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Short communication

Plasmodium blood parasite found in endangered Galapagos penguins (*Spheniscus mendiculus*)

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ABSTRACT

This is the first report of a *Plasmodium* blood parasite found in the Galapagos Archipelago. Phylogenetic analyses place this parasite, recovered from endangered Galapagos penguins (*Spheniscus mendiculus*), within the genus *Plasmodium*, and suggest a close relationship to some of the most dangerous lineages of *Plasmodium* that have been known to cause severe mortality and morbidity in captive penguin populations. Infectious disease is an increasingly important cause of global species extinctions, and extinctions due to avian pox and avian malaria (*Plasmodium relictum*) have been well documented in Hawaiian avifauna. *Plasmodium* blood parasites had not been detected in Galapagos birds until now, despite previous microscopic and molecular screening of many of the species, including the Galapagos penguin. While penguin populations now appear healthy, it is unclear whether this parasite will have an obvious impact on their survival and reproduction, particularly during El Niño events, which cause stress due to reduced food availability. It is possible that this parasite arrived with or shortly after the recent arrival of an introduced mosquito, *Culex quinquefasciatus*, known elsewhere as a competent vector of *Plasmodium* blood parasites.

1. Introduction

The Galapagos Islands are located on the equator approximately 1000 km west of continental Ecuador. Humans have inhabited the archipelago for 200 years, and much of the original biodiversity remains intact, with only 5% species loss (Gibbs et al., 1999). Due to isolation and high endemism, there is concern regarding the introduction of diseases. Island populations are often more susceptible to introduced pathogens, as they have historically been exposed to fewer pathogens than mainland populations (e.g., Fromont et al., 2001). Introduced pathogens, primarily avian pox (*Avipoxvirus*) and avian malaria (*Plasmodium relictum*) are a likely cause of major population declines and extinctions in Hawaiian avifauna (van Riper et al., 1986, 2002). Ongoing disease monitoring is an essential part of conservation efforts in Galapagos (Parker et al., 2006) to prevent extinction due to introduced diseases, increasingly recognized as causes of global wildlife extinctions worldwide (Smith et al., 2006). Here we report a blood parasite in the genus *Plasmodium* found in the endemic Galapagos penguin, which could threaten the health of penguins and other bird species.

Plasmodium, *Haemoproteus* and *Leucocytozoon* (sub-order Haemosporina, phylum: Apicomplexa) are related genera of vector-born protozoan blood parasites commonly found throughout reptiles, birds and mammals. Some *Plasmodium* species are pathogenic and cause disease in wild and captive animals. While *Haemoproteus* parasites appear to have fewer detrimental effects on hosts, some fitness reductions have been documented (e.g., Allander, 1997). Avian malaria, the disease in birds caused by some parasites in the genus *Plasmodium*, causes considerable morbidity and mortality in outdoor penguin exhibits in zoos, where pathogenic species are identified as *P. relictum* and *P. elongatum* (e.g., Fleischman et al., 1968; Stoskopf and Beier, 1979). While many of the world's penguins are distributed in the Antarctic region, some species breed at lower latitudes in temperate environments, where they may naturally encounter these parasites (Graczyk et al., 1995). There are concerns regarding *Plasmodium* parasites in penguins, due, in part, to the acute infections found in captive populations (Fleischman et al., 1968; Stoskopf and Beier, 1979; Fix et al., 1988; Cranfield et al., 1994). There are few reports of blood parasites in wild penguins (e.g., Jones and Shellam, 1999), but the potential for *Plasmodium* to cause disease in endangered or geographically isolated bird populations is grounds for concern and monitoring (Jones and Shellam, 1999; Miller et al., 2001).

The Galapagos penguin (*Spheniscus mendiculus*) is endemic to the Galapagos Islands and classified as Endangered (BirdLife

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International, 2008) due to small population size and restricted geographical range. El Niño events reduce populations of the Galapagos penguin by as much as 50% (Vargas et al., 2006), as warmer waters disrupt upwelling of nutrient-rich cold water that supports the marine ecosystem. The current population of Galapagos penguins is approximately 1500 individuals (Jiménez-Uzcátegui and Vargas, 2008). Galapagos penguins exhibit low levels of genetic diversity (Nims et al., 2008) and very low variation in major histocompatibility complex (MHC) genes (Bollmer et al., 2007), which could contribute to the susceptibility of the population to infectious disease. Overall, the Galapagos penguin population appears healthy, based on surveys of hematology, serum chemistry and serology (Travis et al., 2006). No intraerythrocytic blood parasites were found in microscopic screens of blood smears (Travis et al., 2006). Galapagos penguins ($n = 94$) sampled in 1996 were tested for *Plasmodium* using a molecular screening technique (polymerase chain reaction (PCR)), and no penguins tested positive (Miller et al., 2001).

2. Materials and methods

2.1. Sample collection

Between August 2003 and March 2005, a total of 401 samples were collected from 362 Galapagos penguins captured during four field seasons at 29 sites from seven islands of the Galapagos Archipelago (Table 1, Fig. 1). Due to close proximity and small area, the three Mariela islands are considered here as one Island (Marielas). All tested penguins were marked with microchips (PIT tags) for identification and assessment of survivorship in subsequent field seasons. Details on sample collection, processing and analysis, can be found in Travis et al. (2006).

2.2. Molecular screening

DNA was extracted from blood using a standard phenol–chloroform extraction protocol (Sambrook et al., 1989), and PCR was used to amplify a region of the parasite mitochondrial cytochrome *b* gene. Positive and negative controls were always used and test samples were only run with other Galapagos penguin samples to avoid interspecific contamination. A subset of positive samples were re-amplified to confirm that the first test showed true positive and not contamination. Primers included an initial outer reaction (DW2 and DW4) followed by an internal re-amplification (HaemoR and DW1; Perkins and Schall, 2002). Reaction conditions for DW2 and DW4 were identical to Perkins and Schall (2002) except for the addition of an initial dwell at 94° for 2 min and an annealing temperature of 55° instead of 60° C. Touchdown reaction conditions for HaemoR and DW1 are: initial dwell at 94° for 2 min, followed by 20 cycles of 94° for 30 s, 54° for 30 s (decreasing by 0.5° each cycle) and 72° for 90 s. The program then has 25 cycles of 94° for 30 s, 44.5° for 30 s and 72° for 90 s and a final extension for 15 min. PCR reactions were performed using Takara Ex taq polymerase (Takara Bio Inc.). One microliter of stock DNA was used in the initial reaction, and 0.5 μl of product from the initial reaction was used as a template for the internal re-amplification reaction. Approximately 600 base pairs of double-stranded sequence were obtained on an Applied Biosystems 3100 DNA Analyzer at the University of Missouri – St. Louis.

2.3. Phylogenetic analysis

Sequences were edited in Seqman 4.0, added to a larger dataset containing additional cytochrome *b* sequence data obtained from GenBank (Appendix A, electronic supplement), and aligned using BioEdit (Version 7.0.9.0). Using parameters estimated from the data, the HKY85+I+Γ (Hasegawa et al., 1985) model of nucleotide substitution was used to reconstruct a maximum clade credibility phylogeny (BEAST, 10,000 trees; Drummond and Rambaut, 2007) with maximum likelihood branch lengths (PAUP 4.0) and in a ML bootstrap analysis (500 pseudoreplicates) (Treefinder, Jobb, 2008). BEAST initiates a pre-burn-in to stabilize likelihood values, after which it begins sampling. Parameters in BEAST allow for mutation rate heterogeneity among branches of the phylogeny, in which any biases due to disproportionately long branches are reduced (relaxed clock: uncorrelated lognormal). Priors for the model were optimized by the program using the Yule tree option. Unlike coalescent approaches in which only some lineages are assumed to leave descendants, the Yule tree option assumes that such lineages have already been pruned (Drummond and Rambaut, 2007). The likelihood stationarity of sampled trees was determined graphically via a log-likelihood frequency histogram in Tracer (v1.4; Rambaut and Drummond, 2007).

3. Results

The PCR screen identified 19 (5%) of 362 penguins as positives for *Plasmodium*. The prevalence of the parasite in the four field seasons ranged from 3% to 7% and did not show a tendency to increase from 2003 to 2005 (Table 1). Most positive penguins were found on

Table 1
Number of samples and Plasmodium prevalence in 362 PIT-tagged penguins studied during four field seasons in the Galapagos Islands between 2003 and 2005. Number in parenthesis indicates number testing positive for *Plasmodium*.

Island	Field seasons				Total
	August 2003	March 2004	August 2004	February–March 2005	
Isabela	36 (4)	80 (4)	65 (3)	61 (2)	242 (13)
Marielas	12	20	25	37 (2)	94 (2)
Fernandina	26 (1)	7	1	6	40 (1)
Bartolomé				14 (2)	14 (2)
Santiago				7 (3)	7 (3)
Floreana				3	3
Santa Cruz				1	1
Total samples	74 (5)	107 (4)	91 (3)	129 (9)	401 (21)
Prevalence %(+)	7	4	3	7	5
Penguins (−) recaptured	0	7	8	22	37
Penguins (+) recaptured	0	1	1	0	2[a]
Total penguins	74	99	82	107	362

(+) *Plasmodium* positive.
(−) *Plasmodium* negative.
[a] Tested positive for the first time in August 2003.

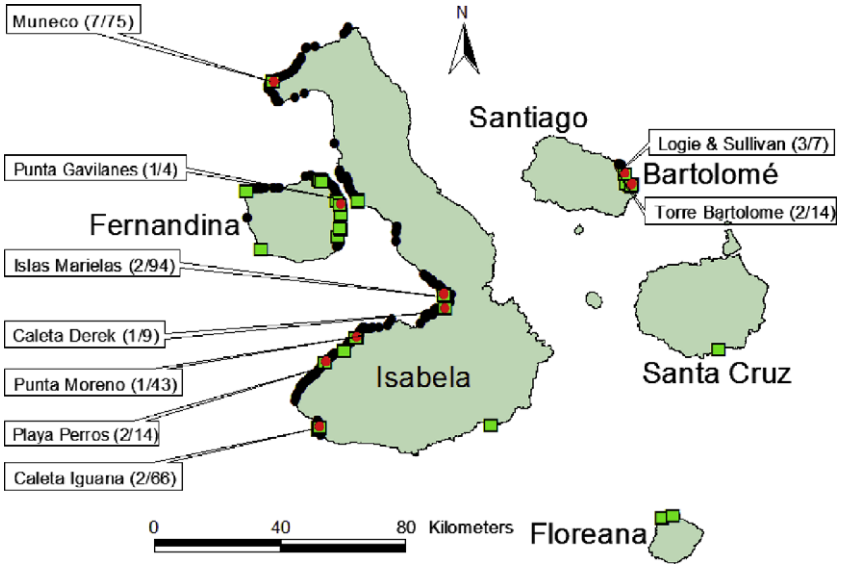

Fig. 1. Spatial distribution of *Plasmodium* in the Galapagos Islands in 2003–2005 based on GPS locations. Red dots indicate locations of positive samples. Green squares are sampling sites. Black dots show distribution of the penguin population during the annual census in September 2005. Penguins are not resident breeders on Santa Cruz. Numbers in parentheses show prevalence of *Plasmodium* at each site (number of positive samples/number of total samples).

northern and western Isabela as well as on Santiago and Bartolomé Islands (Fig. 1). Two penguins that tested positive in the first sampling season were in good health conditions when recaptured in subsequent sampling seasons after seven and 12 months, respectively, and still tested positive (Table 1). Based on molecular sexing data, the 19 positive penguins consisted of 14 adult males and 5 females, three of which were juveniles.

Because screening primers amplify both *Haemoproteus* and *Plasmodium* parasites, DNA sequencing and phylogenetic analysis were used for identification. Phylogenetic analyses place all but one of the Galapagos penguin parasite sequences within a large clade containing all *Plasmodium* parasites (Fig. 2). Galapagos penguin *Plasmodium* sequences are distinct from any other available sequences, and form their own evolutionary unit or clade. Their position within the larger *Plasmodium* clade is near a *P. elongatum* sequence and sequences belonging to the subgenus, *P. huffia*, which includes *P. elongatum*, although this placement does not have strong support. While nearly all of the sequences from this parasite can be unequivocally assigned to the genus *Plasmodium*, one parasite sequence from a Galapagos penguin sequence clustered with *Haemoproteus* (*Haemoproteus 11*).

4. Discussion

This is the first time a blood parasite in the genus *Plasmodium* has been identified in a Galapagos bird. Our phylogenetic inference places this parasite within the genus *Plasmodium* and sister to a clade containing *P. elongatum*, a parasite known to cause avian malaria in penguins and *P. huffia*, the subgenus that contains *P. elongatum* (Fleischman et al., 1968; Cranfield et al., 1994). There is strong support for the inclusion of the blood parasite in Galapagos penguins within *Plasmodium*, but weaker support for a particular sister clade within *Plasmodium*. More sequence data from additional genes and longer sequences could help resolve some of these relationships. One sequence recovered from penguins clustered with *Haemoproteus* sequences, and, to our knowledge, is the first reported *Haemoproteus* parasite in a penguin.

Despite the lack of resolution within *Plasmodium* and uncertainty of the exact sister taxa, we recommend that management strategies consider that this *Plasmodium* is closely related to a species that causes acute avian malaria in captive penguins. Penguins appear susceptible to serious infection by *P. relictum* and *P. elongatum*, and the Galapagos penguin is likely immunologically naïve since it evolved in an isolated island system. Immunological naïveté has been implicated as an important factor in the loss of Hawaiian avifauna due to introduced avian malaria and avian pox (van Riper et al., 1986). If this parasite is recently introduced, it could have disastrous consequences due to the lack of immunity or past exposure that would protect populations from serious infection. Our only evidence suggesting it might not be a pathogenic parasite under benign circumstances is that none of the penguins testing positive in our study showed any clinical indication of illness (see Travis et al., 2006).

The only arthropod present in Galapagos that is known to be a competent vector for *Plasmodium* elsewhere is the mosquito *Culex quinquefasciatus*, first reported in 1989 and well established by 2003 (Whiteman et al., 2005). Miller et al. (2001) suggest there could be a connection between the introduction of *C. quinquefasciatus* and the disappearance of resident penguins on the north shore of the human-inhabited island of Santa Cruz. The other bird-biting mosquito in the archipelago is a native, brackish-water mosquito, *Ochlerotatus taeniorhynchus* (sometimes called *Aedes taeniorhynchus*). Extensive sampling of mosquito populations around penguin colonies is necessary in order to further characterize this parasite, identify its vector, and develop an appropriate management strategy. The *Plasmodium* sequences recovered from Galapagos penguins belong to one phylogenetic lineage whose members are genetically similar, which also suggests a recent arrival with insufficient time for further differentiation. A final piece of evidence suggesting this is a newly introduced parasite is that Miller et al. (2001) found no infected penguins of 96 tested in 1996 using a similar PCR protocol. Based on our prevalence estimates, we would have detected approximately five positive birds with a similar sample size.

The 19 positive penguins were widely distributed across 9 sites of five islands in the Archipelago (Fig. 1). No *Plasmodium* parasites were detected in sites of the southern portion of the penguin distribution and this may be related to the low sample sizes (1 from Santa Cruz, 3 from Floreana and 12 from Puerto Villamil in southern Isabela) and low densities of penguins that limited capturing success. It is possible that the parasite will soon become wide-

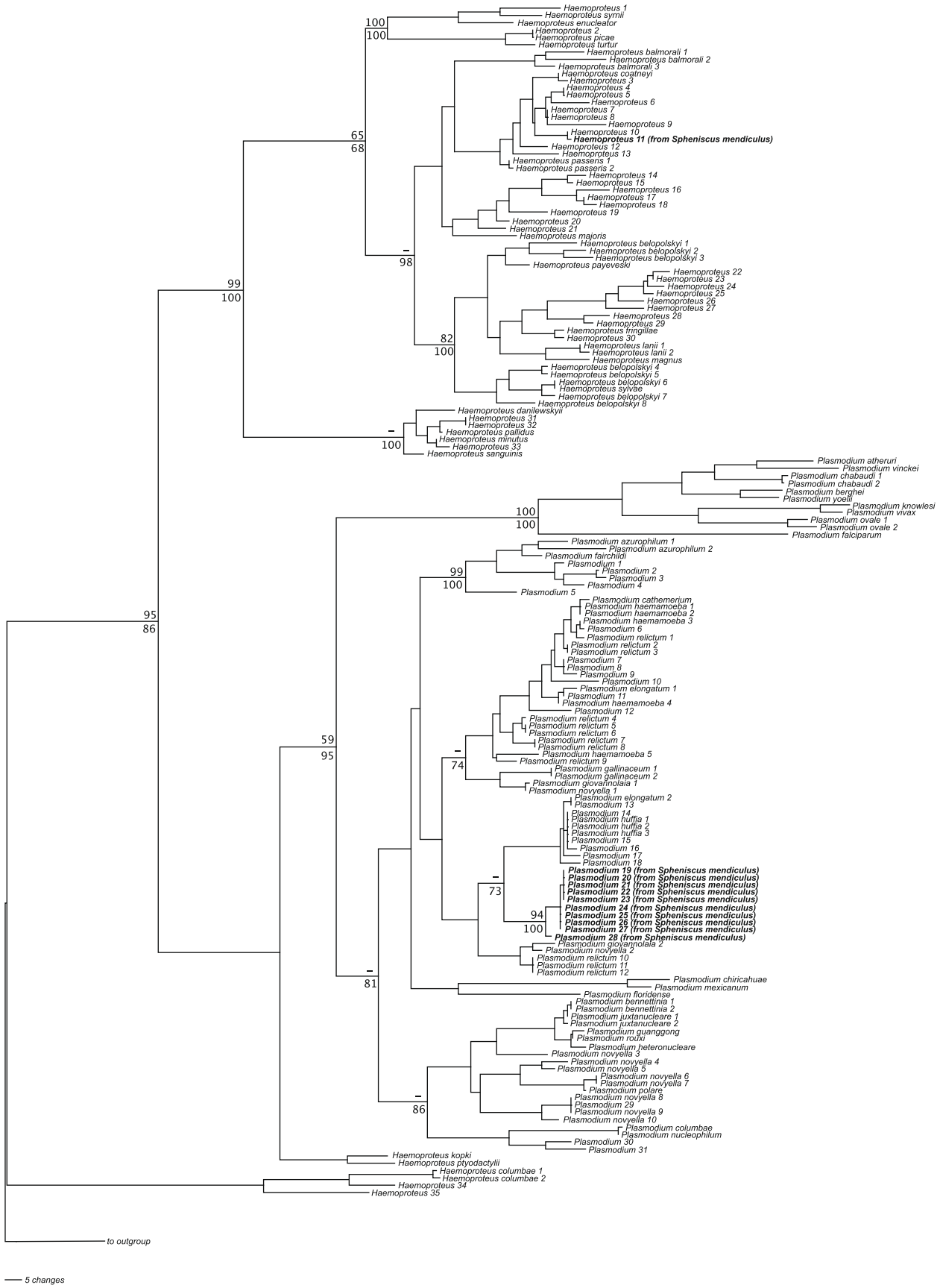

Fig. 2. Maximum clade credibility phylogenetic hypothesis of haemosporidian parasites based on mitochondrial *cytochrome b*. ML bootstrap values appear above nodes and Bayesian posterior probabilities appear below nodes. Parasite lineages are detailed in Appendix A and listed in the order within the phylogeny (top to bottom).

spread along the whole distributional range of the penguin population as recent genetic evidence suggests that the penguins may move long distances (Nims et al., 2008), at least during some part of their lives, and infections can be long-lasting. This also suggests that locations of infected penguins in this study may tell us little about where those infections were contracted.

Given that Galapagos penguins are severely affected by El Niño events, the additional stress caused by an infection with *Plasmodium* could lead to a more serious population decline. Stress has been demonstrated to be positively correlated with *Plasmodium* prevalence (Richner et al., 1995). In experimentally enlarged broods, male Great tits (*Parus major*) increased their feeding effort by 50% and had significantly higher prevalence of *Plasmodium* parasites than males attending control broods (Richner et al., 1995). The last El Niño event occurred in 1997–1998, and based on Miller et al.'s (2001) 1996 sampling and findings, we have no evidence to believe that *Plasmodium* parasites were infecting penguins during this stressful El Niño event. Therefore, the combined effects of *Plasmodium* parasitism and stronger (and more stressful) El Niño events in light of future climate change scenarios could place this endangered population at an even greater risk of extinction (see Vargas et al., 2007). We recommend immediate action to identify the vector for this parasite, and continued monitoring of penguin populations as well as other bird populations at risk of infection.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocon.2009.06.017.

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