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North American Transmission of Hemosporidian Parasites in the Swainson's Thrush (*Catharus ustulatus*), a Migratory Songbird

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ABSTRACT: The geographic structuring of parasite communities across the range of a single host species can illuminate patterns of host-population connectivity. To determine the location of parasite transmission in a Neotropical migrant bird species, we sampled adult and hatch-year (HY) birds across the breeding and wintering range of the Swainson's thrush (SWTH), an abundant passerine with a migratory divide. We examined the phylogenetic relationships among cytochrome *b* lineages of the avian blood parasite genera *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* and determined the transmission location of unique lineages. We found that *Haemoproteus* and *Plasmodium* lineages are transmitted on California breeding grounds, whereas *Leucocytozoon* transmission occurs on Alaskan breeding grounds. The presence of hemosporidians on wintering grounds and shared lineages between the SWTH and resident species suggests that transmission of some of these lineages occurs on both breeding and wintering grounds. We emphasize that the sampling of HY birds and local resident heterospecifics will supplement vector studies to determine the key players in hemosporidian host switching and range-expansion events.

The study of hemosporidian parasites has revealed astounding levels of diversity and spatial variation (Bensch et al., 2000; Ricklefs and Fallon, 2002; Beadell et al., 2004; Valkiūnas, 2005; Loiseau et al., 2010). In some cases, parasite assemblages across different regions of an avian host's range have revealed little geographic structuring (Fallon et al., 2006; Pagenkopp et al., 2008), and, in others, very informative patterns of host population connectivity (Kimura et al., 2006). The study of parasite distribution becomes even more intricate in migratory birds because they may share lineages with resident species on both breeding and wintering grounds (Webster et al., 2002).

Previous work has shown that parasites can be transmitted on wintering grounds (Waldenström et al., 2002), on breeding grounds (Ricklefs et al., 2005; Pagenkopp et al., 2008) and, also, year round at both breeding and wintering sites (Hellgren et al., 2007). On breeding grounds, hatch-year (HY) birds are only exposed to parasites transmitted at that location. Therefore, the sampling of HY birds allows us to infer which lineages are actively transmitted on breeding grounds (Waldenström et al., 2002). When parasites are shared between breeding and wintering grounds, the occurrence of parasite lineages in resident species can similarly give evidence of parasite-transmission location. Year-round parasite transmission increases opportunities for the emergence of disease (Waldenström et al., 2002; Perez-Tris and Bensch, 2005), increases its spread in natural populations (Altizer et al., 2003), and is thought to be common among widespread or generalist parasites, such as certain lineages of avian *Plasmodium* species (Perez-Tris and Bensch, 2005).

Some migratory bird species can exhibit a zone of contact between parapatric subspecies populations. They utilize different migratory routes, which forms a migratory divide (Bensch et al., 1999; Ruegg and Smith, 2002; Ruegg, 2008). Dispersal of parasites appears to be facilitated by migration (Harvell et al., 2009). In fact, higher hemosporidian species richness was found in European birds with migratory divides than those without them (Møller et al., 2011). This finding is not surprising, considering that migratory divides act as barriers to dispersal, causing reduced gene flow and increased genetic differentiation between divided avian populations and, therefore, the parasites they harbor. In addition, a study at the moving contact zone between 2 parapatric sibling passerines in Europe revealed that the host contact zone mainly acts as a barrier to parasite expansion (Reullier et al., 2006). However, recent work on sympatric populations of blackcaps (*Sylvia atricapilla*) with different migratory routes did not find clear parasite community differences between populations (Santiago-Alarcon et al., 2011).

Another passerine with a migratory divide is the Swainson's thrush (SWTH, *Catharus ustulatus*), a long-distance Nearctic-Neotropical migratory songbird comprised of 2 subspecies. The coastal, russet-backed group (*C. ustulatus ustulatus*) winters in Central America and Mexico and migrates along a Pacific coastal route to breeding grounds between coastal northern California and the Alaska panhandle. The inland, olive-backed, subspecies (*C. ustulatus swainsoni*) winters in Panama and South America and migrates along an eastern route to breed in the eastern and interior of the United States as far north as the Arctic Circle (Fig. 1). The migratory divide of SWTH has been well characterized by banding and mitochondrial data, which established that the circuitous migratory route of the eastern, olive-backed, subspecies is a remnant of the historical expansion route of its ancestors as glaciers receded after the Late Pleistocene period (Ruegg and Smith, 2002). A sparsely populated and geographically static hybrid zone, where mtDNA genotypes of both subspecies are found, follows the geographical barrier formed by the coast range in western Canada and Alaska, the Cascade range in Oregon and Washington, and the Sierra Nevada mountain range in California (Ruegg, 2008). Ecological restrictions enforced by these geologic features as well as the habitat differences between these 2 distinct climatic regions have kept the subspecies relatively reproductively isolated (Ruegg, 2008).

A previous study of the hemosporidian parasites of the 2 subspecies of SWTH across this migratory divide revealed a surprisingly unstructured geographical distribution, with the most prevalent *Haemoproteus* lineages shared across the divide, whereas *Plasmodium* lineages were found mostly in the coastal subspecies (Svensson et al., 2007). Here, we sought to confirm the patterns of demographic connectivity presented by Ruegg and Smith (2002). Across 3 distinct regions of the range of the SWTH (Alaska, California, and Costa Rica), we aimed (1) to investigate the lineage diversity of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp.; and (2) to determine transmission location of these parasites.

During the breeding season of 2010 (June and July), we sampled 15 adult birds and 26 HY birds from northern California coastal sites with the help of the Point Reyes Bird Observatory. Over the same time period in Fairbanks, Alaska, 21 adult birds and 37 HY birds were sampled. In Costa Rica, the SWTH wintering grounds, sampling took place between February and March of 2005, 2007, and 2008 and produced a total of 150 adult bird samples. Birds were captured with mist nets and banded, and approximately 50 μl of whole blood was drawn by brachial venipuncture and stored in lysis buffer for subsequent molecular analysis (Sehgal et al., 2001).

From each bird captured in California and Alaska, 2 blood films were prepared on glass slides and fixed in absolute methanol. Details of preparation and staining of blood films are described by Valkiūnas (2005).

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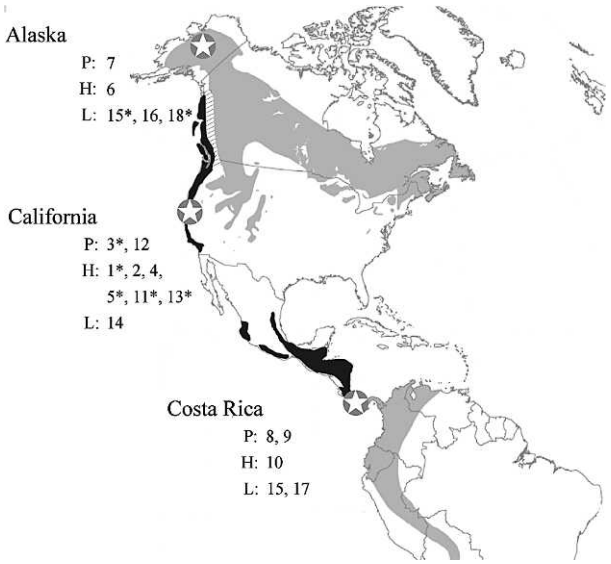

FIGURE 1. Range map of Swainson's thrush subspecies ranges (gray indicates the distribution of the inland form and black indicates the range of the coastal form). Lineage descriptions are noted for each sampling location where H = *Haemoproteus*, P = *Plasmodium*, and L = *Leucocytozoon*. Lineages marked with asterisks were isolated from hatch-year birds. Map from Ruegg (2008).

The blood films from each infected bird were examined with the use of a Nikon E200 compound microscope (Nikon, Melville, New York).

DNA was extracted from whole blood with the use of the Wizard® SV Genomic DNA Purification System (Promega, Madison, Wisconsin). Extraction success was verified by PCR with the use of primers that amplify the gene encoding the brain-derived neurotrophic factor (Sehgal and Lovette, 2003).

Haemoproteus and *Plasmodium* spp. were detected by 2 polymerase chain reactions (PCRs), both amplifying sections of the mitochondrial cytochrome *b* gene. This served to increase sequence length for analysis. Primers for the first reaction were L15183 and H15730, described previously (Fallon et al., 2003; Szymanski and Lovette, 2005). For the second PCR, the primers HaemNF and HaemNR2 were used for the first reaction and HaemF and HaemR2 were used for the second, nested, reaction (Waldenstrom et al., 2004). An overlap of over 200 base pairs between sequences from the 2 reactions allowed the sequences to be combined, yielding a cyt *b* sequence of up to 750 base pairs.

Leucocytozoon spp. presence was determined by a nested PCR following the protocol described by Hellgren et al. (2004), with 2 modifications. The annealing temperature was adjusted to 54.5 C and reactions were performed in Accupower® PCR PreMix (Bioneer, Inc., Alameda, California).

All reactions were performed in 20- or 25-µl volumes and were accompanied by negative (ddH_2O) and positive controls (samples from infected birds as confirmed by microscopy) to control for any contamination and to confirm success of the PCR. Products were run out on a 1.8% agarose gel with the use of 1× TBE and visualized by an ethidium bromide stain under ultraviolet light.

PCR products were purified with the use of Exosap according to the manufacturer's instructions (United States Biochemical Corporation, Cleveland, Ohio); they were sequenced to identify parasite lineages (BigDye® version 1.1 sequencing kit, Applied Biosystems, Foster City, California) on an ABI Prism 3100™ automated sequencer (Applied Biosystems). Sequences were aligned with the use of the program Sequencher 4.8 (Gene Codes, Ann Arbor, Michigan). In 6 instances, "double peaks" were identified on chromatograms. Those individuals were suspected of harboring coinfections with multiple parasite lineages and were omitted from analysis. The BLAST algorithm was used to compare the sequences of new lineages to known *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* spp. lineages deposited in GenBank. All novel sequences were deposited in GenBank (JN792135- JN792152, see Table 1). The new lineages identified in this study from California and Alaska were each found in only 1 individual, whereas lineages from Costa Rica, where sampling was more extensive, were each found in 2–3 individuals.

We used 12 mitochondrial cyt *b* sequences of avian *Haemoproteus* and *Plasmodium* species from our survey (fragment lengths between 459 and 750 base pairs [bp]), 8 sequences that were identified in SWTH by Svensson et al. (2007) and 13 reference sequences from GenBank, which were carefully chosen to correspond to positive morphological identifications (Valkiūnas, 2005). *Leucocytozoon sabrezi* (AB299369) served as outgroup for the *Haemoproteus* and *Plasmodium* phylogenetic analysis. Similarly, *Leucocytozoon* phylogenetic analyses included 4 novel sequences (fragment lengths between 439 and 482 bp), and 6 reference sequences from GenBank, with *Plasmodium relictum* serving as the outgroup.

The appropriate model of sequence evolution was determined by the software MrModelTest (Nylander et al., 2004) to be GTR + I for species of *Plasmodium* and *Haemoproteus* and GTR + Γ for species of *Leucocytozoon*. Phylogenies of cyt *b* lineages were generated in MrBayes version 3.1 (Ronquist and Huelsenbeck, 2003) with the use of 1 cold of 2 hot Monte Carlo Markov chains, which were sampled every 200 generations over 10 million generations for a total of 100,000 generated trees. In all, 25% of these were discarded as burn-in and the remaining 75,000 trees were used to construct a majority consensus tree and to calculate posterior probabilities of the individual clades. Phylogenetic analyses were also implemented with the use of maximum-likelihood (ML) techniques. ML and sequence divergence algorithms were computed with the use of PAUP (Swofford, 2003). Individual branch support was estimated with the use of ML bootstrap analyses with 100 replicates.

HY data from California confirm that *Plasmodium* spp. lineages in clade A (Fig. 2) are transmitted on the breeding grounds of the russet-backed California SWTH. Other members of clade A included SWTH.P.2, which was found in the related but nonmigratory orange-billed nightingale-thrush (OBNT), *Catharus aurantiirostris*, in Costa Rica and in the hybrid breeding zone of the russet-backed and olive-backed SWTH populations by Svensson et al. (2007). Therefore, members of clade A are present in all 3 of the regions sampled and are transmitted on wintering grounds in Costa Rica as well as California breeding grounds.

We identified 3 lineages of *Haemoproteus* spp. in our study that are transmitted on California breeding grounds, as evidenced by their presence in HY birds (Fig. 2, clades C and D). We found 1 lineage in an adult bird from Alaska that is closely related to the 3 *Haemoproteus* lineages identified by Svensson et al. (2007) across russet-backed, olive-backed, and hybrid zones of the SWTH breeding range (Fig. 1, clade B). Thus, *Haemoproteus* lineages appear not to be geographically structured. We confirm previous results (Svensson et al., 2007) showing that, although geologic and climate factors limit gene flow between SWTH subspecies (Ruegg and Smith, 2002; Ruegg, 2008), their hemosporidian parasites appear to bridge this migratory divide.

Evidence of *Leucocytozoon* spp. transmission on Alaskan breeding grounds comes from 3 lineages isolated from HY birds (Fig. 3). The pairwise genetic distance between them ranged from 6.6 to 8.2%, suggesting that *Leucocytozoon* spp. diversity is high on Alaskan breeding grounds and, moreover, transmission takes place there. This result agrees with previous work showing that higher latitudes can be favorable to the transmission of *Leucocytozoon* spp. (Haas et al., 2012).

From this preliminary survey of SWTHs, we conclude that some *Haemoproteus* and *Plasmodium* spp. are transmitted on California breeding grounds of the russet-backed SWTH. There is also evidence of *Plasmodium* spp. transmission on Costa Rican wintering grounds of the olive-backed SWTH because the same lineages are found in resident species, such as the OBNT. OBNT harbored lineages SWTH.P.1 and P.2, which were also isolated from northerly SWTH (Svensson et al., 2007). This is further evidence that some host-generalist *Plasmodium* spp. are

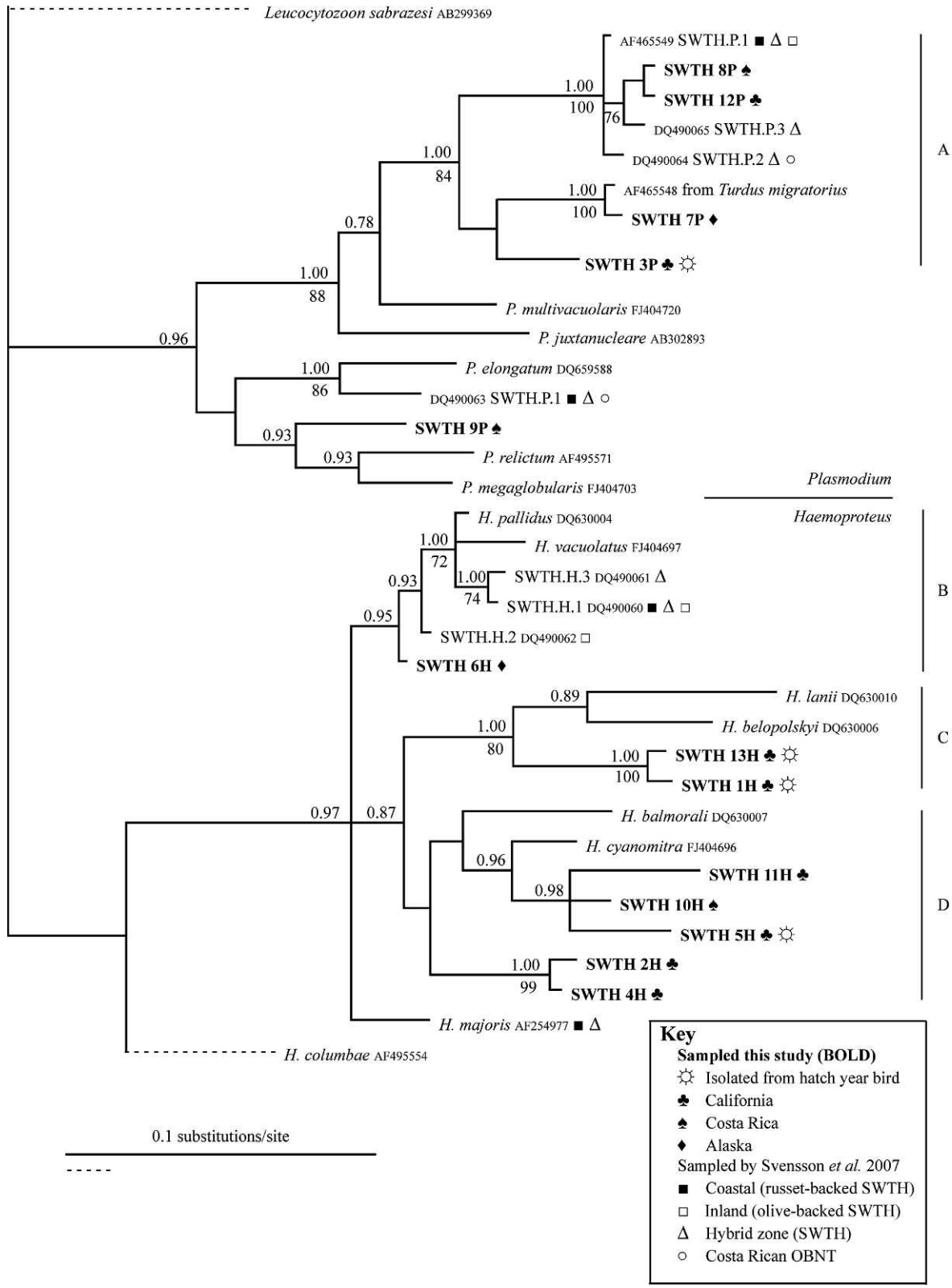

FIGURE 2. Phylogenetic tree of *Haemoproteus* (H) and *Plasmodium* (P) parasite lineages based on 459–750 base pairs of the mitochondrial cytochrome *b* gene. Bayesian posterior probabilities greater than 0.75 are represented by the numerator at each node, and maximum-likelihood bootstrap values greater than 70 are represented by the denominator. Lineage number and symbols representing location of sample collection are indicated for novel lineages.

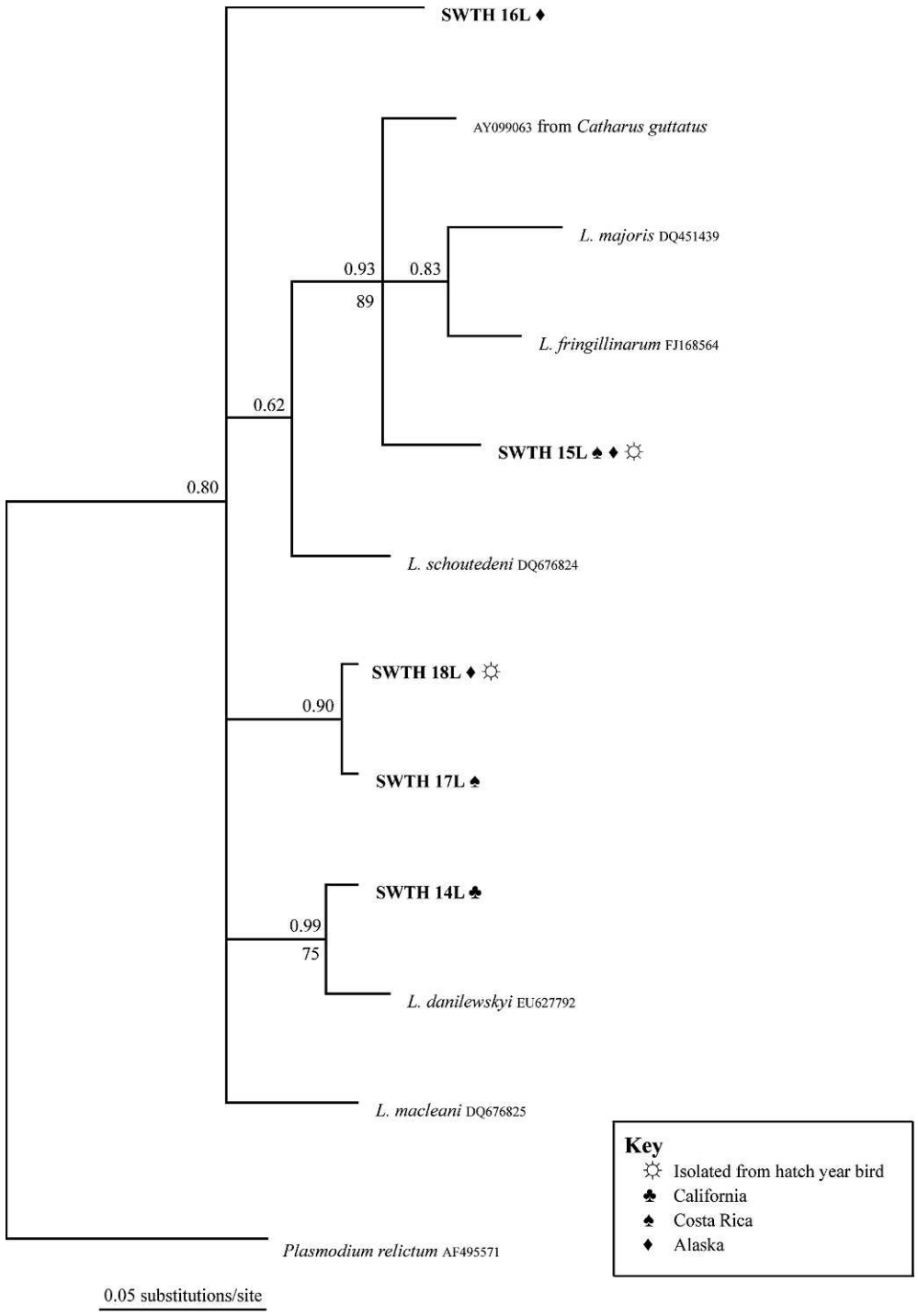

FIGURE 3. Phylogenetic tree of *Leucocytozoon* (L) parasite lineages based on 439–482 base pairs of the mitochondrial cytochrome *b* gene. Bayesian posterior probabilities greater than 0.75 are represented by the numerator at each node, and maximum-likelihood bootstrap values greater than 70 are represented by the denominator. Lineage number and symbols representing location of sample collection are indicated for novel lineages.

capable of transmission on both breeding and wintering grounds (Perez-Tris and Bensch, 2005). Resident species may be susceptible to *Plasmodium* parasites that SWTH individuals carry from their summer breeding grounds.

Evidence of *Leucocytozoon* spp. transmission on Alaskan breeding grounds confirms the need for surveys of black flies (Simuliidae) in this region. Previous work has shown that these vectors can exhibit strong avian host-family preference (Hellgren et al., 2008) and are, therefore, a key player in determining *Leucocytozoon* spp. transmission and host specificity.

Leucocytozoon LIN 15 was present in olive-backed SWTH on both wintering grounds and breeding grounds, with evidence of transmission on Alaskan breeding grounds. LIN 15 confirms connectivity between Alaskan breeding and Costa Rican wintering populations and suggests

TABLE I. Geographical origin and age class of Swainson's thrush sampled and parasite lineages (with GenBank numbers) listed in Figures 2 and 3.

			Lineage (GenBank)		
Location	Age	n	*Plasmodium*	*Haemoproteus*	*Leucocytozoon*
Alaska	AHY	21	SWTH7P (JN92135)	SWTH6H (JN792152)	SWTH16L (JN792149)
	HY	37			SWTH15L (JN792150)
					SWTH18L (JN792138)
California	AHY	15	SWTH12P (JN792146)	SWTH2H (JN792142)	SWTH14L (JN792137)
				SWTH4H (JN792140)	
				SWTH11H (JN792136)	
	HY	26	SWTH3P (JN792148)	SWTH1H (JN792139)	
				SWTH5H (JN792147)	
				SWTH11H (JN792136)	
				SWTH13H (JN792141)	
Costa Rica	AHY	150	SWTH8P (JN792145)	SWTH10H (JN792143)	SWTH15L (JN792150)
			SWTH9P (JN782144)		SWTH17L (JN792151)

that transmission to other species on breeding grounds may be possible if the appropriate vectors are present. Further screening of resident and migratory species in these mixed-species wintering locations will determine whether *Leucocytozoon* spp., apparently common on breeding grounds of the olive-backed SWTH, are also capable of infecting heterospecifics on SWTH wintering grounds.

In the present study, we did some preliminary examination of blood smears. However, the quality of the slides was not sufficiently high to identify parasite species. In addition, with the slides from Alaska, we were unable to detect *Plasmodium* parasites. Two possible reasons for this are, first, that the parasitemia was very low, and slides were not of sufficient quality to detect infections (Valkiūnas et al., 2008); or second, that the parasites are not capable of developing to the gametocyte stage in the SWTH (Ferrell et al., 2007; Olias et al., 2011). In the latter case, PCR may yield product perhaps from infected tissue-stage cells, or possibly from sporozoites (Valkiūnas et al., 2009). In either case, because HY birds acquire infection, we can maintain that transmission of these lineages does occur in Alaska, even though SWTH may not be the preferred host.

Our study highlights the importance of sampling HY as well as resident birds on both breeding and wintering grounds for the deduction of parasite transmission locations in Neotropical migrant birds. Paired with studies of hemosporidian vectors across breeding and wintering grounds, such methods will surely uncover the key players in the spread of hemosporidian parasites over a broad geographical scale.

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