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Tree-based delimitation of morphologically ambiguous taxa: A study of the lizard malaria parasites on the Caribbean island of 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, and this 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 with a molecular phylogenetic approach for assessing the taxonomy of these parasites. We surveyed for blood parasites from 677 *Anolis* lizards, representing 26 *Anolis* spp. 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 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 for only 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 recognised 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.

1. Introduction

The taxonomy of the malaria parasites in the genus *Plasmodium* has typically 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* spp., 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 since 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 from

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☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession number(s): JN187863–JN187938.

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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 the 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* sp. 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., 1988), 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 also 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 is 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 (Fig. 1). Moreover, any specific identification that employs these features must assume that the size distributions of each of the four species are well characterised. Generally speaking, a gametocyte that is moderate in size, oval in shape, contains 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 characterises 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 and 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 determine species limits. This approach been used for the malaria parasites of birds (e.g., Bensch et al., 2004, 2007; Sehgal et al., 2006; 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, noting 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* spp.

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, 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, 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; Wiens and Penkrot, 2002; Funk and Omland, 2003). 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, 2011).

The purpose of this study was to evaluate the utility of a molecular phylogenetic approach in assessing the phylogenetics and taxonomy of saurian malaria parasites on Hispaniola. We employed 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 with those based on molecular data. We estimated phylogenetic trees using nucleotide data from the mitochondrial genes cytochrome *b* (*cytb*) and cyto-