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Authors: Martínez-Renau, Ester, Rojas-Estévez, Natalia, Friis, Guillermo, Hernández-Montoya, Julio C., Elizondo, Pablo, et al.

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RESEARCH ARTICLE

Haemosporidian parasite diversity and prevalence in the songbird genus *Junco* across Central and North America

Ester Martínez-Renau,[1,a] Natalia Rojas-Estévez,[1] Guillermo Friis,[1] Julio C. Hernández-Montoya,[2] Pablo Elizondo,[3] and Borja Milá[1,*]

[1] Department of Biodiversity and Evolutionary Biology, National Museum of Natural Sciences, Spanish National Research Council (CSIC), Madrid, Spain
[2] Grupo de Ecología y Conservación de Islas, Ensenada, BC, Mexico
[3] Costa Rica Bird Observatories, Heredia, Costa Rica
[a] Current address: Departamento de Ecología Funcional y Evolutiva, Estación experimental de Zonas Áridas, Consejo Superior de Investigaciones Científicas (CSIC), Almería, Spain
*Corresponding author: b.mila@csic.es

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ABSTRACT

The evolution of host–parasite interactions as host lineages colonize new geographic regions and diversify over evolutionary time is poorly understood. To assess whether haemosporidian parasite diversity has changed during the diversification of an avian host, we surveyed the diversity and prevalence of blood parasite lineages (genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) across the range of the songbird genus *Junco*, which has diversified recently as it recolonized North America following the last glacial maximum ~18,000 years ago. We report the diversity and prevalence of parasites in junco taxa sampled from Costa Rica to Canada, and examine the influence of local avian species richness in the prevalence and diversity of parasites in junco samples. We screened for parasites in each individual by sequencing a fragment of their cytochrome *b* gene, identifying the different lineages, and quantifying the prevalence per junco taxon and locality. Of 304 juncos sampled, 178 tested positive for 1 or more parasite genera (58.5% overall prevalence). We found high parasite diversity in genera *Haemoproteus* and *Leucocytozoon* and much lower diversity in *Plasmodium*. Among the 63 parasite lineages detected, 32 of which have not been previously described, we found generalist lineages with widespread but low prevalence in *Junco*, but also some that appear to have remained specialized on this genus as it diversified across North America over thousands of years. Our results suggest a range of parasitic strategies, ranging from specialized to generalist lineages within single parasite genera.

Keywords: haemosporidian parasites, New World, songbirds, speciation, specificity

LAY SUMMARY

- In this study we describe patterns of haemosporidian blood parasite diversity and prevalence across the range of the genus *Junco*.
- We surveyed blood parasites in 304 juncos from across the range, and looked at parasite prevalence and diversity.
- We found high parasite diversity in genera *Haemoproteus* and *Leucocytozoon* and much lower diversity in *Plasmodium*. Both the prevalence and diversity of *Haemoproteus* and *Leucocytozoon* were positively correlated with local avian species richness.
- We also found that some parasites are specialized on juncos, whereas others can also be found in other landbird species.
- Some parasites appear to have remained restricted to juncos even when the recolonization of North America caused juncos to adapt to many different habitats and even diversify into different evolutionary lineages.

Diversidad y prevalencia de parásitos hemosporidios en el género paseriforme *Junco* en Centro y Norteamérica

RESUMEN

La evolución de las interacciones entre hospedadores y parásitos cuando linajes de hospedadores colonizan nuevas regiones geográficas y se diversifican a lo largo del tiempo evolutivo es un proceso poco conocido. Para determinar si la diversidad de parásitos hemosporidios ha cambiado durante la diversificación de su hospedador aviar, muestreamos la diversidad y prevalencia de linajes de parásitos sanguíneos (géneros *Plasmodium*, *Haemoproteus* y *Leucocitozoon*) a lo largo de la distribución del género paseriforme *Junco*, el cual se ha diversificado recientemente al recolonizar Norteamérica tras el último máximo glacial ocurrido hace unos 18.000 años. Reportamos la diversidad y prevalencia de parásitos en juncos muestreados desde Costa Rica hasta Canadá, y examinamos la influencia de la riqueza local de especies de aves sobre la prevalencia y diversidad de parásitos en muestras de juncos. Detectamos los parásitos en cada individuo secuenciando un fragmento de su gen Cyt-b, identificando los diferentes linajes, y cuantificando la prevalencia por taxon y por localidad. De los 304 juncos muestreados, 178 dieron positivo para uno o más géneros parásitos (58,5% de prevalencia total). Encontramos alta diversidad parasitaria en los géneros *Haemoproteus* y *Leucocytozoon*, y una diversidad mucho menor en *Plasmodium*. Entre los 63 linajes parasitarios detectados, 32 de los cuales no habían sido descritos previamente, encontramos linajes generalistas con amplia distribución pero baja prevalencia en juncos, pero también algunos que parecen haber permanecido especializados en este género aviar a medida que se diversificaba por Norteamérica a lo largo de miles de años. Nuestros resultados sugieren la existencia de un abanico de estrategias parasitarias, incluyendo desde linajes especializados a generalistas dentro de un mismo género hemosporidio.

Palabras clave: aves canoras, especiación, especificidad, Nuevo Mundo, parásitos hemosporidios

INTRODUCTION

Parasites play an important role in shaping ecological communities and driving evolutionary processes (Ricklefs 2010). The distribution and prevalence of parasites vary depending on a range of ecological and environmental conditions such as abundance of hosts and vectors (in the case of vector-borne parasites; Ellis et al. 2017, Fecchio et al. 2017, 2019), transmission rate, climatic conditions and distance to water bodies (Gonzalez-Quevedo et al. 2014, Padilla et al. 2017, Fecchio et al. 2020, McNew et al. 2021), landscape features (Fecchio et al. 2020), host phylogeny (Barrow et al. 2019) and host life history traits (Matthews et al. 2016, Barrow et al. 2019). In turn, host–parasite interactions are strongly influenced by the parasites' host specificity, which refers to the diversity of host species that a single parasite can infect, and how phylogenetically distant they are from each other. This characteristic can vary geographically as ecological and environmental conditions can modulate the range of host species a parasite can infect (Wells and Clark 2019). Parasites infecting a single or a few closely related species are considered to be more specialized than a parasite lineage capable of infecting a broad range of species, including phylogenetically distant taxa, which would be considered a more generalist parasite (Futuyma and Moreno 1988, Bensch et al. 2000, Moens and Pérez-Tris 2016). Moreover, a parasite's host range, or the number of host species infected by a given parasite (Lymbery 1989), is influenced by the diversity of the local bird community (Keesing et al. 2006, Lima and Bensch 2014).

When an avian host colonizes new habitats and speciates into new allopatric lineages over time, these lineages are likely to be exposed to different biotic and abiotic conditions and different avian communities, so that host-specific parasites present with the ancestral avian host could evolve in different ways: (1) the parasite lineages could remain specialized on the newly speciated, closely related new host taxa without diversifying themselves; (2) they could co-speciate with the new hosts; or (3) they could become more generalist in the newly colonized habitats and spread through the new bird community (host switching), evolving in some cases into new parasite lineages (Hellgren et al. 2007, Ricklefs et al. 2014, Santiago-Alarcon et al. 2014, Fecchio et al. 2018). Phylogenetic approaches which take into account the phylogenetic dispersion of parasite lineages on host phylogenies can be used to test these alternative hypotheses (Clark and Clegg 2017).

Haemosporidians are protozoan blood parasites of the phylum Apicomplexa found in reptiles, birds, and mammals. Those infecting birds are in the genera *Plasmodium, Haemoproteus, Leucocytozoon,* and *Parahaemoproteus*, each transmitted by a different family of dipteran vector, causing disease and thus affecting the host's fitness (Atkinson and Van Riper III 1991, Valkiūnas 2005, Romano et al. 2019). These parasites have been found across every continent apart from Antarctica (Valkiūnas 2005, Clark et al. 2014), and are abundant in many bird species (Valkiūnas 2005), which make them a good model to study host–parasite interactions. These interactions have been studied mostly in specific geographic localities or ecological communities (Njabo et al. 2011, Oakgrove et al. 2014, Matthews et al. 2016, Walther et al. 2016, Jones et al. 2018, Fecchio et al. 2019), and in large-scale comparative studies, including the entire range of host species (Barrow et al. 2019, Fecchio et al. 2017, McNew et al. 2021), yet this relationship has rarely been explored focusing on a recently diversified avian genus throughout its entire distribution range. Surveying parasites across the entire range of a host taxon can reveal patterns of geographic variation in diversity and prevalence and help us understand how they vary with habitat, between closely related host species, and with local avian diversity across the range.

Juncos are New World sparrows in the family Passerellidae, with several species ranging from Costa Rica through Canada. North American forms underwent a rapid and recent radiation following the Last Glacial Maximum ~18,000 years ago (Milá et al. 2016). When conditions became favorable following the retreat of the ice sheets, the Yellow-eyed Junco (*Junco phaeonotus*) from southern Mexico expanded northward into North America and diversified into no less than 7 phenotypically distinct and geographically structured Dark-eyed Junco (*J. hyemalis*) lineages (Milá et al. 2007, Friis et al. 2016), which are currently classified as subspecies (see Methods section). Junco taxa have thus diversified across a broad range of habitats and avian communities. Species in tropical and subtropical latitudes occupy very high elevations in isolated mountain ranges, or "sky islands", where bird diversity tends to be low relative to the lowlands (Sánchez-Ramos et al. 2018). However, as juncos expanded and diversified into the temperate zone, they were able to colonize low altitude environments where avian diversity can be high. The recent and rapid nature of the junco radiation in North America provides the opportunity to study the parasite lineages in both the recently formed junco lineages in the north and in the older ancestral lineages in Central America, from which northern juncos originated just a few thousand years ago. Haemosporidian diversity in juncos has been examined at the local scale in the context of comparing migratory and sedentary populations (Slowinski et al. 2018, Becker et al. 2019, 2020) but not at large geographic and phylogenetic scales. The sampling in the present study provides a broad overview of haemosporidian diversity in the entire genus and can help shed light on the coevolution of hosts and parasites as it provides a system with a known phylogenetic history of old and recently diverged host lineages distributed across a steep ecological cline. Here we sequenced the mitochondrial DNA cytochrome *b* gene (cyt *b*) of hemosporidian parasites of the genera *Haemoproteus*, *Leucocytozoon* and *Plasmodium* found in junco blood samples collected across the range of the genus, and used a phylogenetic approach to understand the relationships among parasite lineages. Our specific objectives are (1) to describe the diversity and composition of parasite assemblages across the range of different specific and subspecific junco taxa from Costa Rica through Canada; (2) to examine patterns of parasite diversity as junco hosts speciated and diversified into different lineages; and (3) to assess the role of local avian diversity in affecting host–parasite interactions as juncos joined increasingly diverse avian communities as they expanded north.

METHODS

Study Species

The current taxonomic treatment of juncos includes 5 species (Gill et al. 2022). The Central American taxa include the divergent Volcano Junco (*J. vulcani*) in Costa Rica; Baird's Junco (*J. bairdi*) from the southern tip of the Baja California Peninsula; the Island junco (*J. insularis*) on Guadalupe Island in the Mexican Pacific; and two closely related Yellow-eyed Juncos in the highlands of Chiapas (Mexico) and Guatemala, currently classified as *J. phaeonotus fulvescens* and *J. p. alticola*, respectively. Post-glacially radiated lineages across the North American continent comprise two more Yellow-eyed Junco taxa in mainland Mexico, *J. p. phaeonotus* and *J. p. palliatus*, and at least 6 forms currently grouped within the Dark-eyed Junco complex: the Red-backed Junco (*J. h. dorsalis*) from southwestern USA; the Gray-headed Junco (*J. h. caniceps)* in the Rocky Mountains; the Oregon Junco (*J. h. oreganus*) group across the West, composed in turn of several distinct subspecific forms from northern Baja California to Alaska including *townsendi*, *pontilis*, *thurberi*, *pinosus*, *montanus*, *shufeldti* and *oreganus*; the Pink-sided Junco (*J. h. mearnsi*) in the northern Rocky Mountains; the White-winged Junco (*J. h. aikeni*) in the Black Hills of South Dakota; and the Slate-colored Junco group in eastern and boreal North America, comprising *J. h. hyemalis*, *J. h.carolinensis* and *J. h. cismontanus* (Miller 1941, Sullivan 2020, Nolan et al. 2020) (see approximate distribution ranges in Figures 1–3). Despite marked divergence in phenotype (plumage, beak, and iris color) and genetic markers (Friis et al. 2016, 2018), several of these forms are considered to be subspecies, as some of them can interbreed at contact zones (Milá et al. 2016).

Field Sampling

Juncos were sampled across Central and North America over several years as part of a long-term study aimed at understanding the evolution of the group using phenotypic and genetic data. To increase sampling efficiency, at each locality we captured between 10 and 30 territorial males (Table 1) using a single mist net and song playbacks to attract them, thus no females nor juveniles were sampled. Due to this very targeted sampling method, no other bird species were typically captured in the nets, so that only junco blood samples were available to conduct the present study. Information on age and condition was collected for each individual, and birds were ringed with permanent aluminum bands to avoid resampling. Small blood samples (~100 uL per bird) were collected by venipuncture of the brachial vein and stored in 100% ethanol. Field sampling took place during the breeding season (April–July) between 2001 and 2017 at 14 different localities (Table 1).

DNA Extraction and Sequencing

Genomic DNA was extracted from blood samples using a Qiagen DNeasy Blood Tissue Kit (Qiagen), and we amplified 479 base pairs (bp) of the parasite cyt *b* gene using a nested polymerase chain reaction (PCR) protocol. In the first reaction 3 hemosporidian genera were amplified using

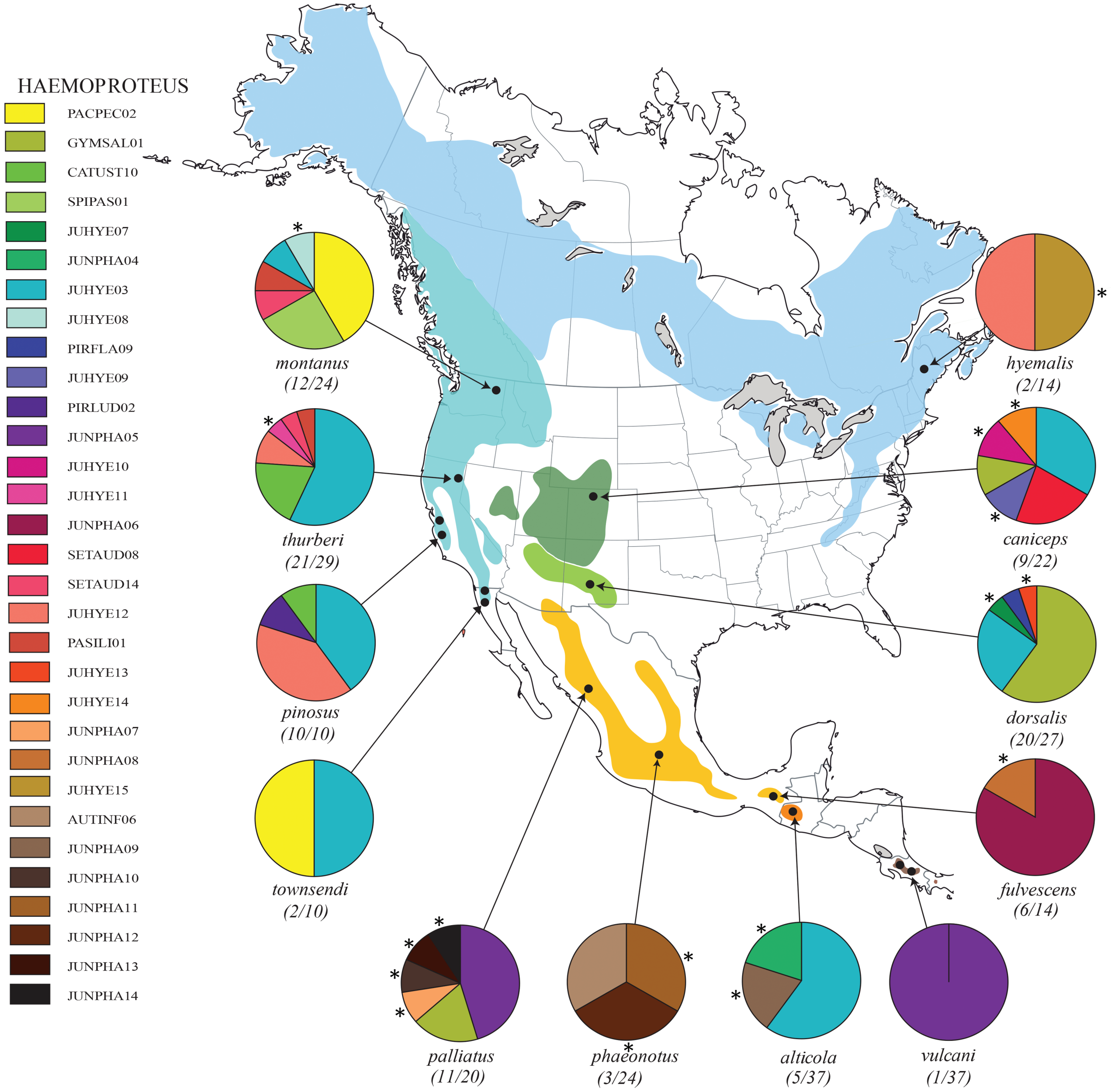

FIGURE 1. Diversity and prevalence of *Haemoproteus* lineages in the genus *Junco*. Lineages and frequency of *Haemoproteus* in each *Junco* taxon. Prevalence per locality is shown in parentheses (infected birds per total sample). Asterisks identify the parasite lineages that have been found in a single junco taxon. Colors on the map represent the 6 *Junco* taxa distributions.

HaemNF1 (5’-CATATATTAAGAGAAITATGGAG-3’) (I = inosine) and HaemNR3 (5’-ATAGAAAGATAAGAAATACCATTC-3’). The second reaction was performed with the primers HaemF (5’-ATGGTGCTTTCGATATATGCATG-3’) and HaemR2 (5’-GCATTATCTGGATGTGATAATGGT-3’) for *Plasmodium* spp. or *Haemoproteus* spp., and HaemFL (5’-ATGGTGTTTTAGATACTTACATT-3’) and HaemR2L (5’-CATTATCTGGATGAGATAATGGIGC-3’) for *Leucocytozoon* spp., following Hellgren et al. (2004). The first PCR was performed in a volume of 25 μL including 2 μL of DNA, 1× MyTaq Reaction Buffer, 5 mM dNTPs and 15 mM $MgCl_2$, 0.6 μM of each primer, and 0.6 units of MyTaq DNA polymerase. The nested PCR was performed with the same proportions and 2 μL of the first PCR product as the template. The following conditions were used to run both PCRs: an initial denaturation of 1 min at 95°C was followed by 34 cycles of 45 s at 94°C, 45 s at 50°C, and 1 min at 72°C, with a final 10 min extension at 72°C. Amplified fragments were precipitated and sequenced in both

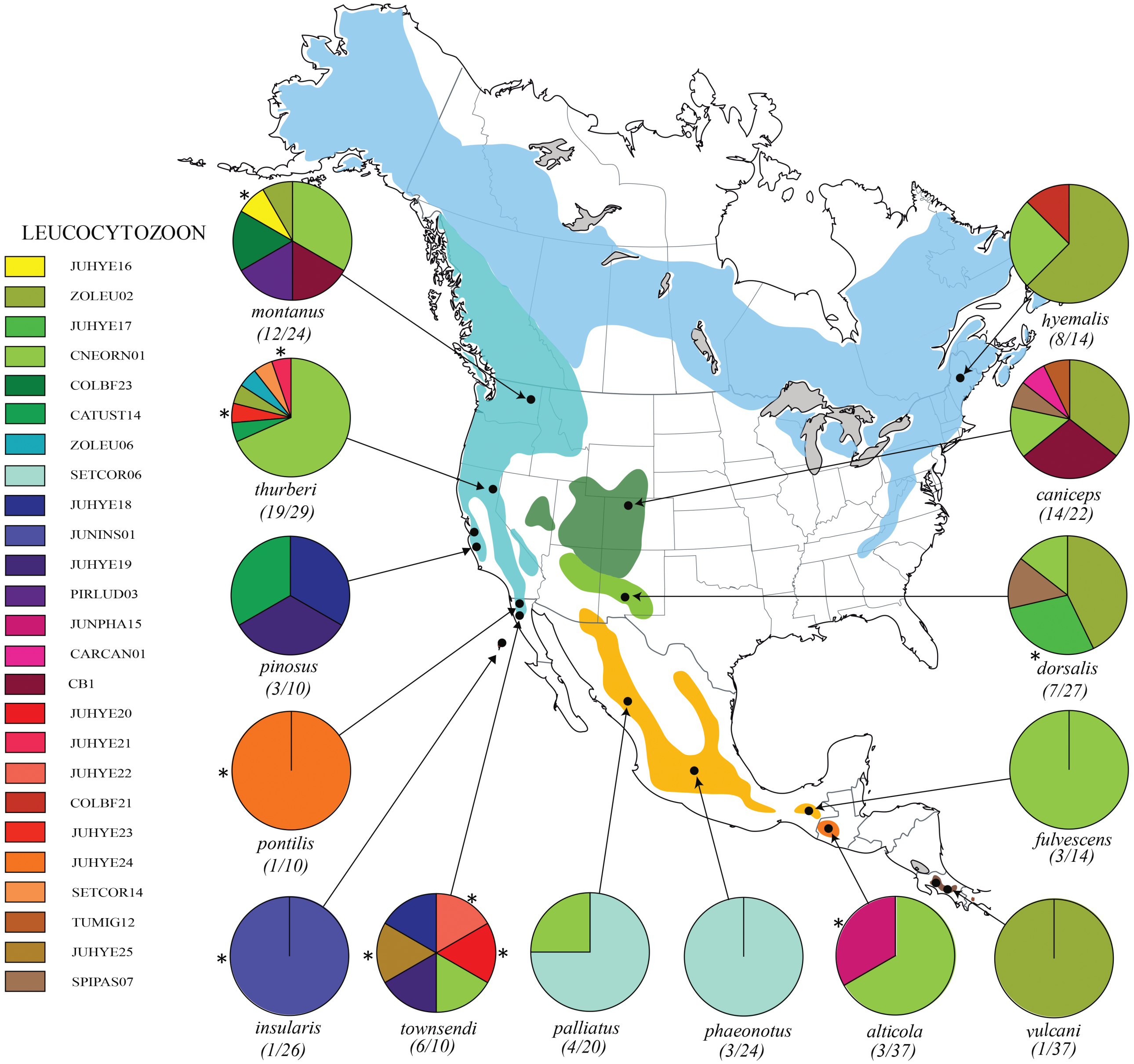

FIGURE 2. Diversity and prevalence of *Leucocytozoon* lineages in the genus *Junco*. Lineages and frequency of *Leucocytozoon* in each *Junco* taxon. Prevalence per locality is shown in parentheses (infected birds per total sample). Asterisks identify the parasite lineages that have been found in a single junco taxon. Colors on the map represent the 6 *Junco* taxa distributions.

directions in an ABI 3730xl DNA automated sequencer at Secugen S.L. (Madrid). Of the total 304 birds sampled, and out of the 608 amplification reactions conducted, we obtained 246 parasite sequences, of which 202 were complete and were used in estimates of diversity and prevalence, and 44 were incomplete (lacked 15 base pairs at the 5' end) and were used in prevalence (presence/absence) analyses only. The nested PCR does not allow us to separate coinfections of *Plasmodium* and *Haemoproteus*, or coinfections with the same parasite genera, since they are amplified using the same pair of primers, so that 40 possibly coinfected sequences (21 *Haemoproteus/Plasmodium* and 19 *Leucozytozoon*) contained double peaks and base ambiguities and were excluded from the analyses. To reduce the probability of false negatives, we repeated those amplifications that did not work the first time, and 17% were positive the second time.

Sequences were aligned automatically using Sequencher 4.1.4 (Gene Codes Corporation) and all variable sites were confirmed by eye on the chromatographs. Sequences differing by one substitution were considered new lineages (Bensch et al. 2004). The software DnaSP v6.11.01 (Librado and Rozas 2009) was used to identify the different parasite lineages and easily determine which lineage was found in

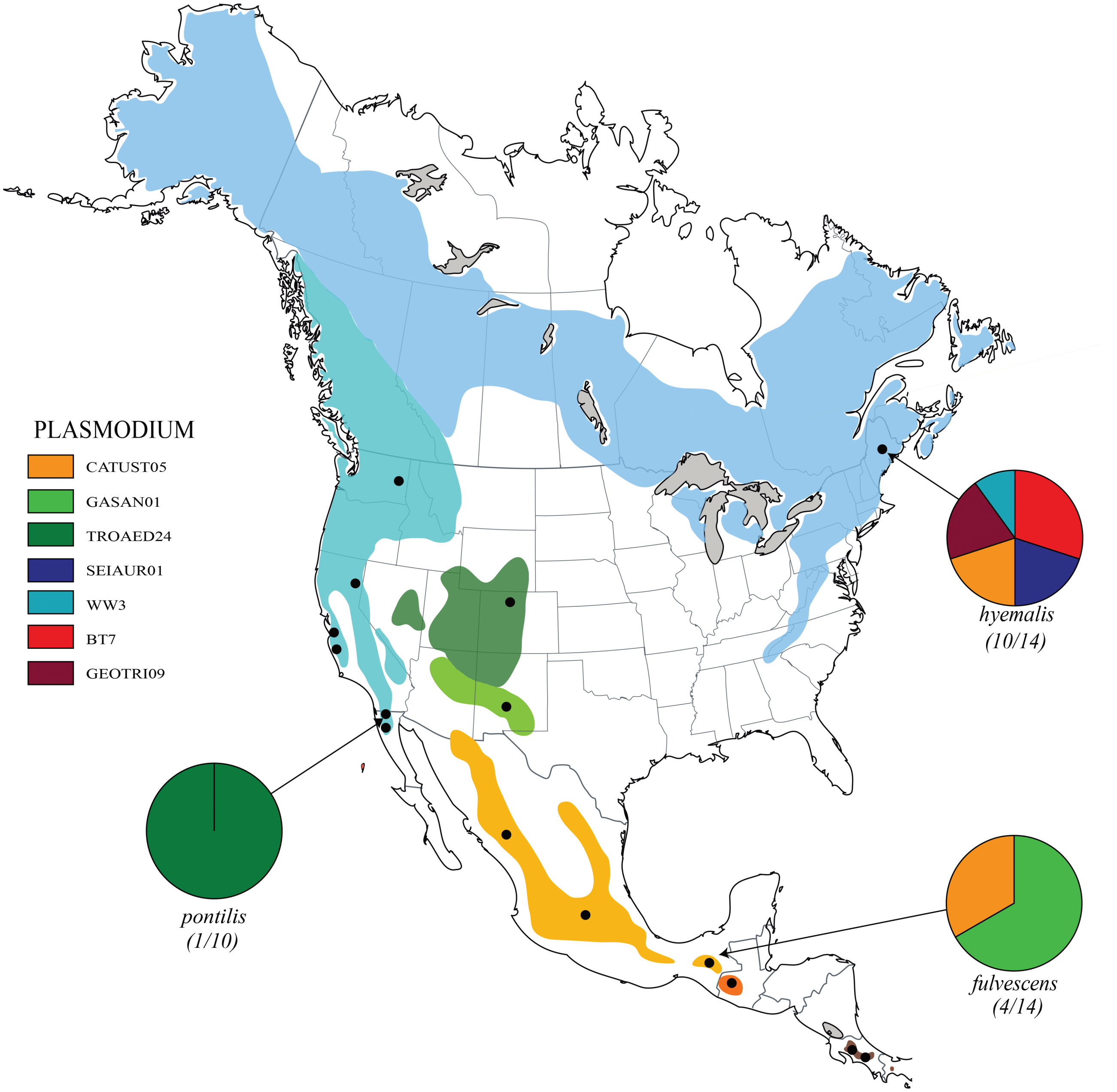

FIGURE 3. Diversity and prevalence of *Plasmodium* lineages in the genus *Junco*. Lineages and frequency of *Plasmodium* in each *Junco* taxon. Prevalence per locality is shown in parentheses (infected birds per total sample). Colors on the map represent the 6 *Junco* taxa distributions.

each sample of juncos. BLAST searches were performed in the MalAvi database (Bensch et al 2009) and GenBank to identify lineages that had been described previously. New lineages were assigned names following the MalAvi nomenclature.

Parasite Prevalence and Diversity

Parasite prevalence per locality was determined as the proportion of individuals infected in the total number of individuals sampled, and parasite diversity was defined as the number of different lineages in each junco taxon. To account for differences in sample size among junco taxa, we used the iNEXT R-package to conduct an extrapolation of the diversity data to a common sample size of 40. The extrapolation was conducted with an endpoint of 40, using 1,000 bootstrap replicates and 95% upper and lower confidence intervals, and taxon-specific rarefaction curves were generated to assess the completeness of the sampling.

TABLE 1. Prevalence and diversity of lineages in each *Junco* taxon. Parasite diversity was calculated with complete, good quality sequences, whereas parasite prevalence was calculated using also partly incomplete sequences but sufficient to determine parasite genus (see Methods). Extrapolated diversity values are shown in parentheses. Note that one individual can be infected by two different parasite genera, so total prevalence is not the sum of parasite prevalence across genera. NM: New Mexico, CO: Colorado, BCN: Baja California Norte, CA: California, OR: Oregon, NH: New Hampshire

Junco taxa	Locality	Country	Elevation (m)	Number of sequences used prevalence	Number of sequences used diversity	Individuals sampled	Total diversity	*Haemoproteus* diversity	*Plasmodium* diversity	*Leucocytozoon* diversity	Total prevalence (%)	*Haemoproteus* prevalence (%)	*Plasmodium* prevalence (%)	*Leucocytozoon* prevalence (%)
vulcani	Cerro de la Muerte and Irazu Volcano	Costa Rica	3,400	5	2	37	2 (2.15)	1(1)	0(0)	1(1)	13.5	10.8	0	2.7
insularis	Guadalupe Island	Mexico	1,095	8	1	26	1(1)	0(0)	0(0)	1(1)	26.9	15.4	0	15.4
alticola	Chichim	Guatemala	3,440	20	8	37	5 (5.24)	3(3.146)	0(0)	2(2.07)	43.2	29.7	0	24.3
fulvescens	Chiapas	Mexico	2,169	15	13	14	6(9.04)	2(2)	3(4.59)	1(1)	78.6	50	28.6	28.6
palliatus	Durango	Mexico	2,505	15	15	20	8(12.05)	6(9.07)	0(0)	2(2)	60	55	0	20
phaeonotus	Mexico City	Mexico	2,755	6	6	24	4(5.42)	3(4.42)	0(0)	1(1)	20.8	12.5	0	12.5
dorsalis	Sacramento Mts. (NM)	USA	2,760	27	27	27	9(11.16)	5(6.12)	0(0)	4(4.75)	77.8	74.1	0	25.9
caniceps	Aspen (CO)	USA	2,242	24	23	22	12(16.48)	6(8.64)	0(0)	6(7.85)	77.3	45.5	0	63.6
townsendi	San Pedro Martir Mts. (BCN)	Mexico	2,842	12	8	10	8(23.33)	2(2.98)	0(0)	6(15.84)	90	50	0	70
pontilis	Juarez Mts. (BCN)	Mexico	1,624	2	2	10	2(2.89)	0(0)	1(1)	1(1)	20	0	10	10
pinosus	Santa Cruz Mts. (CA)	USA	319	13	13	10	7(14.22)	4(4.89)	0(0)	3(5.59)	100	100	0	30
thurberi	Tahoe (CA)	USA	2,050	43	40	29	13(16.26)	6(6.99)	0(0)	7(9.09)	86.2	75.9	0	72.4
montanus	Wallowa N. F. (OR)	USA	1,787	34	24	24	12(14.84)	6(8.11)	0(0)	6(6.55)	100	70.8	4.2	66.7
hyemalis	White Mts. (NH)	USA	833	22	20	14	10(11.817)	2(2.91)	5(5.16)	3(3.45)	100	14.3	71.4	71.4

We also calculated the Shannon diversity index using the same parameters.

We tested the relationship between local bird diversity and both parasite prevalence and parasite extrapolated diversity using linear regression in R (R Core Team 2015). Bird diversity was calculated as the approximate number of species potentially coexisting with juncos at each locality, and was extracted from various published references, using only the number of breeding and wintering species and excluding transients and rarities. Coexistence of a given species was assumed if its range overlapped the junco sampling locality (Supplementary Material Table 1). For North American localities we used the Birds of the World database (Del Hoyo et al. 2018), except for localities for *J. vulcani*, for which diversity data was extracted from Garrigues and Dean (2014), and for Yellow-eyed Juncos *J. p. phaeonotus*, *J. p. palliatus*, *J. p. alticola*, and *J. p. fulvescens*, for which species diversity data were extracted from Howell and Webb (1995).

Host Specificity

Lineage specificity was estimated calculating the host specificity index (S_{TD}) for each parasite lineage. Because host specificity is not just a function of the number of species it can infect, but also of how closely related those species are to each other, the S_{TD} index measures the phylogenetic distinctness among host species used by a given parasite lineage (Poulin and Mouillot 2003). We used this method because it provides a general measure of lineage specificity, taking into account the taxonomic distances among taxa, yet is not as focused on data from the local avian community at a given locality as other methods like the mean phylogenetic distance (MPD) (Svensson-Coelho et al. 2013), given that our sampling was restricted to juncos. To calculate the global specificity of each parasite lineage, avian host species described by other authors were extracted from the MalAvi database. Singleton lineages (those appearing only in one avian host species) were excluded from S_{TD} analysis, as they could influence the results by making the lineages seem more species-specific than they may actually be, and more sampling effort is required to make sure these lineages are only infecting a single host (Moens and Pérez-Tris 2016). Parasite lineages found only in *Junco* taxa had an S_{TD} value lower than 3, and the rest of lineages found in more than one genus had S_{TD} values higher than 3. Therefore, we considered generalist lineages those with S_{TD} values above 3.

Phylogenetic Analysis

To visualize relationships among parasite lineages detected in juncos, we constructed haplotype networks for each genus using a median-joining algorithm (Bandelt et al. 1999) as implemented in PopART (Leigh and Bryant 2015). In addition, to place parasite lineages found in juncos in a broad phylogenetic context, we generated a dataset including lineages found in this study together with all available New World lineages deposited in MALAVI (560 sequences for *Haemoproteus*, 497 sequences for *Leucocytozoon,* and 666 sequences for *Plasmodium*). We then constructed a phylogenetic tree for each of the 3 parasite genera using the neighbor-joining algorithm in MEGA X (Tamura et al. 2007) and modified in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). A sequence from *Leucocytozoon fringillinarum* was used as the outgroup in *Plasmodium* and *Haemoproteus* trees, and *Plasmodium gallinaceum* was used as the outgroup in the *Leucocytozoon* tree. Finally, to visualize the relationship between the junco phylogeny and that of *Haemoproteus* lineages, we generated neighbor-joining trees as above and linked them by means of a tanglegram constructed by hand.

RESULTS

Parasite Prevalence

Out of 304 birds sampled, 178 tested positive for one or more parasite genera, resulting in an overall prevalence of 58.5% (Table 1). Out of the total, 126 were positive for *Haemoproteus* (41.4%), 104 for *Leucocytozoon* (34.2%), and 16 for *Plasmodium* (5.3%). Parasite prevalence varied considerably among junco taxa: from 0% to 100% in *Haemoproteus*, 2.7% to 72.4% in *Leucocytozoon*, and from 0% to 71.4% in *Plasmodium* (Table 1).

Lineage Diversity

Thirty-one *Haemoproteus* lineages were found among the 102 juncos of 12 taxa that tested positive for this parasite (Table 1). Out of those 31 lineages, 20 are reported here for the first time. Lineages CATUST10 (GenBank accession: MG726181) and JUHYE03 (GenBank accession: KF314764) had been detected previously in *J. hyemalis* individuals sampled in Alaska and California, respectively (Oakgrove et al. 2014, Walther et al. 2016). According to the MalAvi database, JUHYE03 has also been recorded in *Sitta pygmaea* and *Sialia mexicana* (Barrow et al. 2021). In our study it was the most common lineage, detected in 29 out of 102 individuals (28.43%) in seven *Junco* taxa: *alticola*, *caniceps*, *dorsalis*, *townsendi*, *thurberi, pinosus*, and *montanus* (Figure 1). There were 17 *Haemoproteus* lineages restricted to single *Junco* taxa. It is worth mentioning that lineage JUNPHA06, which appears for the first time in this study, was detected in 5 individuals of a single taxon (*J. ph. fulvescens*). Taxa *palliatus* and *caniceps* showed the highest diversity after extrapolation, and they also present the highest Shannon indices, indicating that lineage evenness was greater in those juncos (Supplementary Material Table 2).

TABLE 2. Host specificity index (S_{TD}) values for each parasite haplotype detected in juncos across their range. Parasite lineages are ordered according to increasing S_{TD} value.

Parasite genus	Lineage	S_{TD} value
Haemoproteus	JUHYE12	1.43
	JUNPHA05	2.00
	JUYHE03	2.10
	CATUST10	2.89
	SETAUD08	2.99
	GYMSAL01	3.10
	PIRLUD02	3.41
	SETAUD14	3.50
	SPIPAS01	3.64
	PACPEC02	3.83
	PASILI01	3.96
Leucocytozoon	JUHYE19	1.00
	JUHYE18	1.00
	TUMIG12	2.57
	CARCAN01	3.00
	PIRLUD03	3.25
	ZOLEU02	3.27
	CNEORN01	3.66
	SETCOR06	3.76
	SPIPAS07	3.83
	CB1	3.86
	COLBF21	3.89
	CATUST14	3.94
Plasmodium	BT7	3.77
	GASAN01	3.86
	TRAED24	3.91
	GEOTRI09	3.92
	SEIAUR01	3.95
	WW3	4.07
	CATUST05	4.12

From 85 positive samples for *Leucocytozoon*, 25 lineages were identified. According to the MalAvi and GenBank databases, 12 of these lineages have not been described previously. The lineages ZOLEU02 (GenBank accession: MG726144.1), CB1 (GenBank accession: MG726102), CNEORN01 (GenBank accession: MG726148), and TUMIG12 (GenBank accession: MG726105) were detected in *Junco hyemalis* from Alaska by Galen and Witt (2014) and Oakgrove et al. (2014). CNEORN01 and ZOLEU02 are the most common lineages in our samples, infecting 29 birds from 9 taxa (34.11%) and 16 birds (18.82%) from 6 taxa, respectively (Figure 2). Furthermore, 10 of the *Leucocytozoon* lineages were infecting single *Junco* taxa.

Plasmodium was rare in our samples, and only 7 lineages were detected in 15 birds of 3 junco taxa (Figure 3), 5 of them in a single junco taxon (*J. h. hyemalis*). The lineages GEOTRI09 and SEIAUR01 (GenBank accession: MG726173) were already found in *Junco hyemalis* in Alaska (Oakgrove et al. 2014) and Virginia (Slowinski et al. 2018). No new *Plasmodium* lineages were discovered.

Host Specificity

Of 31 *Haemoproteus* lineages found in juncos, 17 were singletons (and thus not included in this analysis), 2 (JUHYE12 and JUNPHA05) had S_{TD} values below or equal to 2.0 (1.43 and 2.0, respectively), and JUHYE03 had a S_{TD} value of 2.1. The latter value means this parasite lineage infects closely related hosts separated by small phylogenetic distances (Table 2), although it was recently found to infect other bird species besides juncos. According to the MalAvi database, these 4 lineages appear to infect mostly juncos and are more specific than the rest of lineages. JUHYE03 is the most prevalent *Haemoproteus* lineage in juncos, infecting 29 individuals of 7 different taxa (50%) (Figure 4). Five other lineages (JUNPHA04, JUNPHA08, JUNPHA09, JUHYE11, and JUHYE15) were found to differ from JUHYE03 by only one or two base pairs, suggesting they have derived from it recently (Figure 5A). Together, this group of closely related haplotypes is found in 9 of the 14 *Junco* taxa, from the older Central American taxa (*fulvescens* and *alticola*) to the more recently diverged dark-eyed junco taxa (Figures 1 and 4). We found 25 lineages of *Leucocytozoon*, of which 10 were found only in 1 *Junco* taxon (and thus were excluded from the host-specificity analysis) (Figure 2). Two of the lineages had low S_{TD} values (S_{TD} = 1) and appeared for the first time in this study (JUHYE19 and JUHYE18) (Table 2). The remaining lineages tended to be more generalist, showing S_{TD} values above 2.56. *Plasmodium* lineages had S_{TD} values slightly higher than those appearing in *Leucocytozoon* and *Haemoproteus* (from 3.77 to 4.12), showing a more generalist pattern (Table 2).

Phylogenetic Analysis

Haplotype networks showing relationships among parasite lineages as well as the frequency of each lineage across the junco range, revealed different patterns across parasite genera (Figure 5). In *Haemoproteus* and *Leucozytozoon*, some haplotypes are separated by long branches, whereas others form groups of closely related haplotypes. Among the latter, 3 cases stand out where a high-frequency haplotype is surrounded by closely related, low frequency haplotypes (*Haemoproteus* GYMSAL01 and JUHYE03, and *Leucozytozoon* CNEORN01), a “starlike” pattern that is typically associated with a rapid population expansion by the high-frequency, ancestral haplotype, followed by recent mutation. In contrast, the few *Plasmodium* lineages sampled were found to be very divergent from each other (Figure 5B), with a high average number of substitutions separating them (13.8 ± 6.8).

When we placed parasite lineages found in juncos in a broad phylogenetic context using all New World lineages previously reported in the MalAvi database, most lineages detected in juncos showed high phylogenetic diversity

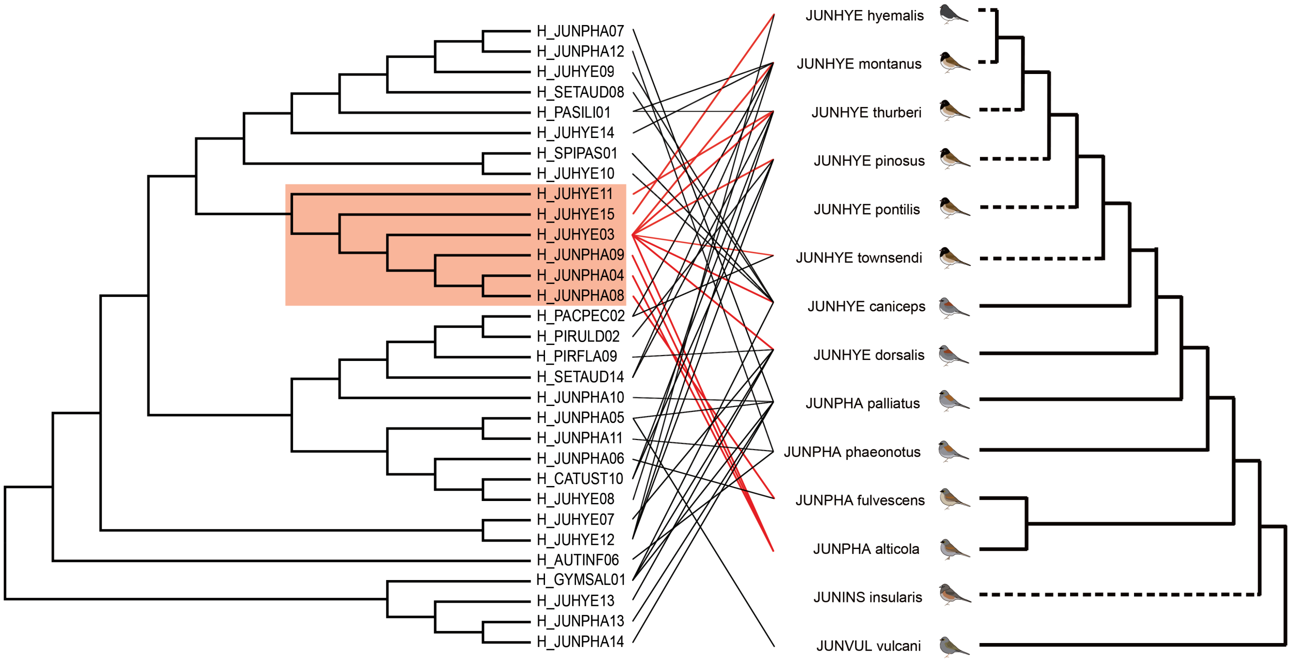

FIGURE 4. Diversity and specificity of *Haemoproteus* lineages infecting junco taxa. Connecting lines between parasite lineages (left) and junco lineages (right) represent confirmed infection in at least one case. Lineages inside the red box on the *Haemoproteus* phylogeny (and associated red connecting lines) are those differing in just one or two base pairs from the JUHYE03 (see also Figure 5A). Junco taxa are ordered according to their phylogenetic relationships, as depicted by the phylogenetic tree on the right (modified from Friis et al. 2016). Dashed branches represent those predicted or with low node support due to recent divergence.

and were spread across the phylogeny of their respective genera, and were not found to cluster together in a single clade (Supplementary Material Figure 1).

Local Avian Diversity and Parasite Prevalence

The total prevalence of infection was positively correlated with the number of species in the bird community of each junco taxon ($F = 15.71$, $P = 0.002$, $R^2 = 0.61$). Per-genus tests revealed a positive correlation between prevalence and avian diversity in *Haemoproteus* ($F = 9.16$, $P = 0.013$, $R^2 = 0.478$; Figure 6A) and *Leucocytozoon* ($F = 20.45$, $P = 0.0011$, $R^2 = 0.671$; Figure 6B), but not in *Plasmodium* ($F = 0.33$, $P = 0.57$, $R^2 = 0.032$; Figure 6C). The correlation between parasite lineage diversity and local avian diversity was significantly positive for *Haemoproteus* (extrapolated diversity: $F_{1,10} = 5.047$, $P = 0.048$, $R^2 = 0.34$; Shannon index: $F_{1,10} = 2.90$, $P = 0.119$; $R^2 = 0.22$; Figure 6D) but not for *Leucocytozoon* (extrapolated diversity: $F_{1,10} = 2.52$, $P = 0.14$, $R^2 = 0.20$; Shannon index: $F_{1,10} = 0.22$, $P = 0.65$, $R^2 = 0.022$; Figure 6E), nor *Plasmodium* ($F_{1,10} = 0.01$, $P = 0.90$, $R^2 = 0.001$; Shannon index: $F_{1,10} = 0.01$, $P = 0.92$, $R^2 = 0.001$; Figure 6F). Although correlations in *Leucocytozoon* tend to be positive (Figure 6E), they were not significant due to the diversity of *pontilis* and *townsendi*, which despite living in low avian richness habitats, presented high parasite diversity. If these isolated Baja California populations were excluded, results were also positively correlated with local avian species richness (extrapolated diversity: $F_{1,9} = 38.89$, $P = 0.00$, $R^2 = 0.81$; Shannon index: $F_{1,9} = 14.87$, $P = 0.00$; $R^2 = 0.623$). As can be seen on the graphs (Figure 6), most of these correlations are partly affected by a bimodal pattern of species richness, which results in the segregation of data points at opposite extremes of the regression line. This is mainly caused by the fact that juncos at lower latitudes tend to occupy high-elevation habitats, where species richness is low, so that correlation results must be interpreted with caution. Rarefaction curves for haemosporidian diversity in most junco taxa failed to reach an asymptote, suggesting that additional sampling will be necessary to properly document the existing haemosporidian diversity in the genus (Supplementary Material Figure 2).

DISCUSSION

Our results represent a first attempt at documenting the distribution of haemosporidian parasites across the range of the genus *Junco*, which spans Central and North America. In our study, haemosporidian prevalence and diversity in the genus *Junco* did not follow a standard latitudinal diversity gradient (greater diversity near the equator) as described in previous studies (Fecchio et al. 2019). Instead, we found greater parasite diversity and abundance at higher latitudes. This is likely due to the fact that at tropical latitudes juncos are restricted to very high

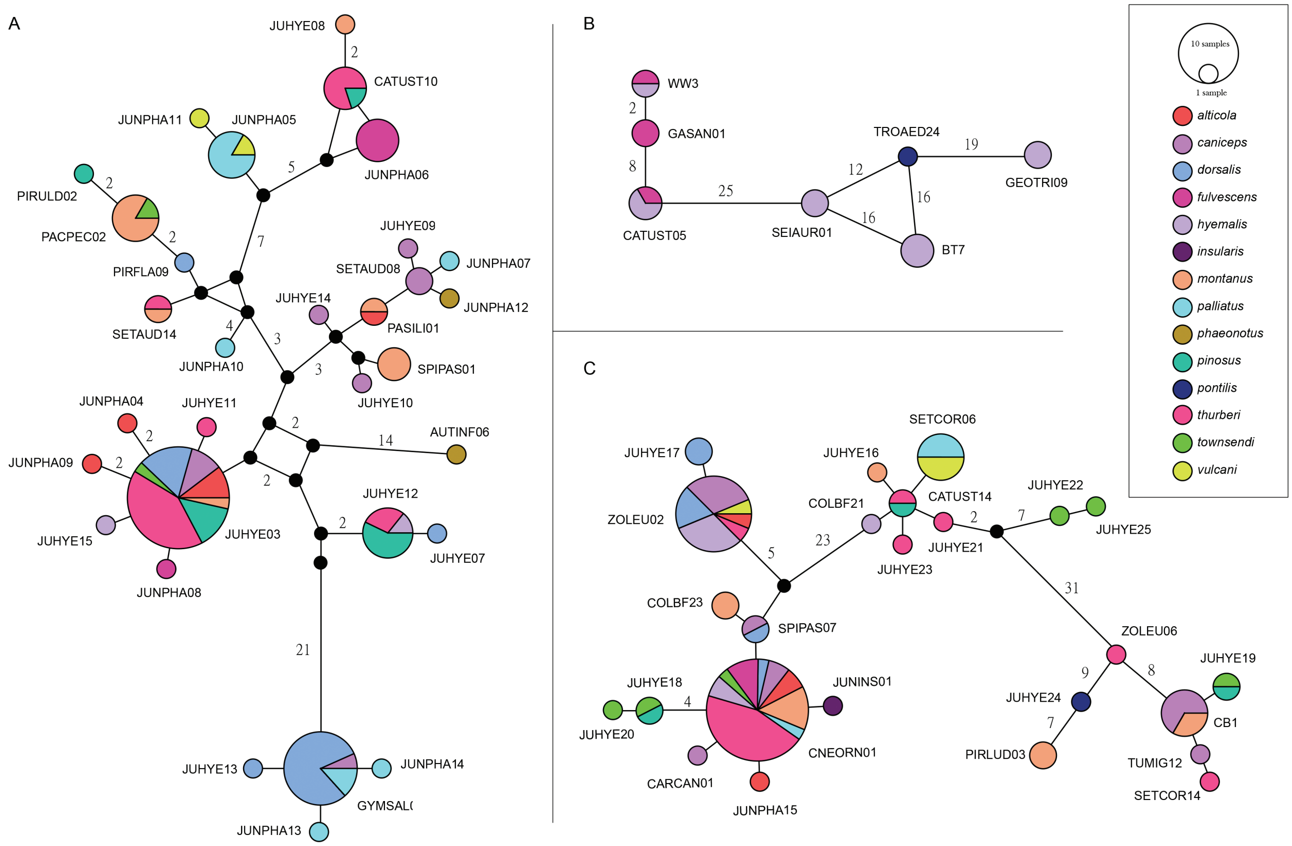

FIGURE 5. Phylogenetic relationships among haemosporidian lineages found in juncos sampled across their range. Median-joining networks of *Haemoproteus* (**A**), *Plasmodium* (**B**), and *Leucocytozoon* lineages (**C**), where circles represent parasite lineages (individually labeled), the size of the circle is proportional to the lineage's frequency in the sample, and small black dots represent unsampled or extinct haplotypes. Branches correspond to a single nucleotide change, and numbers indicate the number of changes if more than one. Colors correspond to the different junco taxa where the parasites were detected, as shown in the legend.

elevations, where avian diversity is low, whereas at higher latitudes they can occupy lower elevations where the avian community is richer. Another factor that may contribute to the low diversity of parasites in the Central American taxa (*vulcani*, *alticola*, and *fulvescens*) is the fact that the high-elevation junco populations are small and highly isolated, and have suffered population bottlenecks that have purged genetic diversity (Milá et al. 2007, Friis et al. 2016), which may have contributed to the loss of parasite lineages as well. Analysis of haemosporidian diversity and prevalence in high-elevation tropical habitats at the avian community level will be necessary to disentangle the ecological underpinnings of host–parasite dynamics there.

Many lineages found in juncos were also found in other bird species, suggesting they may have been acquired by host shifts from the bird community to juncos (Barker 1991, Bensch et al. 2000, Ricklefs and Fallon 2002). Yet an interesting finding from our study is that a small group of *Haemoproteus* lineages including JUHYE03 and its close relatives, were found to be present across the junco range, from the old and divergent Central American taxa (*fulvescens* and *alticola*) to the more recently diverged ones in North America, with JUHYE03 remaining unchanged through the diversification of the genus *Junco*. The high prevalence of JUHYE03 in juncos and the apparent low incidence in other bird species (at least in North America and as determined from records in public databases), suggests that this lineage could be a junco specialist that colonized North America as juncos diversified across the continent. Being specific to a host or a group of closely related hosts, can have some benefits such as higher fitness in the hosts that they exploit, and higher ability to face changes in host defense as parasites adapt to their immune system (Poulin and Mouillot 2004, Beadell et al. 2009). Our preliminary data suggest that this group of parasite lineages may have remained largely specialized in juncos as new junco species evolved, in spite of the broad range of latitudes, altitudes, and habitat types colonized by juncos within the last 18,000 years. Moreover, the star-like phylogenetic pattern shown by JUHYE03 and its close relatives in the phylogenetic network, with JUHYE03 occupying a central position, surrounded by low-frequency lineages of recent

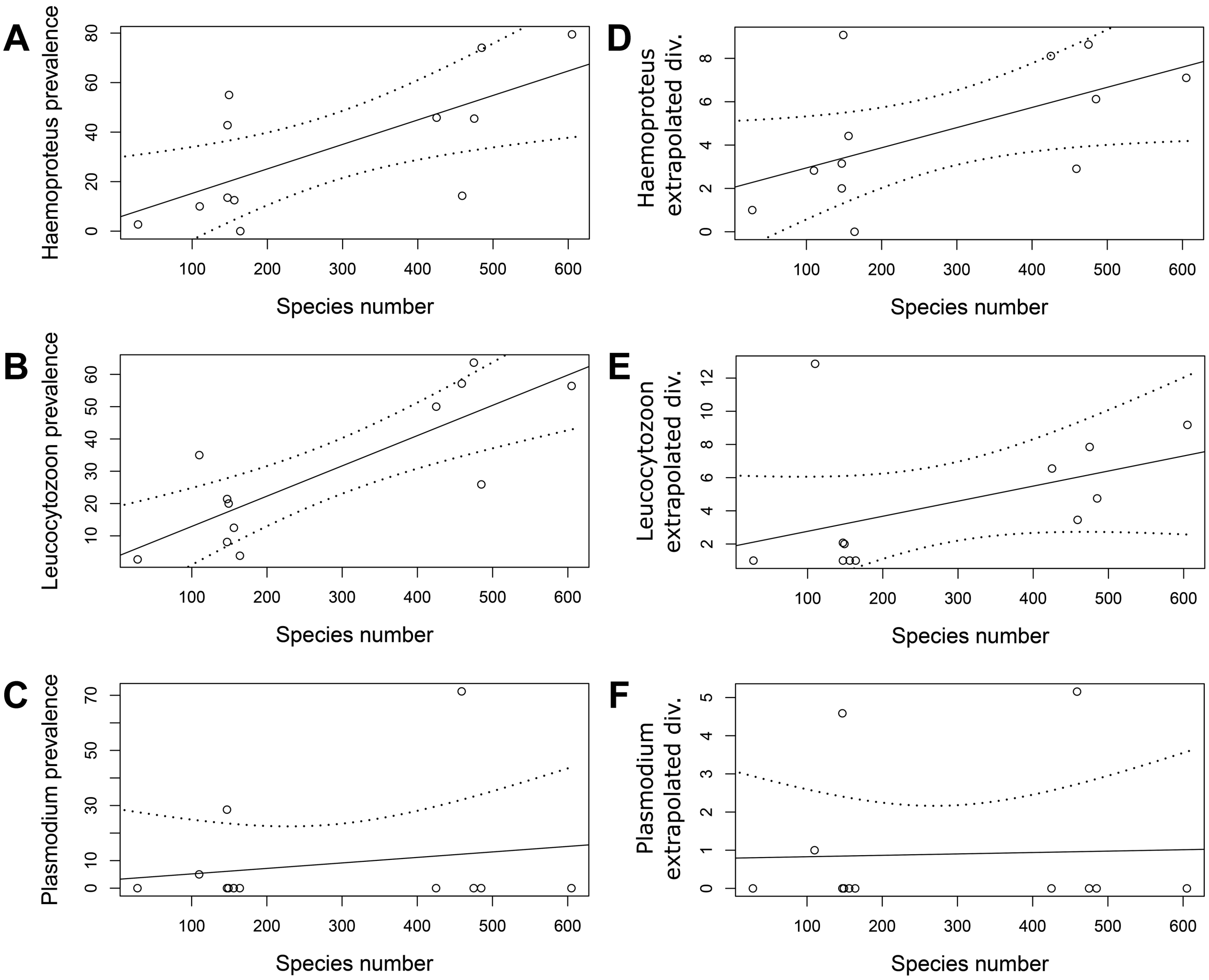

FIGURE 6. Relationship between parasite prevalence and extrapolated parasite diversity and local avian species diversity. Shown are linear regressions between the number of bird species in the local community and *Haemoproteus* prevalence (**A**), *Leucocytozoon* prevalence (**B**), *Plasmodium* prevalence (**C**), *Haemoproteus* extrapolated diversity (**D**), *Leucocytozoon* extrapolated diversity (**E**), and *Plasmodium* extrapolated diversity (**F**). *Leucocytozoon* extrapolated diversity statistics excluding Baja California juncos are as follows: $F_{1,9} = 38.48$, $P = 0.001$, $R^2 = 0.790$. Dotted lines correspond to 95% confidence intervals.

origin, suggests that the parasite itself may have diversified within the junco host, an evolutionary phenomenon that has been previously reported in blackcaps (Pérez-Tris et al. 2007), although further sampling will be necessary to confirm this hypothesis.

The apparent absence of JUHYE03 in central and northern Mexico (*palliatus* and *phaeonotus*) could be due to the relatively dry habitats there, which could lead to lower fitness in the parasite or its vector, although additional sampling would be necessary to confirm this. An alternative explanation for the presence of JUHYE03 in both old and young junco lineages is that this parasite underwent an independent recent expansion across the continent, and that its association with juncos is not the result of an old host–parasite relationship. However, given the distances involved and the broad range of habitat types, climatic conditions, and vector abundances across the region, such an expansion would more likely be undertaken by a generalist parasite than an apparent specialist like JUHYE03, and thus we find this to be a less parsimonious hypothesis given current data. Importantly, since we only surveyed juncos in the field and therefore lack information about prevalence and diversity of parasites in other bird species at our sampling localities, our conclusions on the apparent host-specificity of JUHYE03 and its closely related lineages are necessarily tentative and will require confirmation as the parasite diversity of more avian communities is sampled.

According to presently available data, the rest of *Haemoproteus* lineages found in juncos appeared to have a different strategy from that of JUHYE03 and its relatives, and infected more host species from different avian families. The generalist strategy may be less vulnerable to extinction, since the parasite does not depend on a single host to survive (Beadell et al. 2009). However, a parasite lineage may appear to be generalist and instead have cryptic diversity, with narrower host range or with recently evolved lineages in the process of specialization (Stireman III 2005). *Plasmodium* lineages are thought to be more generalist than *Haemoproteus* (Atkinson and Van Riper III 1991) which is congruent with our results in juncos. *Plasmodium* lineages in this study have higher S_{TD} values than *Haemoproteus* and *Leucocytozoon*, and every *Plasmodium* lineage found in our junco samples has been previously described in several other bird species from different avian families and even orders, as is the case of BT7, which has been previously found in 4 different orders (namely Passeriformes, Anseriformes, Falconiformes and Charadriiformes) (Yohannes et al. 2009, Ramey et al. 2016, Huang et al. 2020, DeBrock et al. 2021). Regarding *Leucocytozoon*, the vast majority of lineages in the present study are known to be generalists in birds, although it remains to be seen whether the newly described lineages found here in juncos show the same pattern. However, two generalist lineages were found to be widespread across *Junco* taxa in contrast to *Haemoproteus* generalist lineages, which were found only in a few hosts. As described in other studies (Beadell et al. 2009), our results suggest different levels of host specificity even among closely related host lineages. These differences in host specificity could also be due to differences in vector diversity and abundance across the *Junco* range. The literature on avian malaria is biased towards studying bird-parasite associations, and the effect of vectors in the distribution, prevalence and host-specificity of parasites remains poorly understood (Hellgren et al. 2008, Clark et al. 2014, Ferraguti et al. 2018, Lima and Pérez-Tris 2020, Valkiūnas and Atkinson 2020). Some vectors have broad blood-feeding tendencies promoting host switching in generalist parasites, while parasites with specialized vectors tend to have narrower host ranges, although some parasites can remain avian generalists if their single vector has broad blood-feeding tendencies (Njabo et al. 2011). More studies focused on the entire bird-parasite-vector network are needed to better understand host-specificity patterns.

Parasite Diversity and Avian Species Richness

We found that in *Haemoproteus* and *Leucozytozoon*, parasite prevalence and diversity in juncos is positively correlated with the species richness of the local bird community (once Baja California localities are excluded; see Results), as shown in previous studies on other host species (Holt et al. 2003, Hechinger and Lafferty 2005, Ellis et al. 2017, Jones et al. 2018, Fecchio et al. 2019). Given that hosts are the "habitat" of parasites, a greater host richness should lead to higher abundance and diversity of parasites (Poulin and Morand 2000, Anderson and Sukhdeo 2013, Fecchio et al. 2019, Williamson et al. 2019, McNew et al. 2021). Furthermore, with higher abundance, the ability of vectors to find hosts and transmit parasites is increased, which in turn increases the prevalence of the parasite. This transmission rate can be increased at high latitudes, where many of the species are migratory and interact with additional species in the non-breeding grounds (Waldenström et al. 2002, Pérez-Tris and Bensch 2005, Altizer et al. 2011, Ricklefs et al. 2017). Our results show a higher prevalence of *Haemoproteus* parasites at lower elevations with a noticeable higher number of bird species in the communities. Furthermore, our results also show an increase of total parasite diversity, *Haemoproteus* diversity and *Leucocytozoon* diversity when host species richness increases. This phenomenon can be partly explained by the specialization of parasites. As some parasite lineages tend to specialize on one or a few host species (trade-off hypothesis; Futuyma and Moreno 1988, Lima and Bensch 2014), a higher number of host species could result in a higher number of specialized lineages, thus driving up parasite diversity (Anderson and Sukhdeo 2013, Hechinger and Lafferty 2005).

Overall, our study provides a first attempt at describing haemosporidian diversity in a single bird genus across a broad geographic region. We document patterns of parasite diversity and prevalence across junco taxa, and provide evidence for the effect of local bird diversity in shaping the parasite community. In addition to the biotic conditions affecting haemosporidian ranges, abiotic factors such as temperature and precipitation can play an important role as well (Zamora-Vilchis et al. 2012, Harrigan et al. 2014, Padilla et al. 2017, Barrow et al. 2019, Williamson et al. 2019, Fecchio et al. 2020, McNew et al. 2021,), and have not been taken into account here as it would require additional sampling that is beyond the scope of our surveys to date. However, we are confident that our publicly available data on prevalence will be useful in future analyses that take into account environmental variables at large geographic scales. Also, given the small sample sizes from some localities, and the fact that we only captured and sampled juncos at any given locality, and not the avian community at large, results must be interpreted with caution. Larger sample sizes and samples from a larger proportion of species in the local avian community will be necessary to confirm some of our conclusions, particularly those regarding host specificity. Our study underscores the importance and utility of public repositories of genetic information such as MalAvi and GenBank, yet proper geographic and species sampling will be essential in further advancing our understanding of host–parasite dynamics in avian communities.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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Ethics statement: All sampling activities were conducted in compliance with Animal Care and Use Program regulations at the University of California Los Angeles, and with state and federal scientific collecting permits in the USA, Mexico, Guatemala and Costa Rica.

Author contributions: B.M. and E.M.R. designed the study; B.M., E.M.R., N.R.E., G.F., J.H.M. and P.E. conducted the field sampling; E.M.R. conducted the genetic analyses in the molecular laboratory; E.M.R. and B.M. analyzed the data; E.M.R. and B.M. wrote the manuscript with input from all authors.

Data deposits: The analyses reported in this article can be reproduced using the data deposited in public databases. All sequences have been deposited in GenBank (accessions MT350642-MT350686) and have also been submitted to the MalAvi public database (http://130.235.244.92/Malavi/).

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