.37234

BVI = British Virgin Islands
USVI = U.S. Virgin Islands

Table 4.4. Observed host prevalence for *Spauligodon anolis* and *Parapharyngodon cubensis*. We list the total number of hosts (N) and the number of infected hosts for each parasite. For *P. cubensis*, this includes the total number found, the number of host individuals infected with each OTU (Figure 4.2), and the number of infections that we did not identify.

Host	N	*Spauligodon anolis*	*Parapharyngodon cubensis*				
			Total	A	B	C	Unknown
Anolis acutus	84	56	6	0	4	2	0
Anolis cristatellus	421	40	179	53	98	0	28
Anolis evermanni	18	2	5	4	0	0	1
Anolis gundlachi	32	1	17	10	0	0	7
Anolis krugi	7	0	0	0	0	0	0
Anolis pulchellus	49	0	10	4	4	0	2
Anolis stratulus	30	1	4	1	3	0	0
Hemidactylus mabouia	3	0	0	0	0	0	0
Sphaerodactylus macrolepis	7	0	1	0	1	0	0
Total	651	100	222	72	110	2	38

Table 4.5. Number of *Spauligodon anolis* and *Parapharyngodon cubensis* sequenced for *coxI* at each locality. *Parapharyngodon cubensis* samples are identified by OTU (Figure 4.2). Localities are identified by a number that corresponds with the map in Figure 4.1 and locality information in Table 4.3.

Locality	Island	*Spauligodon anolis*	*Parapharyngodon cubensis*	
			A	**B**
1	Puerto Rico	2	2	0
2	Puerto Rico	0	0	1
3	Puerto Rico	4	0	0
4	Puerto Rico	2	8	0
5	Puerto Rico	4	0	0
6	Puerto Rico	0	0	8
7	Puerto Rico	3	3	1
8	Puerto Rico	0	*	4
9, 12	Puerto Rico	5	4	0
10, 11	Puerto Rico	5	1	0
13	Puerto Rico	0	0	5
14	Puerto Rico	1	2	0
15	Puerto Rico	2	2	0
16	Puerto Rico	2	*	2
17	Puerto Rico	1	2	0
18	Puerto Rico	1	3	0
19	Puerto Rico	0	1	0
20	Puerto Rico	0	3	0
21-30	Vieques	3	4	17
31-35	St. Thomas	8	8	12
36-38	St. Croix	11	0	6
39, 41	St. John	1	14	5
40	Jost Van Dyke	0	0	20
42-44	Tortola	0	9	11
45	Virgin Gorda	0	6	0
46	Anegada	0	0	14
	Total	55	72	106

*Locality presence inferred using *18s* sequence, but unable to amplify *coxI*.

Table 4.6. Summary statistics and estimates of population divergence for *Parapharyngodon cubensis* A, *Parapharyngodon cubensis* B, and *Spauligodon anolis* using 640-bp of *coxI*. Haplotype diversity is similar among OTUs, though nucleotide diversity (π) and the population mutation rate (θ_W) are highest in *S. anolis*. A greater proportion of variation is between - rather than within - populations in *S. anolis*.

OTU		N	H_N	HD	π	θ_W	Tajima's D	Exact Test of Non-differentiation	Φ_{st}
P. cubensis A	PRB only	72	47	0.977	0.01878	0.03063	-1.3151 ns	P = 0	0.34, P = 0
P. cubensis B	All	106	85	0.996	0.02076	0.03581	-1.3845 ns	P = 0	0.35, P = 0
	PRB only	103	83	0.996	0.02117	0.03601	-1.3613 ns	P = 0	0.35, P = 0
S. anolis	All	55	37	0.980	0.04941	0.04262	0.5629 ns	P = 0	0.76, P = 0
	PRB only	44	29	0.974	0.03951	0.03694	0.2518 ns	P = 0	0.65, P = 0

N = number of samples
H_N = number of haplotypes
HD = haplotype diversity
π = nucleotide diversity
θ_W = population mutation rate (Watterson's estimator)

Table 4.7. Isolation-by-distance results for *Spauligodon anolis*, *Parapharyngodon cubensis* A, and *Parapharyngodon cubensis* B. All except *P. cubensis* A exhibit significant isolation-by-distance, with the highest observed coefficient values in *S. anolis*. Both *S. anolis* and *P. cubensis* B occur on both the PRB and St. Croix, and each exhibit a lower correlation coefficient when the datasets are restricted to the PRB only.

	PRB + St. Croix		PRB only	
OTU	Coefficient	P-value	Coefficient	P-value
S. anolis	0.3334	0.001	0.3019	0.01
P. cubensis A	n/a	n/a	0.003843	0.45
P. cubensis B	0.2426	0.013	0.2040	0.04

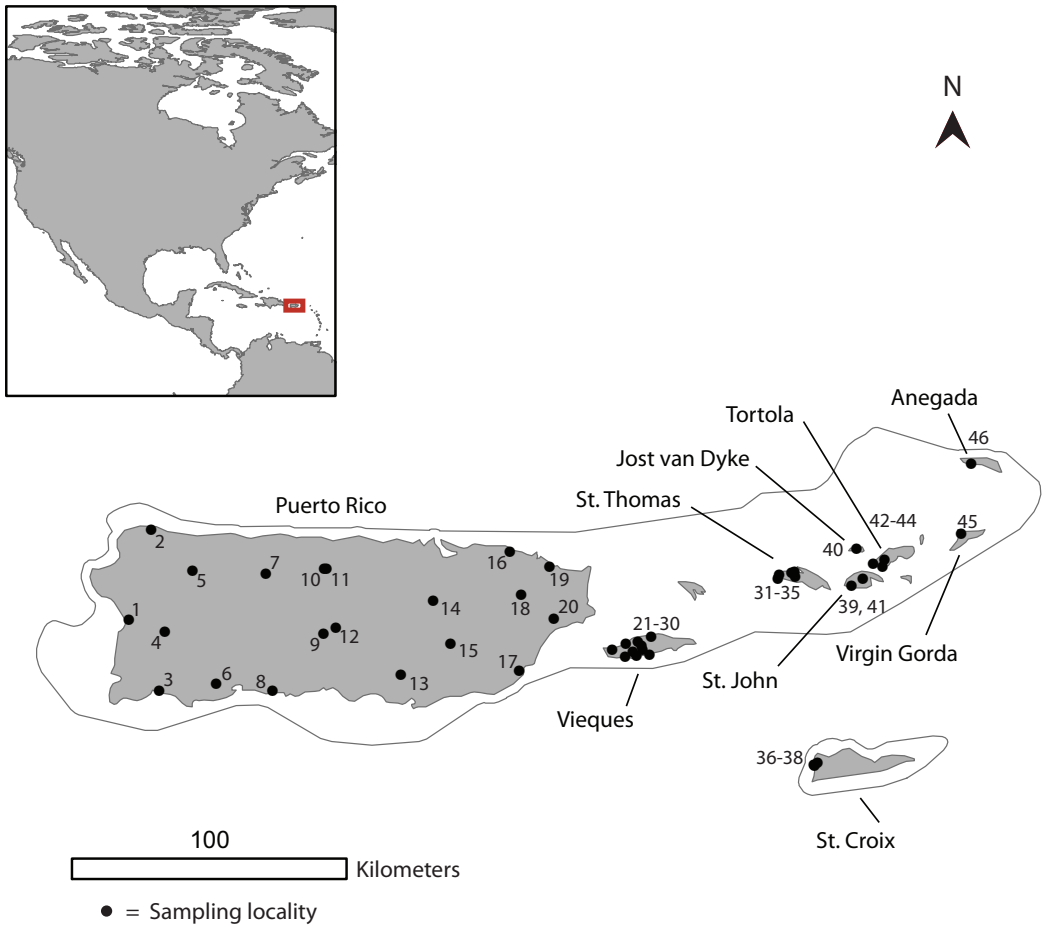

Figure 4.1. Map of sampling localities on the Puerto Rican Bank and St. Croix. The estimated emergent areas of the Puerto Rican Bank and St. Croix during the Pleistocene glacial maxima are delimited by the ~120m bathymetric level. Sampling localities are labeled with numbers that and correspond to locality information in Table 4.2 and prevalence data in Table 4.3.

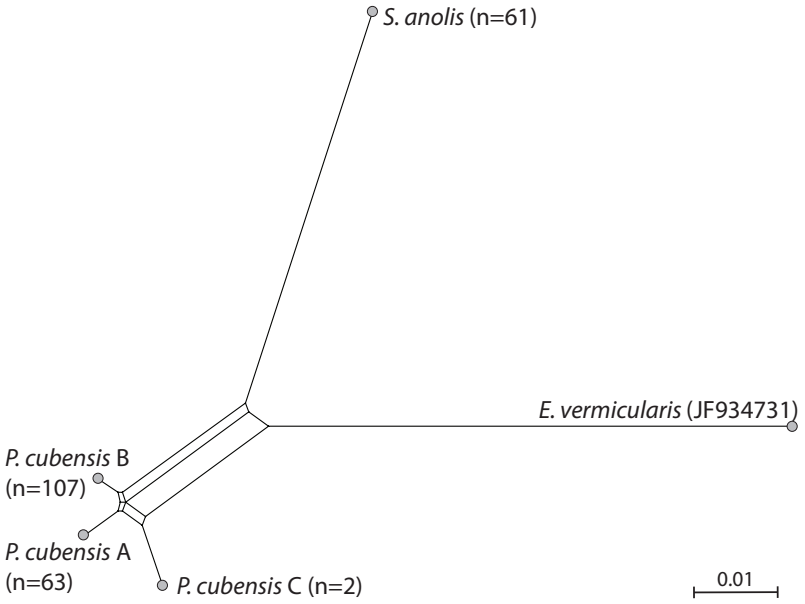

Figure 4.2. Neighbor-Net haplotype network inferred from 233 *18s* sequences of *Parapharyngodon cubensis* and *Spauligodon anolis*, along with a GenBank sample of *Eutrombicula vermicularis*. All *S. anolis* samples share a single haplotype. All *P. cubensis* samples share one of three haplotypes, and these are arbitrarily named A, B, and C. Scale bar shows the number of differences per site.

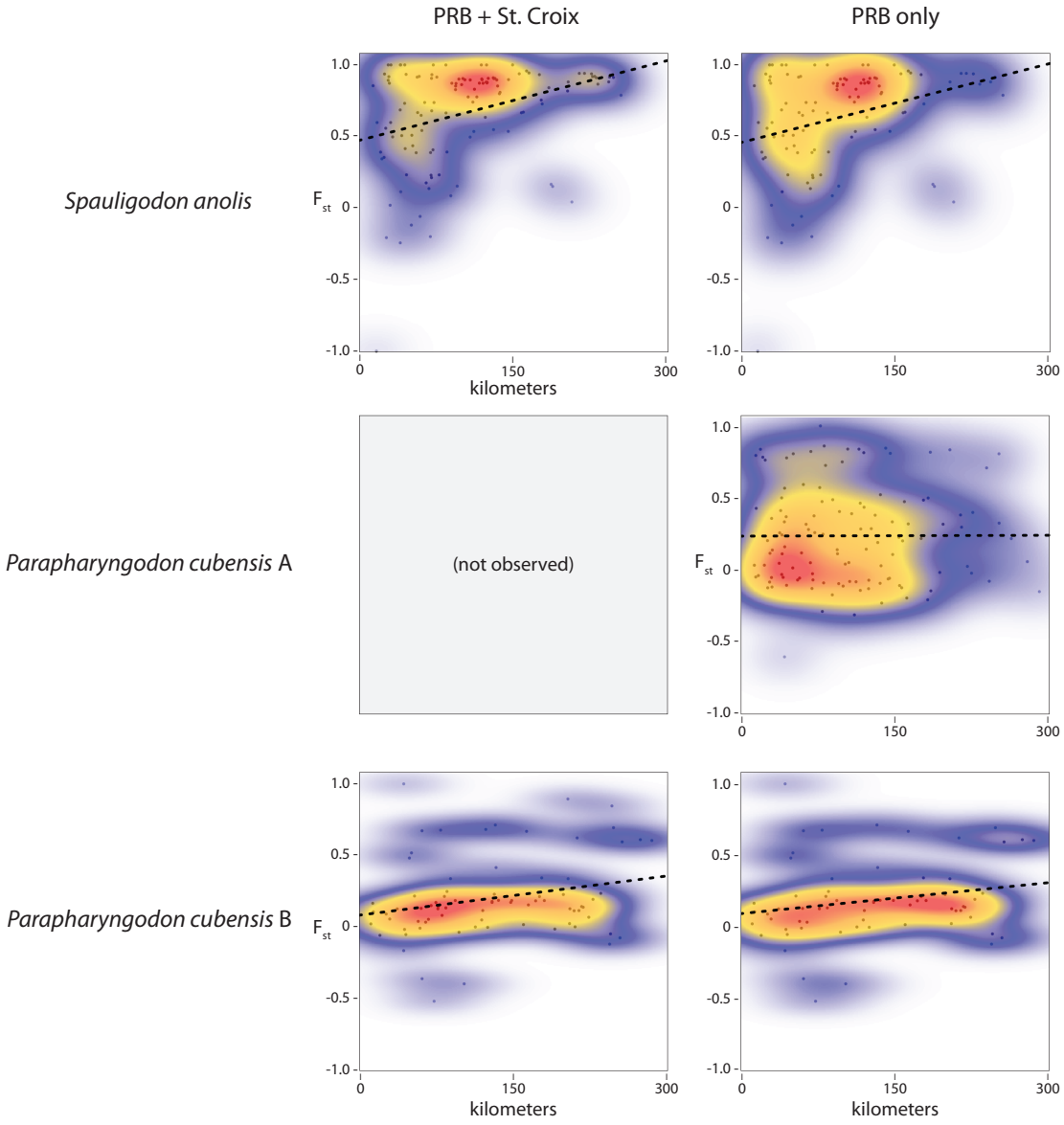

Figure 4.3. Density scatterplots of isolation-by-distance. Geographic distance between localities is on the X-axis, and genetic distance (F_{st} estimates) between localities is on the Y-axis. Color represents the relative density of points, with warmer colors indicating higher densities. A single high-density nucleus indicates clinal variation, and multiple high-density nuclei indicate an island model of differentiation. The dotted line shows the correlation between the two distance matrices.

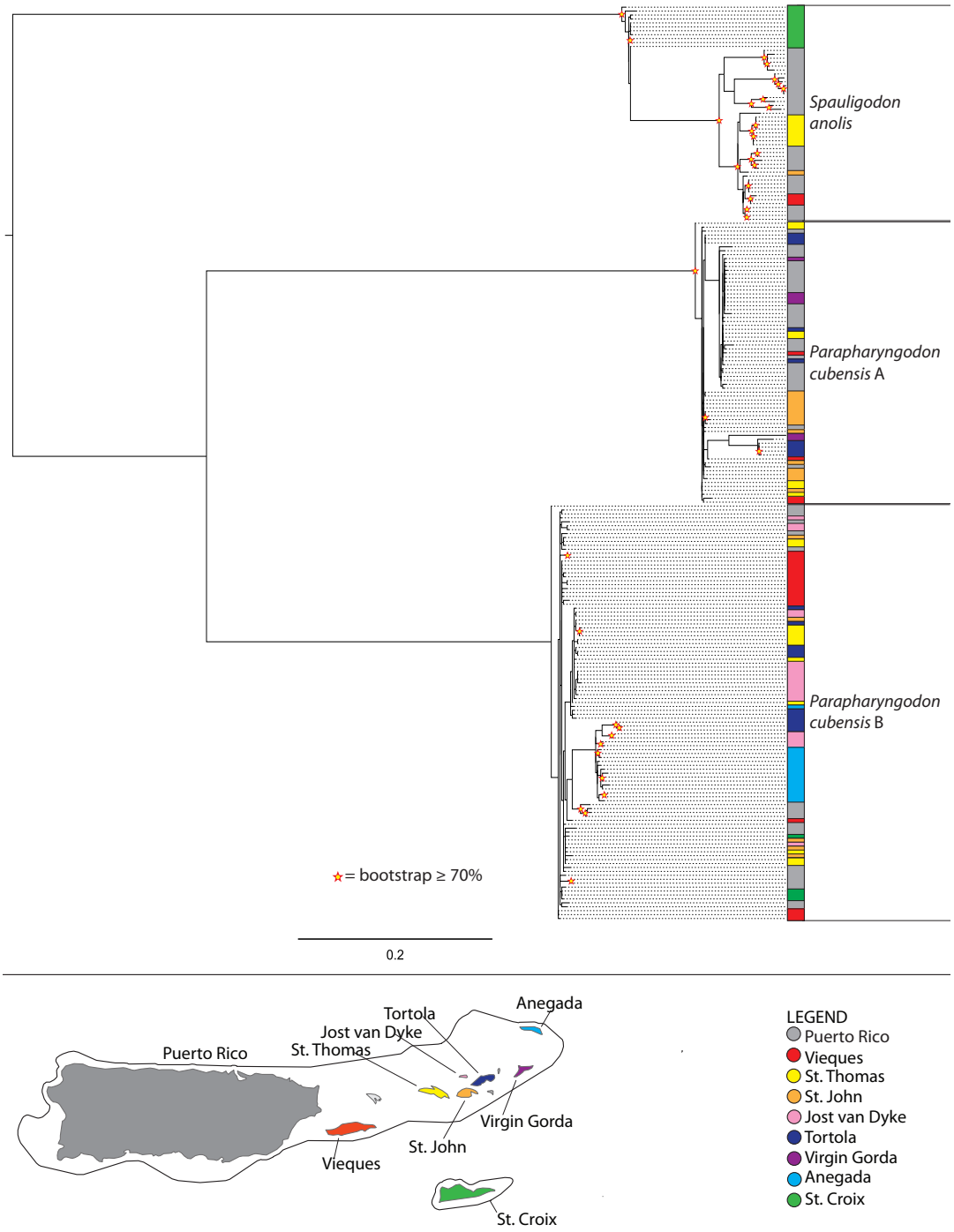

Figure 4.4. ML phylogenies of *Spauligodon anolis*, *Parapharyngodon cubensis* A, and *Parapharyngodon cubensis* B inferred using 640-bp of *coxI* and a GTR + Γ substitution model in RAxML. Samples of *S. anolis* collected from each of the smaller islands generally form well supported, monophyletic groups. In contrast, within-island samples for each of the *P. cubensis* OTUs are scattered throughout the phylogeny, and few clades are well supported.

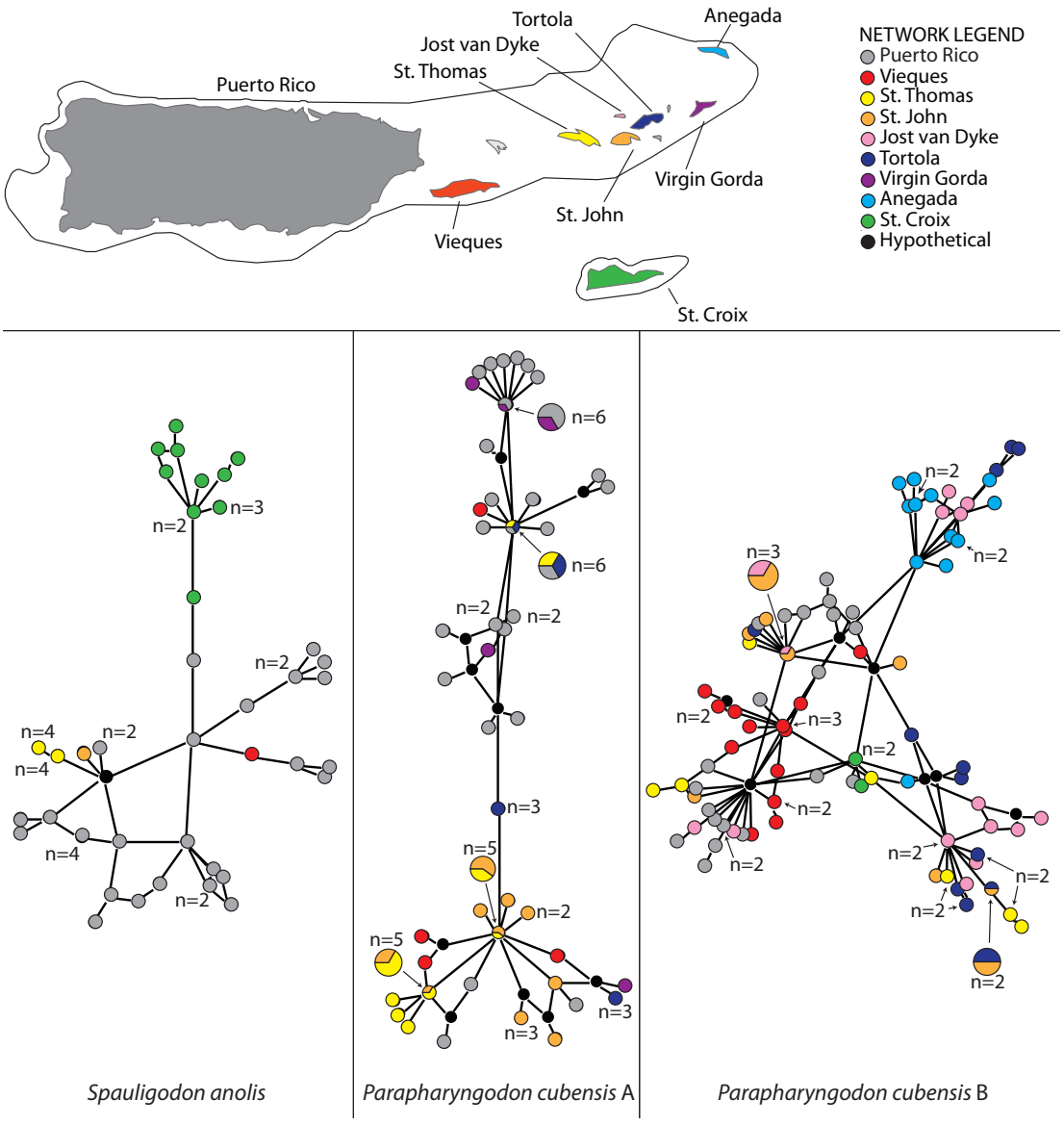

Figure 4.5. Median-joining haplotype networks of *Spauligodon anolis*, *Parapharyngodon cubensis* A, and *Parapharyngodon cubensis* B inferred using 640-bp of *coxI* in SPLITSTREE. *Spauligogodon anolis* haplotypes collected from each of the smaller islands are clustered together, and when more than one individual shares a single haplotype, these are always from the same island. Within-island samples in the *P. cubensis* OTUs are generally not clustered together, and in many cases, individuals from different islands share a single haplotype.

CHAPTER V

CONCLUSION

The primary goal of my dissertation research was to characterize the malaria and pinworm parasite diversity of Caribbean *Anolis* lizards. I compared taxonomic hypotheses in malaria parasites using morphological *vs*. molecular data (Chapter II), made predictions about malaria parasite diversification and tested them (Chapter III), and tested the hypothesis that host range in multi-host parasites influences population structure (Chapter IV). My general conclusions are:

- Traditional morphological criteria fail to delimit lizard malaria species on Hispaniola. This casts doubt on the validity on many lizard malaria parasite species that are delimited using these morphological characters, particularly in the Neotropics. Molecular data, on the other hand, are consistently useful in malaria parasite species identification and delimitation.
- Several taxonomic changes are made for malaria parasite species. *Plasmodium fairchildi hispaniolae* is elevated to *Plasmodium hispaniolae*, and this species is in general morphologically indistinguishable from *Plasmodium floridense*, with which it co-occurs on Hispaniola and the Puerto Rican Bank. *Plasmodium minasense anolisi* and *Plasmodium tropiduri caribbense* are not valid.
- Prevalence of lizard malaria parasites on Hispaniola is variable among species. *Anolis* lizards belonging to the crown-giant and grass-bush ecomorphs were not infected, suggesting that host ecology or phylogeny may play a role in parasite infection.

- Prevalence of lizard malaria parasites is generally low in Caribbean *Anolis* lizards, and is variable over the landscape. For example, overall prevalence of *P. floridense* on Hispaniola is ~5%, but it exceeds 50% prevalence in several localities.
- Diversification in *P. floridense* is shaped by the malaria parasite life cycle and transmission dynamics, where low prevalence favors inbreeding. It contains at least 11 independently evolving lineages in the Caribbean and southeastern North America. These lineages are characterized by low population sizes, have diverged very recently (some diverged ~0.11 million years ago), and most variation is contained between lineages (e.g., most polymorphic sites are fixed between lineages). This pattern is shared with human malaria parasite species, and may be common to all malaria parasites.
- Three operational taxonomic units (OTUs) are identified in the pinworm *Parapharyngodon cubensis*, suggesting it may be a complex of several species.
- Two *P. cubensis* OTUs are distributed on the Puerto Rican Bank, but these rarely co-occur at the same locality. This suggests that their distributions are mediated by competition, and are consistent with patterns of low-intensity among hosts.
- Transmission is important in shaping the population structure in the pinworms *Spauligodon anolis* and *P. cubensis* on St. Croix and the Puerto Rican Bank. Both are multi-host parasites found in *Anolis* lizards, but whereas *S. anolis* is found only in anoles, *P. cubensis* is found in anoles and many other squamate species. *Spauligodon anolis* exhibits greater population structure and isolation-by-distance than each of two *P. cubensis* OTUs.

APPENDIX:

SUPPLEMENTARY MATERIAL FOR CHAPTER III

(LIFE CYCLE AND TRANSMISSION SHAPE DIVERSIFICATION IN THE LIZARD MALARIA PARASITE *PLASMODIUM FLORIDENSE*)

Table S3.1. Primer information and PCR conditions used to generate sequence data in this study. All PCRs are nested and have a 25µl volume. Initial reactions use 2µl template (DNA extraction), and nested reactions use 1µl template (product from first reaction). All thermocyler protocols begin with a 90°C melting step for 4-minutes, then 40 cycles of a 90°C melting step for 30-seconds, an annealing step for 30-seconds, and a 68°C extension step with time depending on fragment length. Step-up protocols use the first temperature for the first five cycles, and the second temperature for the remaining 35 cycles. All protocols finish with a 10-minute, 68°C extension step.

Table S3.1.

Name	Locus	5'-Sequence-3'	PCR conditions
aslF aslR	*Adsl*	AGAGTGAAAAAAATTGAAGAAGAGAC GCTAAATGTAAATTACCTTCTGCATTTTC	46°C anneal 60-sec extension
aslFin aslRin	*Adsl* – nested	AAGAGACAAATCACGATGTGAAAGC ACCTTCTGCATTTTCAAAATCAATAGG	49°C anneal 60-sec extension
TubAF TubAR	*Atub*	CAGTCGGGCGTCATCAGGATTACAAGGATTTTTGATGTTT[a] GGAAACAGCTATGACCATCAGCTCTCATAACTTTTGCTAAATC[a]	44/55°C step-up 60-sec extension
TubAFin TubARin	*Atub* – nested	AGGAGGAGGTACTGGAAGCG ATCCGGTAGGGCACCAATCA	53/56°C step-up 60-sec extension
coxIF coxIR	*coxI*	CGAATCTTACTCATTCATATCCAAGCC GTATTTTCTCGTAATGTTTTACCAAAGAA	50°C anneal 90-sec extension
coxIF Pf.coxImR	*coxI* – nested 1	CGAATCTTACTCATTCATATCCAAGCC CTGGATGACCAAAAAACCAGAATAA	50°C anneal 60-sec extension
Pf.coxImF coxIR	*coxI* – nested 2	CAACATTTATTCTGGTTCTTTGGACATC GTATTTTCTCGTAATGTTTTACCAAAGAA	50°C anneal 60-sec extension
DW2 DW4	*cytb*	TAATGCCTAGACGTATTCCTGATTATCCAG TGTTTGCTTGGGAGCTGTAATCATAATGTG	52°C anneal 60-sec extension
DW2 3932R	*cytb* – nested 1	TAATGCCTAGACGTATTCCTGATTATCCAG GACCCCAAGGTAATACATAACCC	50°C anneal 60-sec extension
3932F DW4	*cytb* – nested 2	GGGTTATGTATTACCTTGGGGTC TGTTTGCTTGGGAGCTGTAATCATAATGTG	50°C anneal 60-sec extension
clpCF clpCR	*clpC*	GTTGGATTTTATGTGGDCCTAGTGG AAWGGACGWGCWCCATATAAAGG	49°C anneal 60-sec extension
clpCFin clpCRin	clpC – nested	TCTATTTCTAGATTAATAGG AAGGATTATAAGATAATTTAG	37/40°C step-up 60-sec extension
EF2F EF2R	*EF2*	CATGGAAAATCAACATTAACAGATTCT CAGGATATACTTGAATATCACCCAT	46°C anneal 60-sec extension
EF2Fin EF2Rin	*EF2* – nested	AGACAAGATGAACAAGAAAGATGT TCACCCATTAATTTATCTGTGTATGT	47°C anneal 60-sec extension
HH3F HH3R	HisH3	CAGTCGGGCGTCATCATTATGATCTTTCTCCACG[a] GGAAACAGCTATGACCATGAAAATCCACMGGAGGA[a]	41/55°C step-up 60-sec extension
HH3Fin HH3Rin	HisH3 – nested	AGCCCCAAGAAAGCAATTAG GATCTTTCTCCCAGAATACGTC	47/50°C step-up 60-sec extension
HSP70F HSP70R	HSP70	GGAACTATTGAACCATGTGAAAAAT TTAATACTAACTTGTGTTTGATTATCAGC	46°C anneal 60-sec extension
HSP70Fin HSP70Rin	HSP70 –nested	CTTGTGTTTGATTATCAGCTGCTG CCATGTGAAAAATGCATTAAAGATGC	49°C anneal 30-sec extension

[a] Contains CAG/UNI tag to facilitate annealing

Table S3.2. GenBank accession numbers and collecting localities for samples included in the identification phylogeny.

Species	Collecting locality	GenBank Number
Plasmodium azurophilum	Dominica	AY099055
Plasmodium azurophilum	Hispaniola	JN187894
Plasmodium berghei	n/a	AF014115
Plasmodium fairchildi	Costa Rica	AY099056
Plasmodium floridense	Dominica	AY099059
Plasmodium floridense	Florida	NC_09961
Plasmodium floridense	Hispaniola, sample "DR401"	JN187899[a]
Plasmodium floridense	Hispaniola, sample "DR453"	JN187902[a]
Plasmodium floridense	Hispaniola, sample "DR522"	JN187916[a]
Plasmodium floridense	Hispaniola, sample "M1064"	JN187935[a]
Plasmodium hispaniolae	Hispaniola	JN187914
Plasmodium hispaniolae	Hispaniola	JN187890
Plasmodium knowlesi	n/a	AY598141
Plasmodium leucocytica	Dominica	AY099058
Plasmodium leucocytica	Hispaniola	JN187938
Plasmodium mexicanum	Southwestern USA	NC_09960

[a]*Plasmodium floridense* samples for which we previously identified and submitted partial *cytb* sequences to GenBank. We sequenced the remainder of the locus and partial *coxI* for this analysis.

Table S3.3. Host and locality information for the samples included in this study. Parasite species identification is as inferred using the phylogeny of *cytb* and partial *coxI* data. General collecting region is noted along with exact GPS coordinates (in decimal degrees) of the collecting locality.

Table S3.3.

Species	Sample	Region	Host	Latitude	Longitude
Plasmodium fairchildi	MX12	Mexico	*Anolis uniformis*[a]	18.5851	-95.07451
Plasmodium floridense	REG2223	Cuba	*Anolis sagrei*	22.4012	-81.4277
	REG2251	Cuba	*Anolis sagrei*	22.1668	-81.1375
	REG2611	Cuba	*Anolis sagrei*	19.9144	-77.2011
	REG2840	Cuba	*Anolis sagrei*	21.8128	-78.1381
	REG2939	Cuba	*Anolis sagrei*	22.4994	-79.4574
	REG2940	Cuba	*Anolis sagrei*	22.4994	-79.4574
	REG2941	Cuba	*Anolis sagrei*	22.4994	-79.4574
	FL1101	Florida	*Anolis sagrei*	27.04453	-82.27919
	FL1111	Florida	*Anolis sagrei*	27.04453	-82.27919
	FL1181	Florida	*Sceloporus undulatus*	30.33554	-84.44075
	FL179	Florida	*Anolis sagrei*	27.04453	-82.27919
	FL509	Florida	*Anolis sagrei*	26.68313	-81.91175
	FL513	Florida	*Anolis sagrei*	26.68313	-81.91175
	FL519	Florida	*Anolis sagrei*	27.04453	-82.27919
	DR158	Hispaniola	*Anolis distichus*	18.05374	-71.2898
	DR159	Hispaniola	*Anolis cybotes*	18.05374	-71.2898
	DR171	Hispaniola	*Anolis cybotes*	18.05374	-71.2898
	DR199	Hispaniola	*Anolis cybotes*	18.91468	-70.72939
	DR216	Hispaniola	*Anolis cybotes*	18.8801	-69.11956
	DR221	Hispaniola	*Anolis cybotes*	18.8801	-69.11956
	DR232	Hispaniola	*Anolis cybotes*	18.51567	-68.36947
	DR233	Hispaniola	*Anolis cybotes*	18.51567	-68.36947
	DR235	Hispaniola	*Anolis cybotes*	18.51567	-68.36947
	DR401	Hispaniola	*Anolis cybotes*	19.86266	-70.96433
	DR453	Hispaniola	*Anolis cybotes*	19.30035	-69.17233
	DR522	Hispaniola	*Anolis cybotes*	18.77687	-71.19923
	M1064	Hispaniola	*Anolis cybotes*	18.96772	-72.72537
	JA.004.01	Jamaica	*Anolis lineatopis*	18.08762	-76.31215
	JA.042.05	Jamaica	*Anolis lineatopis*	18.08121	-76.31284
	JA.059.06	Jamaica	*Anolis valencienni*[a]	18.07865	-76.31339
	JA.082.08	Jamaica	*Anolis lineatopis*	18.38966	-77.05101
	JA.084.10	Jamaica	*Anolis lineatopis*	18.38966	-77.05101
	JA.122.15	Jamaica	*Anolis grahami*	18.45763	-77.27183
	JA.123.16	Jamaica	*Anolis lineatopis*	18.45763	-77.27183
	JA.124.17	Jamaica	*Anolis lineatopis*	18.45763	-77.27183
	JA.125.18	Jamaica	*Anolis lineatopis*	18.45763	-77.27183
	JA.126.19	Jamaica	*Anolis lineatopis*	18.45763	-77.27183
	JA.128.20	Jamaica	*Anolis lineatopis*	18.45763	-77.27183
	JA.130.21	Jamaica	*Anolis grahami*	18.45763	-77.27183
	JA.219.22	Jamaica	*Anolis grahami*	18.04946	-77.85984
	JA.220.23	Jamaica	*Anolis valencienni*[a]	18.05033	-77.87384
	JA.222.24	Jamaica	*Anolis lineatopis*	18.05033	-77.87384
	PR.022.279	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*	18.36646	-66.68867
	PR.024.280	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*	18.36646	-66.68867

	PR.133.288	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*	18.16853	-66.49990
	VI.037.51	Puerto Rican Bank (St. John)	*Anolis cristatellus*	18.32669	-64.76401
	VI.045.52	Puerto Rican Bank (St. John)	*Anolis cristatellus*	18.32669	-64.76401
	VI.047.53	Puerto Rican Bank (St. John)	*Anolis cristatellus*	18.32669	-64.76401
	VI.360.93	Puerto Rican Bank (St. John)	*Anolis cristatellus*	18.34926	-64.72855
	VI.333.89	Puerto Rican Bank (St. Thomas)	*Anolis cristatellus*	18.37065	-64.95063
	VI.338.90	Puerto Rican Bank (St. Thomas)	*Anolis cristatellus*	18.37065	-64.95063
	VI.352.92	Puerto Rican Bank (St. Thomas)	*Anolis cristatellus*	18.35664	-64.95004
	VI.227.82	Puerto Rican Bank (Virgin Gorda)	*Anolis cristatellus*	18.49719	-64.40387
	VI.234.83	Puerto Rican Bank (Virgin Gorda)	*Anolis pulchellus*	18.49719	-64.40387
	VI.242.84	Puerto Rican Bank (Virgin Gorda)	*Anolis cristatellus*	18.49719	-64.40387
	VI.245.85	Puerto Rican Bank (Virgin Gorda)	*Anolis cristatellus*	18.49719	-64.40387
	VI.249.86	Puerto Rican Bank (Virgin Gorda)	*Anolis cristatellus*	18.49719	-64.40387
	VI.252.87	Puerto Rican Bank (Virgin Gorda)	*Anolis cristatellus*	18.49719	-64.40387
	SAB1344	Saba	*Anolis sabanus*	17.63337	-63.22742
	SAB1361	Saba	*Anolis sabanus*	17.63354	-63.22411
	SAB6120	Saba	*Anolis sabanus*	17.63037	-63.25434
	SAB6123	Saba	*Anolis sabanus*	17.64001	-63.25322
	SAB6133	Saba	*Anolis sabanus*	17.63039	-63.25255
Plasmodium hispaniolae	PR.146.290[b]	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*[a]	18.13538	-66.08157
	PR.179.295	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*[a]	18.03267	-66.24347
	PR.234.298	Puerto Rican Bank (Puerto Rico)	*Anolis cristatellus*[a]	18.38813	-65.75592
	PR.243.299	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.15762	-65.42194
	PR.245.300	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.15762	-65.42194
	PR.266.301	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.09369	-65.50729
	PR.268.302	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.10911	-65.48299
	PR.269.303	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.10911	-65.48299
	PR.271.304	Puerto Rican Bank (Vieques)	*Anolis cristatellus*[a]	18.10911	-65.48299

[a]New host/parasite record.
[b]PR.146.290 (*P. hispaniolae*) was sequenced at all loci and used as an outgroup taxon when applicable.