J. Parasitol., 99(2), 2013, pp. 270–276

DIVERSITY AND PHYLOGENETIC RELATIONSHIPS OF HEMOSPORIDIAN PARASITES IN BIRDS OF SOCORRO ISLAND, MÉXICO, AND THEIR ROLE IN THE RE-INTRODUCTION OF THE SOCORRO DOVE (*ZENAIDA GRAYSONI*)

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ABSTRACT: The Socorro dove *Zenaida graysoni*, endemic to Socorro Island, was last reported in the wild in 1972. Fortunately, the species has been propagated in zoos in Europe and the United States, and plans are under way to re-introduce it to its native habitat. This will be the first known attempt to return a bird species extinct in the wild to its ancestral island. In order to assess the disease threats the Socorro dove may face, the avifauna of Socorro Island, with a specific focus on Socorro ground doves *Columbina passerina socorroensis* and mourning doves *Zenaida macroura*, as well as Socorro doves in captivity, were screened for blood parasites of the genera *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, and *Trypanosoma* spp. We found *Haemoproteus* spp. in 17 (74%) of 23 Socorro ground doves, 23 (92%) of 25 mourning doves, and 3 (14%) of 21 northern mockingbirds; none of the other bird species showed infections. Here, we report the phylogenetic analysis of 19 distinct lineages of *Haemoproteus* spp. detected in birds of Socorro Island and compare their evolutionary relationships to parasites detected in the avifauna of the Galápagos Islands, continental Latin America, and Europe. Microscopic examination revealed 1 mourning dove infected with *Plasmodium* (*Haemamoeba*), thus underscoring the importance of using both PCR and microscopy when analyzing avian blood samples for hemosporidian parasites. The study confirms that the Socorro dove will most likely be exposed to *Haemoproteus* spp. that currently infect mourning doves and Socorro ground doves of Socorro Island. A monitoring program for both birds and vectors should be implemented to establish the prevalence of *Plasmodium* sp. and as a necessary conservation measure for critically endangered birds on the island.

Socorro Island is the largest of the 4 Revillagigedo Islands, located approximately 700 km west of the port city of Manzanillo, Colima, México. Because of its isolation from the mainland, there is a high degree of biotic endemism (Brattstrom, 1990). Vertebrate wildlife diversity is low, with terrestrial birds predominating among a few reptiles and very few non-endemic mammals (Brattstrom, 1990). A major concern for vertebrate populations on Socorro Island, as for many other island populations, is the introduction of pathogens to immunologically naive species. For example, the introduction of avian malaria (*Plasmodium relictum*) contributed to major declines and extinctions in the avifauna of Hawaii (Warner, 1968; Van Riper et al., 1986; Van Riper et al., 2002), and may negatively affect the avifauna of the Galápagos Islands in the future (Padilla et al., 2004; Parker et al., 2006; Levin et al., 2009; Santiago-Alarcón et al., 2012).

Disease monitoring is essential for the conservation of island populations in order to prevent the spread of introduced pathogens that may cause the extirpation of one or more species. Such efforts are extremely important on Socorro Island because of the concentration of critically endangered bird species (Martínez-Gómez and Curry, 1996; Martínez-Gómez et al., 2001; Martínez-Gómez and Jacobsen, 2004; Martínez-Gómez et al., 2010). Socorro Island's terrestrial avifauna includes 8 endemic species, i.e., the green parakeet (*Aratinga holochlora brevipes*), Socorro red-tailed hawk (*Buteo jamaicensis socorroensis*), Socorro ground dove (*Columbina passerina socorroensis*), Socorro wren (*Troglodytes sissonii*), Socorro mockingbird (*Mimus graysoni*), Socorro towhee (*Pipilo maculatus socorroensis*), tropical parula (*Parula pitiayumi graysoni*), and the yellow-crowned night heron (*Nyctanassa violacea gravirostris*) (Rodriguez-Estrella et al., 1996). Two avian species arrived on the island recently, the mourning dove (*Zenaida macroura*) and the northern mockingbird (*Mimus polyglottos*) (Jehl and Parkes, 1982), while 2 endemic taxa are no longer on the island, the Socorro dove (*Zenaida graysoni*) and the elf owl (*Micrathene whitneyi graysoni*) (Jehl and Parkes, 1982; Rodríguez-Estrella et al., 1996).

The extinction of the endemic elf owl is definitive, but the Socorro dove currently survives in captivity (Martínez-Gómez et al., 2010). Cats and humans contributed to the decline of the endemic dove, last reported on the island in 1972 (Velasco-Murgia, 1982; Jehl and Parkes, 1983). Ongoing collaborative efforts are in place to achieve its re-introduction. At this point in time, several threats remain that could hinder these efforts, including the presence of potential predators (introduced cats and native hawks), the presence of the mourning doves (which may hybridize with the Socorro dove), and pathogens currently found on the island. Although there is no evidence that blood parasites contributed to the extinction of the Socorro dove, they could potentially impede their successful re-introduction (e.g., Marzal et al., 2005; Donovan et al., 2008; Puente et al., 2010; Olias et al., 2011). This is especially true when considering that animals that have been bred in captivity may not be immunologically competent to resist an infection caused by parasites or viruses (Viggers et al., 1993).

Hemosporidians, including species of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, are related vector-borne blood parasites typically found in reptiles, birds, and mammals (Valkiūnas, 2005). *Plasmodium* species are pathogenic and often cause disease in wild birds (Warner, 1968; Van Riper et al., 1986; Atkinson, 1999; Kilpatrick et al., 2006; Atkinson and LaPointe, 2009; Knowles et al., 2009, 2010). *Haemoproteus* species, which in some cases appear to be less pathogenic than *Plasmodium*, may still reduce host fitness (Allander, 1997; Knowles et al., 2009; Puente et al., 2010), cause severe diseases in avian hosts (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002), and even lead to death in wild birds (Valkiūnas, 2005; Donovan et al., 2008).

Received 17 May 2012; revised 1 October 2012; accepted 8 October 2012.

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DOI: 10.1645/GE-3206.1

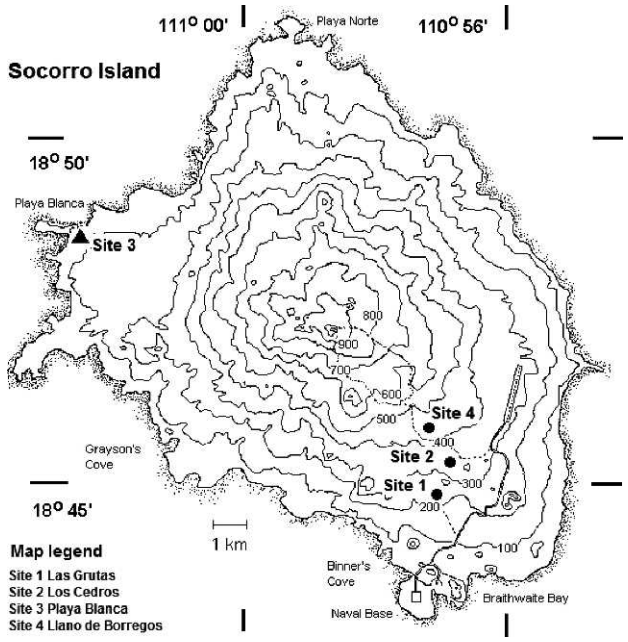

FIGURE 1. Map of Socorro Island showing the geographical distribution of study sites 1–4 where birds were trapped (Base map courtesy of Manuel Escamilla—INECOL, according to the photomap prepared by México's National Institute of Statistics, Geography and Informatics—INEGI).

A preliminary survey was conducted on Socorro Island for avian pathogens in the 2 columbid species currently present on the island (Yanga et al., 2011), i.e., the endemic Socorro ground dove, which is found in small numbers at lower elevations throughout the island (Wehtje et al., 1993), and the mourning dove, a species that arrived to the island in the 1970s (Jehl and Parkes, 1982). The mourning doves are congeneric with the Socorro dove (Johnson and Clayton, 2000) and colonized the island as the Socorro dove population was waning (Jehl and Parkes, 1983); the Socorro ground dove coexisted with the Socorro dove (Jehl and Parkes, 1982).

The objectives of the present study were as follows: (1) to screen the avifauna of Socorro Island for parasites, including species of *Plasmodium*, *Haemoproteus*, *Leucocytozoon*, and *Trypanosoma*; (2) to perform a phylogenetic analysis of the parasites of Socorro Island using a portion of the mtDNA cyt *b* gene to establish lineage relationships with parasites from other regions of the world; and (3) to screen Socorro doves residing at the Albuquerque Zoo in New Mexico, which will be used in the reintroduction program.

MATERIALS AND METHODS

Sample collection

Sampling took place during 3–19 July 2009 at 4 sites on the island (Fig. 1): Site 1 Las Grutas (18°44′4.8″N, 110°56′48.3″W); Site 2 Los Cedros (18°45′28.9″N, 110°56′44.4″W); Site 3 Playa Blanca (18°48′51″N, 111°02′422.5″W); and Site 4 Llano de Borregos (18°45′57.5″N, 110°56′58.5″W). Birds were trapped using 10 to 12 mist nets (12 m long and 3 m tall, with 36 × 36 mm and 60 × 60 mm mesh), which were set up in the morning shortly after sunrise and checked every 20 min until dusk. Upon capture, each bird was identified, measured, and weighed. Additionally, numbered aluminum bands were placed on each bird. Blood samples of 5 to 20 µl were collected by brachial venipuncture (Kerlin, 1964) and immediately stored in lysis buffer (10 mM Tris-HCL, pH 8.0, 100 mM EDTA, 2% SDS) at room temperature while on the island and, upon return to the lab at San Francisco State University, were then stored at –20 C. Additionally, we acquired 12 blood samples from Socorro doves held captive at the Albuquerque Zoo, New Mexico.

Parasite screening using microscopy

Two blood films were prepared from each bird. Blood films were air-dried within 5–10 sec after their preparation; they were fixed in absolute methanol in the field and then stained with Giemsa in the laboratory as described by Valkiūnas (2005). We examined nearly all blood films using microscopy. Some of the blood smears were of insufficient quality to yield reliable results. An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software Analysis Five was used to examine slides. One blood film from each bird was examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1,000). Voucher blood films were deposited in the Nature Research Centre, Vilnius, Lithuania.

Parasite screening using PCR

Parasite DNA was extracted from avian whole blood stored in lysis buffer, including the 12 Socorro dove samples from the zoo, following animal tissue protocols of the Wizard SV Genomic DNA Purification kits (Promega Corporation, Madison, Wisconsin). All polymerase chain reactions (PCR) were carried out in a 25 µl reaction mixture containing 10–100 ng of genomic DNA (2 µl of template DNA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM MgCl, 0.4 mM of each dNTP, 0.4 mM of each primer, 5 µl of CL buffer, and 0.5 units *Taq* (Qiagen, Valencia, California).

The extracted avian blood samples were then screened for species of *Trypanosoma*, *Leucocytozoon*, *Plasmodium*, and *Haemoproteus*. Screening of *Trypanosoma* spp. was conducted by a nested PCR with primers Tryp763/Tryp1016 and Tryp99/Tryp957 designed and used by Valkiūnas et al. (2011). Screening of *Leucocytozoon* spp. was conducted by a nested PCR with primers described in Hellgren et al. (2004) HaemNR3/HaemNFI and HaemFL/HaemR2L. To screen for *Plasmodium* and *Haemoproteus* spp., we amplified ~740 bp of the mitochondrial cytochrome oxidase subunit *b* gene (cyt *b*) using 2 sets of primers. The first set were L15183 and the H15730 developed by Fallon et al. (2003) and Szymanski and Lovette (2005) as employed by Chasar et al. (2009). The second primer set consisted of the nested primers HaemNF/HaemNR2 and HaemF/HaemR2 as described in Waldenström et al. (2004). All PCR products were viewed on 1.8% agarose gels stained with ethidium bromide.

Positive PCR products were then sent to Elim Biopharmaceuticals Inc., Hayward, California, for Bi-directional sequencing and were edited using Sequencher 4.8 (GeneCodes, Ann Arbor, Michigan). Parasite sequences that differed by only 1 to 3 base pairs were considered a separate distinct lineage (Ricklefs and Fallon, 2002). Distinct lineages were then verified by repeating an independent PCR and sequencing analysis. Additionally, chromatograms of all sequences were inspected for double peaks to ensure that no individual harbored multiple infections. If multiple peaks were found in the same chromatograms of 1 blood sample, it was considered to be infected with multiple distinct lineages (Pérez-Tris and Bensch, 2005). In addition to the samples collected in 2009, we obtained 12 DNA positive samples from the study conducted in 2004 by Yanga et al. (2011) from 6 mourning doves and 6 Socorro ground doves; the cyt *b* gene was amplified using the nested PCR protocol from Waldenström et al. (2004). All final sequences were deposited to GenBank with accession numbers (JN788932–JN788950).

Phylogenetic analysis

Phylogenetic relationships were analyzed using the best fit GTR+G model of molecular evolution as calculated with MrModeltest (Nylander, 2004), incorporating results in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). In addition to *Haemoproteus* sequences obtained from Socorro Island, 37 *Haemoproteus* sequences from the Galápagos Islands, North America, and continental Latin America (Valkiūnas et al., 2010) were obtained from Genbank (see Fig. 2 for accession numbers). Two *Leucocytozoon schoutedeni* lineages were used as outgroups. Two Markov Chain Monte Carlo (MCMC) simulations were run simultaneously for 10 million generations with sampling every 200 generations, creating 100,000

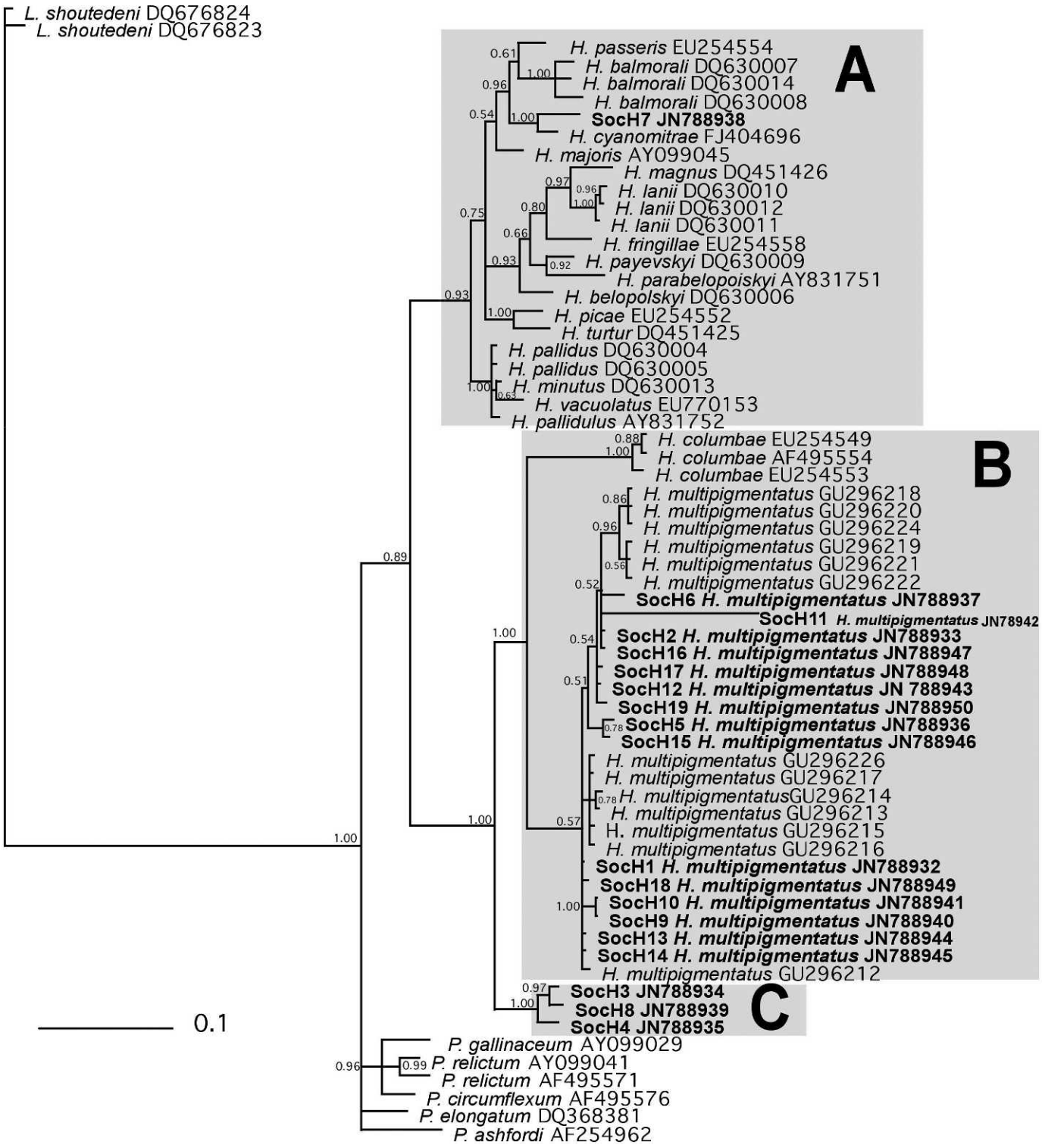

FIGURE 2. Bayesian phylogeny of 45 mitochondrial cytochrome *b Haemoproteus* spp. lineages found in birds from Socorro Island, the Galápagos Islands, and continental Latin America. Two *Leucocytozoon schoutedeni* lineages were used as outgroups. Gray boxes indicate a grouping of closely related lineages of hemoproteids. The Bayesian posterior probabilities are depicted at each node. Lineages obtained from Socorro Island are in bold. All other *Haemoproteus* lineages are delineated by the parasite name followed by the Genbank accession numbers. Clade A, species of the subgenus *Parahaemoproteus*; clades B and C, species of the subgenus *Haemoproteus*.

TABLE I. Sample numbers of *Haemoproteus* spp. lineages found in birds on Socorro Island in 2004 and 2009 (N = overall number of recorded infections, n = numbers of recorded infections in each species).

		Bird species (n)		
Lineages	N	Mourning doves	Socorro ground doves	Mockingbirds
SocH1	13	13	0	0
SocH2	8	3	3	2
SocH3	11	0	11	0
SocH4	8	0	8	0
SocH5	2	2	0	0
SocH6	1	1	0	0
SocH7	1	0	0	1
SocH8	2	0	2	0
SocH9	1	1	0	0
SocH10	1	1	0	0
SocH11	1	1	0	0
SocH12	2	2	0	0
SocH13	2	2	0	0
SocH14	1	1	0	0
SocH15	1	1	0	0
SocH16	2	2	0	0
SocH17	1	1	0	0
SocH18	2	2	0	0
SocH19	2	2	0	0
Total	62	35	24	3

trees. The first 25,000 trees were discarded from the sample as the "burn-in" period that accounted for 25% of the trees. The remaining trees were used to construct a majority rule consensus tree and to calculate the posterior probabilities of the individual clades (Labarthe et al., 1998). The genetic distances between different lineages were calculated in PAUP Version 4.0 (Swofford, 2001).

RESULTS

Parasite diversity and prevalence

In total, 122 birds were caught and sampled for processing by PCR. Included were 21 northern mockingbirds, 7 Socorro wrens, 1 Socorro mockingbird, 40 tropical parulas, 5 Socorro towhees, 25 mourning doves, and 23 Socorro ground doves. We screened all birds for blood parasites and found 46 individuals infected, i.e., 23 (92%) mourning doves, 17 (74%) Socorro ground doves, and 3 (14%) northern mockingbirds. All other species tested negative for parasite DNA. Of the 12 lineages from the 2004 study, 8 aligned with lineages that were detected in the present study (SocH1, SocH2, SocH3, SocH4, SocH5, SocH8, SocH13, and SocH16), while 4 lineages were distinct (SocH9, SocH10, SocH15, and SocH17). We detected 19 distinct lineages of *Haemoproteus* spp. (Table I) in the 58 combined sequences from the 2004 and the 2009 studies. No species of *Leucocytozoon*, *Plasmodium*, or *Trypanosoma* were detected in any bird species by PCR. Microscopic examination confirmed these results, with the following exceptions. Two of the mourning doves, which were negative by microscopy, tested positive by PCR and aligned with the SocH1 lineage. PCR examination revealed that only 6 mourning doves were co-infected with different species of *Haemoproteus*, while microscopic examination revealed that 18 of 21 mourning doves (85%) were co-infected with *Haemoproteus columbae* and *Haemoproteus multipigmentatus*, which included the 6 that were detected by PCR. Blood samples that were deemed to be co-infected by PCR were re-extracted, and PCR and sequencing were conducted as described above. We successfully obtained a clean sequence for 1 of the parasites in the co-infected bird after re-extraction. Most interesting, however, was the finding by microscopy that, in addition to being co-infected with both *H. columbae* and *H. multipigmentatus*, 1 mourning dove also harbored *Plasmodium* (*Haemamoeba*) spp. The 12 Socorro dove blood samples tested negative for all parasites by PCR. Blood slides were not available for these individuals.

Phylogenetic relationships

Phylogenetic relationships among the 19 *Haemoproteus* lineages found in birds of Socorro Island and those found in the Galápagos Islands and in the Americas are depicted in Figure 2. Three distinct clades were observed, i.e., Group A, Group B, and Group C. Group A consisted of 21 closely related lineages belonging to the subgenus *Parahaemoproteus* and 1 lineage from Socorro Island that was obtained from a northern mockingbird (SocH7). Group B consisted of 15 *Haemoproteus* (*Haemoproteus*) spp. lineages obtained from mourning doves of Socorro Island, 3 *H. columbae* lineages, and 13 lineages of *H. multipigmentatus* obtained from doves captured in the Galápagos, México, Guatemala, and Perú. Genetic distances within the highly supported clade containing *H. multipigmentatus* and the *Haemoproteus* lineages from Socorro Island ranged from 0.6 to 4.8%. Upon microscopic examination of blood smears, it was determined that all Socorro Island lineages in Group B belong to morphospecies *H. multipigmentatus* (Valkiūnas et al., 2010). It is interesting to note that lineage SocH2 was the only one found in multiple bird species, i.e., 3 mourning doves, 3 Socorro ground doves, and 2 northern mockingbirds. Finally, Group C consisted of a monophyletic group of 3 *Haemoproteus* lineages that were detected in Socorro ground doves. Based on morphological evidence, parasites of these lineages belong to a previously undescribed species, which will be described elsewhere. Genetic distances between lineages found in Group B and Group C ranged from 7.1 to 9.8% (Valkiūnas et al., 2013).

DISCUSSION

Haemoproteus spp. were identified both by PCR and microscopy from blood samples of 3 avian species, i.e., the Socorro ground dove, the mourning dove, and the northern mockingbird. Our study provides the first documentation of the phylogenetic relationships of hemosporidian parasites in the avifauna populations of Socorro Island. We found 19 lineages of *Haemoproteus* spp., of which the majority were identified via microscopic and sequence analysis as *H. multipigmentatus*. This indicates that the diversity of these parasites on Socorro Island is large and includes at least 1 undescribed *Haemoproteus* parasite (lineages of the clade C).

Group A in Figure 2 depicts an alignment of closely related lineages belonging to the subgenus *Parahaemoproteus*. This grouping also includes a distinct lineage (SocH7) that was detected in a northern mockingbird. Lineage SocH7 fell in a highly supported clade with *Haemoproteus cyanomitrae*, a species described by Iezhova et al. (2010), which was detected in an African olive sunbird. We did not manage to identify this parasite

to species level because of light parasitemia (a few immature gametocytes were seen). In order to understand where this parasite may have originated, additional sampling and identification of morphospecies must be conducted on hemoproteids on the mainland Americas.

Group B (Fig. 2) depicts a grouping of 3 lineages of *H. columbae* with lineages of *H. multipigmentatus* and *Haemoproteus* sp. detected primarily in mourning doves of Socorro Island. The high support of the clade in Group B containing *H. multipigmentatus* lineages found in doves from the Galápagos Islands, continental Latin America, and the Socorro Island *Haemoproteus* lineages suggests that these parasites may be the same species.

Microscopic examination of the slides revealed gametocytes that are indistinguishable from *H. multipigmentatus*. The records of this parasite in Socorro Island represent a new avian host for this species. There is no geographical differentiation between parasites from Socorro Island and those from continental populations and the Galápagos Islands. Santiago-Alarcón et al. (2010) observed a similar pattern in their phylogenetic analyses. Because Socorro Island *H. multipigmentatus* did not form a unique clade, but is intermixed with lineages from the mainland and the Galápagos Island, we can speculate that this morphospecies has a broad distribution and must have been brought recently by mourning doves, northern mockingbirds, or both.

Group C (Fig. 2) depicts a monophyletic group of 3 distinct lineages that were obtained solely from Socorro ground doves. Morphologically, all 3 lineages of clade C represent the same morphospecies, which is different from *H. columbae* and *H. multipigmentatus* and will be described elsewhere (G. Valkiūnas, unpubl. obs.). The placement of this clade in this phylogenetic tree suggests that Socorro ground doves harbor different *Haemoproteus* species than those detected in mourning doves. However, there is 1 exception, SocH2, which was a *Haemoproteus* sp. detected in the Socorro ground dove, the mourning dove, and the northern mockingbird. This suggests that this species of *Haemoproteus* may be a generalist, capable of infecting multiple bird species, as is frequently the case in some avian hemoproteids (Beadell et al., 2004, 2009; Križanauskienė et al., 2010; Loiseau et al., 2010).

The present study, in addition to providing an account of the diversity of *Haemoproteus* spp. on Socorro Island, has provided further evidence of the importance of combining both PCR and microscopy methods when analyzing bird blood samples for hemosporidian parasites (Valkiūnas et al., 2008). No *Plasmodium*, *Leucocytozoon*, or *Trypanosoma* spp. were detected in any of the birds by PCR-based methods. *Plasmodium* (*Haemamoeba*) sp. infection was detected by microscopy in a single mourning dove. However, microscopic examination of the blood smears revealed multiple co-infections and 1 infection by *Plasmodium* sp. The primers used in this study appear to preferentially bind to cyt *b* gene sequences of *H. multipigmentatus* when in the presence of a co-infection of multiple *Haemoproteus* spp. This indicates that when using PCR as a diagnostic method to determine hemosporidian diversity in avian populations, multiple genes of the parasite should be analyzed, and primers that are selective for 1 species over another should be used with caution (Valkiūnas et al., 2010). New methods to address co-infections within bird populations should be considered and explored while still continuing to use both PCR and microscopy methods concurrently to ensure that each hemosporidian species is accounted for.

This study was carried out in conjunction with a study of the mosquito fauna of Socorro Island (Carlson et al., 2011) to determine the diversity of mosquito species and whether or not they harbored hemosporidian parasites. *Haemoproteus* spp. lineages have been detected in wild caught mosquitoes (Ishtiaq et al., 2008; Njabo et al., 2009). However there is no current evidence that mosquitoes can transmit *Haemoproteus* spp. Carlson et al. (2011) found no mosquitoes infected with *Haemoproteus* but did find mosquitoes infected with *Plasmodium* spp. This study underscores the need to monitor the insect vectors as well as the bird hosts; had we relied solely on PCR-based detection of *Plasmodium* spp. in birds, we would have concluded erroneously that *Plasmodium* was not present on the island.

In sum, this phylogenetic analysis of *Haemoproteus* spp. provides a more extensive understanding of the diversity of these parasites in bird populations of Socorro Island and some insight into what the Socorro doves may face upon re-introduction. Based upon the results of this study, as suggested by Yanga et al. (2011), we expect that the Socorro doves will most likely be exposed to the *Haemoproteus* spp. that currently infect the mourning doves and the Socorro ground doves of Socorro Island. This is a plausible scenario when considering earlier documentation of the low host specificity of several lineages of *Haemoproteus* parasites (Križanauskienė et al., 2010) and that lineage SocH2 was recovered from blood samples of mourning doves, Socorro ground doves, and northern mockingbirds. Previous studies have shown that *H. columbae*, a common parasite of birds of the order Columbiformes, can be lethal to doves (Earlé et al., 1993) and that *Haemoproteus* infections can also affect the health and fitness of passerine birds (Merino et al., 2000; Marzal et al., 2005). In addition, mortality due to *Haemoproteus* spp. infections has been reported in birds in American zoos (Ferrell et al., 2007) and captive birds in Europe (Olias et al., 2011). Thus, hemoproteid infections warrant more attention in conservation projects, especially with potentially naive bird populations.

A continuous monitoring program of both the avian and insect vector populations using PCR-based and microscopy techniques on Socorro Island is required to understand how parasites interact with both the host and potential vectors. In conjunction with ongoing programs for population monitoring, this approach would provide a complete picture of all possible threats that could jeopardize the success of the re-introduction program for the Socorro dove and the population viability of other already fragile endemic avifauna.

ACKNOWLEDGMENTS

We would like to offer our sincere gratitude and recognition to the Secretaría de Marina–Armada de México, and, in particular, to Admiral Ezequiel Ramiro Bordonave, Captain Pedro Villaciz León, and the military personnel stationed at Socorro Island for their hospitality and valuable logistical support. Tatjana A. Iezhova is gratefully acknowledged for help with microscopic examination of blood films. Patricia Escalante at IBUNAM kindly provided access to samples collected in 2004. We would like to thank the members of the Island Endemics Foundation for their support and technical advice. We would also like to extend our gratitude to the Albuquerque Zoo in New Mexico for providing blood samples from captive Socorro doves. Funding was provided by the College of Science and Engineering, San Francisco State University, the American Society of Parasitologists Willis A. Reid Student Research Grant, the San Francisco State University Instructionally Related Activities Grant, Conservation International, the American Bird Conservancy, the Islands Endemics Foundation, and INECOL. We would also like to thank Dr. Susan

Perkins and 2 anonymous reviewers whose comments and suggestions improved this manuscript. All research was carried out under permits (SGPA/DGVS/51015/03 and SGPA/DGVS/04005/08) issued by the Secretaría de Medio Ambiente y Recursos Naturales and the Secretaría de Gobernación. Research protocols in this study also comply with IACUC permit A9-002 SFSU.

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