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Speciation in mountain refugia: phylogeography and demographic history of the pine siskin and black-capped siskin complex

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Following Pleistocene glacial maxima, species that adapted to temperate climates in low-latitude refugia had to modify their ranges as climate changed, expanding either latitudinally towards the poles, or altitudinally to higher elevations in mountainous regions. Within just a few thousand years, populations taking alternative routes during interglacials became isolated from each other and subjected to different selection pressures, often leading to lineage divergence and speciation. The pine siskin *Spinus pinus* is a common and widespread songbird showing relative phenotypic uniformity across the North American continent. One exception is the subspecies found in the highlands of northern Central America (*S. p. perplexus*), which shows marked differentiation in plumage color and shares some traits with the endemic and partly sympatric black-capped siskin *S. atriceps*, suggesting potential introgression or even a hybrid origin of *perplexus*. Relationships and species limits among *pinus*, *perplexus* and *atriceps* have been controversial for decades. We provide new molecular evidence to help resolve the evolutionary history of the group. Phylogenetic analysis of mitochondrial DNA and nuclear intron sequences revealed three distinct lineages within the complex, corresponding to: 1) *S. pinus* individuals from Canada through central Mexico (*S. p. pinus* and *S. p. macropterus*), 2) individuals from the highlands of Guatemala and Chiapas (*S. p. perplexus*), and 3) *S. atriceps.* Pine siskins across North America show evidence of a recent postglacial population expansion and extremely low levels of diversity and structure. In contrast, *S. p. perplexus* shows evidence of demographic stasis, reflecting long-term isolation and restricted dispersal. Marked and diagnostic genetic differences among the three lineages in mtDNA and at least one intron, suggest that a hybrid origin of *S. p. perplexus* is unlikely, yet some degree of introgression between *S. p. perplexus* and *S. atriceps* cannot be ruled out in localities where they occur in sympatry.

Pleistocene glacial cycles have played a major role in shaping the evolutionary and demographic history of species at temperate and boreal latitudes (Hewitt 1996, 2000, Johnson and Cicero 2004). Following glacial maxima, species that found refuge at lower latitudes during cold periods took advantage of newly available habitats and expanded their ranges as ice sheets retreated, often undergoing dramatic continent-wide latitudinal expansions toward the poles (Comes 1998, Taberlet et al. 1998, Hewitt 2000, Schmitt 2007). Alternatively, populations at lower latitudes reacted to climate change by tracking the gradual movement of suitable temperate habitats along altitudinal gradients in subtropical mountainous regions. Thus, as interglacials proceeded, montane populations became isolated both from each other and from increasingly distant populations colonizing temperate latitudes, and these isolated populations began to accumulate genetic differences by drift and environmentally driven selection in the process. Although these concurrent latitudinal and altitudinal spatial dynamics have the potential to drive fast lineage divergence and speciation, their actual role in differentiation has been difficult to assess because, at any given interglacial, there are few species with sister lineages in southern mountains and northern temperate latitudes.

In North American birds, postglacial population expansions across the continent have been documented in a number of species, some of which also have retained breeding populations in mountains of southern Mexico and northern Central America. Phylogeographic studies to date on a few of these species have revealed a general pattern of differentiation, but with varying degrees of intraspecific genetic and phenotypic divergence. Thus, in species showing marked genetic divergence in mitochondrial DNA genes, some show consistent phenotypic differentiation like yellow-rumped warblers *Setophaga coronata* (Milá et al. 2011) or hairy woodpeckers *Leuconotopicus villosus* (Klicka et al. 2011), whereas others show more limited differences in phenotype like white-breasted nuthatches *Sitta carolinensis* (Spellman and Klicka 2007) or black-headed grosbeaks *Pheucticus melanocephalus* (van Els et al. 2014). Similarly, in species showing evidence of a very recent colonization of temperate latitudes, we also find cases with limited phenotypic divergence such as chipping sparrows *Spizella passerina* (Milá et al. 2006), or pygmy nuthatches *Sitta pygmaea* (Spellman and Klicka 2006), in contrast to cases of marked differentiation in plumage color and morphology, as exemplified by the rapid radiation of dark-eyed juncos *Junco hyemalis* (Milá et al. 2007a). Therefore,

results to date on the phylogeography of North and Central American birds reflect idiosyncratic evolutionary histories and the need to investigate speciation processes on a case-by-case basis to better understand the relative importance of specific geographic and selective factors.

Here we examine the phylogeography of the widespread North American pine siskin *Spinus pinus* and its sister species the black-capped siskin *S. atriceps* of Central America to infer the role of recent glacial dynamics in driving lineage divergence in the complex. Pine siskins inhabit open coniferous or pine-oak forests across North America and the highlands of northern Central America, undertaking characteristic irruptive movements for up to hundreds of kilometers in search of explosive pine-seed resources (Dawson 1997). Consistent with this high dispersal capacity, pine siskins show relative phenotypic uniformity across their North American range, from Alaska through central Mexico, where two subspecies (*S. p. pinus* in the US and Canada, and *S. p. macropterus* in Mexico) have been described based on slight differences in the amount of streaking, morphology and the size of yellow wing-spots (Ridgway 1901). A more distinct subspecies, *S. p. perplexus*, is found south of the Isthmus of Tehuantepec, in the highlands of southern Mexico and Guatemala, where it is sympatric with its endemic sister species, the black-capped siskin *S. atriceps* (Fig. 1A). *Spinus p. perplexus* individuals are pale gray, with a variable amount of streaking, and have extensive yellow pigmentation on the wings (Dawson 1997). Some individuals show a dark wash on the crown, similar to that of *S. atriceps*, which is otherwise mostly olive and unstreaked (Fig. 1A, Supplementary material Appendix 1, Fig. A1), a pattern that has led some authors to propose that *S. p. perplexus* could be the result of hybridization between *S. pinus* and *S. atriceps* in Chiapas and Guatemala, and may even constitute a lineage of hybrid origin (Van Rossem 1938, Howell and Webb 1995).

The taxonomic history of the complex has been mired in confusion (Vallely et al. 2014) and molecular evidence to date on the relationships and relative divergence among *S. pinus*, *S. p. perplexus* and *S. atriceps* has been inconclusive and controversial. An earlier study of North and Central American siskins concluded that all three taxa form a monophyletic group, but detected no differentiation in mtDNA markers between *S. p. perplexus* and *S. atriceps* (Arnaiz-Villena et al. 2007) thus suggesting either very recent divergence or potentially past mtDNA introgression, which has been previously documented in passerines (Weckstein et al. 2001, Milá et al. 2011). However, small sample sizes, weak resolution in the inferred phylogenies, and a lack of consensus regarding the nomenclature of group members (gray birds with black caps have been portrayed as '*atriceps*' by some authors), renders results published to date inconclusive (Arnaiz-Villena et al. 2007, 2008). In this study we use the taxonomy and nomenclature proposed by Vallely et al. (2014), which is consistent with major reference works (Howell and Webb 1995, Collar and Newton 2010) and observes three main phenotypic groups: mostly streaked birds found in most of North America (*S. p. pinus* and *S. p. macropterus*), mostly gray-breasted birds found south of the Isthmus of Tehuantepec (*S. p. perplexus*), and olive birds with black caps found in the Guatemalan highlands (*S. atriceps*) (Fig. 1A, Supplementary material Appendix 1, Fig. A1).

We use mitochondrial and nuclear DNA sequence data and extensive population-level sampling to address three main questions: 1) is genetic divergence between *S. p. pinus*, *S. p. perplexus* and *S. atriceps* consistent with phenotypic differences among them? 2) Is the timing of divergence among pine siskin lineages in temperate latitudes and subtropical mountains consistent with climatic changes during the late Pleistocene; and 3) is *S. p. perplexus* from Guatemala a lineage of hybrid origin or instead an independent lineage well differentiated from both *S. pinus* and *S. atriceps*?

Methods

Sampling methods

Birds were captured in the field using mist-nets, and each individual was marked with a metal ring, measured and photographed. For genetic analysis we collected a blood sample by venipuncture of the sub-brachial vein and/or two tail feathers. We obtained samples from 86 individuals from 10 localities across the species range in North and Central America (Fig. 1A, Table 1). Genetic samples from Alaska, California, Oregon, Montana and New Jersey were obtained from tail feathers collected by collaborators running constant-effort banding stations as part of the Mapping Avian Productivity and Survivorship (MAPS) program coordinated by the Inst. of Bird Populations (IBP) in California, and samples are deposited at the UCLA Conservation Genetics Resource Center.

To complement our *S. p. perplexus* samples from the highlands of Guatemala, we obtained genetic material from eight museum specimens deposited at the Moore Laboratory of Zoology (MLZ catalog numbers: 50012, 50013, 50014, 56848, 56892, 56895, 57018, 57023, 57024, Supplementary material Appendix 1, Fig. A2), that were collected near San Cristóbal de las Casas, in Chiapas, Mexico (region 2 in Supplementary material Appendix 1, Fig. A3). This area is isolated from the central Mexican highlands north of the Isthmus of Tehuantepec, and from the Guatemalan highlands (Supplementary material Appendix 1, Fig. A3). Because the DNA of old museum specimens is typically degraded, increasing the risk of cross-contamination from fresh DNA samples, we also amplified and sequenced two equally old museum specimens from the *S. p. macropterus* range in Mexico as controls: one from Durango (20566), and one from Jalisco (26769).

Laboratory protocols

We extracted genomic DNA from blood or feather material using a Qiagen™ DNEasy kit, and amplified two mitochondrial DNA (mtDNA) regions and three nuclear introns. The mtDNA subunit 2 of the NADH dehydrogenase gene (ND2) was amplified with primers L5215 (Hackett 1996) and H6313 (Johnson and Sorenson 1998) and ATP-synthase genes 8 and 6 (ATPase) with primers H9855 (Sorenson et al. 1999) and L8950 (García-Moreno et al. 2004). PCR conditions used were 3-min denaturation at 94°C followed by 36 cycles composed of 30 s at 94°C, 45 s at the annealing temperature 56°C, and an extension

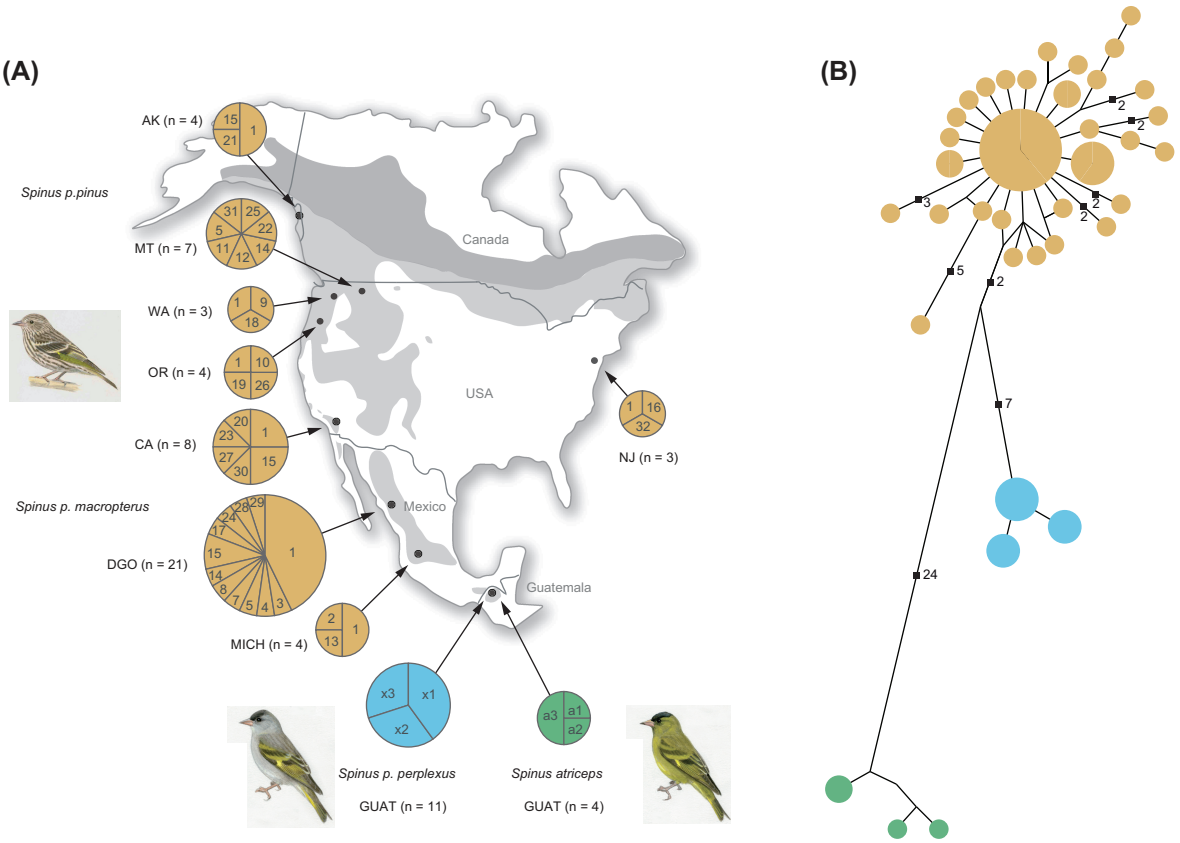

Figure 1. (A) Distribution range, sampling sites, and mtDNA variation for pine and black-capped siskins (*S. pinus* and *S. atriceps*). Light gray areas represent areas where the species is present year-round. Dark gray areas are occupied only in the summer, and wintering areas include most of the continent. Sampling localities are marked by black dots and sample sizes (n) are given for each population. Location codes correspond to Alaska (AK), Washington (WA), Montana (MT), Oregon (OR), California (CA) and New Jersey (NJ) in the USA; Durango (DGO) and Michoacán (MICH) in Mexico, and Huehuetenango (HUE) in Guatemala. Pie diagrams indicate the frequency of all haplotypes detected at each locality, and are color coded by taxon: *S. p. pinus* and *S. p. macropterus* (orange), *S. p. perplexus* (blue), *S. atriceps* (green). For the location of the museum samples from Chiapas used in the study see Supplementary material Appendix 1, Fig. A3. (B) Network of mtDNA haplotypes (concatenated ND2 and ATPase 6&8 genes). Circles correspond to haplotypes, with size being proportional to the haplotype's frequency. Branches correspond to a single nucleotide change, and figures next to black squares indicate additional changes. Color coding as in (A). Bird illustrations by Dale Dyer, reproduced with permission.

of 60 s at 72°C, with a final extension of 5 min at 72°C. The nuclear intron 11 of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was amplified using primers GapdL890 and GapdH950 (Friesen et al. 1997). PCR conditions were set to an initial step of 5 min at 95°C followed by 20 cycles of 30 s at 95°C, 30 s at 55°–65°C and 60 s at 72°C, plus an additional 20 cycles of 30 s at 95°C, 30 s at 55°C and 60 s at 72°C with a final extension of 5 min at 72°C. The aldolase B gene (ALDOB) was amplified using primers AldoB4F (5′-GGCAGGAACAAATGGAGAAACT-3′)

Table 1. Sampling localities in the USA, Mexico (MX) and Guatemala (GT), and sample sizes of pine siskins *S. pinus* and black-capped siskins *S. atriceps*, with corresponding haplotypes for the concatenated mtDNA markers and nuclear intron regions. The frequency of each haplotype is shown in parentheses if greater than one. See Supplementary material Appendix 1–2 for additional details on sampling localities.

Taxa	Locality	n_{mt}	mtDNA haplotypes	GAPDH	BRM15	ALDOB
S. p. pinus	New Jersey, USA	3	1, 16, 32	–	–	–
	Montana, USA	7	5, 11, 12, 14, 22, 25, 31	1(5)	1(5)	1(4)
	Oregon, USA	4	1, 10, 19, 26	–	–	–
	Alaska, USA	4	1(2), 15, 21	–	–	–
	Washington, USA	3	1, 9, 18	–	–	–
	California, USA	8	1(2), 15(2), 20, 23, 27, 30	1(5)	1(5)	1(4)
S. p. macropterus	Durango, MX	21	1(9), 3, 4, 5, 7, 8, 14, 15(2), 17, 24, 28, 29	1(10)	1(9), 2	1(9), 6
	Michoacán, MX	4	1(2), 2, 13	–	–	–
S. p. perplexus	Huehuetenango, GT	11	33(5), 34(3), 35(3)	1(11), 4	1(7), 3(9)	1(6), 5(4)
S. atriceps	Huehuetenango, GT	4	36, 37, 38(2)	1(2), 2(4), 3	4(4)	5(4)

and AldoB4R (5′-GCCAGAACCTGAAAACAGGAG-3′) (Burgess and Penhoet 1985), and intron BRM15, a 350 bp fragment in the region between exons 15 and 16 of the Brahma protein was amplified using primers BRM15F (5′-AGCACCTTTGAACAGTGGTT-3′) and BRM15R (5′-TACTTTATGGAGACGACGGA-3′) (Goodwin 1997). PCR conditions were set to an initial step of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature 56°C and an extension of 60 s at 72°C followed by a final extension of 10 min at 72°C. All products were precipitated with ethanol and sequenced in an ABI 3730X automated sequencer. Sequences were automatically assembled and aligned with Sequencher ver. 4.1.4, and variable sites were checked visually. We assigned haplotypes through DnaSP ver. 5.10 (Librado and Rozas 2009). All mtDNA coding regions were unambiguously translated into their amino acid sequence, suggesting that sequences were of mitochondrial origin and not nuclear copies. Final lengths of sequenced fragments were as follows: 906 bp for ND2, 890 bp for ATPase, 1798 bp for concatenated mtDNA, 306 bp for GAPDH, 350 bp for BRM15, 1017 bp for ALDOB. All sequences have been deposited in Genbank under the following accessions: KT358733–KT358758 (ND2), KT358759–KT358784 (ATPase 6&8), KT358785–KT358791 (BRM), KT358792–KT358799 (GAPDH), and KT358800–KT358806 (ALDOB) (Supplementary material Appendix 2).

Due to the highly degraded DNA encountered in the ten museum specimens obtained from the Moore Laboratory of Zoology at Occidental College, we were only able to amplify and sequence shorter fragments of our target mtDNA genes (93 bp of ND2 and 41 bp of ATPase 6) using primers that were especially designed to target diagnostic regions: ND2F (5′-TCCACCGGCCTCATCCTRTC-3′), ND2R (5′-CYATTCATCCGCCGATAGCT-3′) and ATPaseF (5′-TCAACCTTCTAGGCCTACTACCA-3′), ATPaseR (5′-CATAGGGGGAAAGCCAGTGC-3′). The small fragments were aligned with full-length haplotype sequences in the main dataset and assigned visually to one of the main *S. pinus* haplogroups, but they were not used in the main phylogenetic or genetic diversity analyses.

Phylogenetic analysis and lineage divergence time

Sequences from the two mitochondrial regions and the nuclear introns were concatenated and analyzed using maximum likelihood (ML) and Bayesian inference (BI) algorithms. We used jModelTest ver. 2.0.2 (Posada 2008) to determine the evolutionary model most appropriate for each dataset. We run the BI analysis in MrBayes ver. 3.2 (Ronquist et al. 2012) with data partitioned by gene and by codon. The assigned evolutionary models in ND2 and ATPase were HKY for the 1st position, F81 for the 2nd position and HKY (ND2) and HKY + G (ATPase) for the 3rd position. HKY was the most suitable evolutionary model for all nuclear introns. We used uniform priors and run 4 chains during 2 000 000 generations, sampling every 1000 steps. To check the mixing and convergence of the MCMC runs we used Tracer ver. 1.5 (Rambaut and Drummond 2003), with a burn-in set at 4000 trees and ensuring that ESS values were above 200. Similar tree topologies were recovered from the different partitions and analyses tested. We constructed an ML phylogeny in Mega ver. 6 (Tamura et al. 2013) using the unpartitioned dataset with an HKY + G model of evolution, and 2000 bootstrap replicates to assess node support. As outgroups we used the European siskin *Spinus spinus* (from Genbank accession: HQ915866), which is the sister species to the *S. pinus/atriceps* complex (Nguembock et al. 2009, Zuccon et al. 2012, Beckman and Witt 2015), and the closely related black-headed siskin *S. notatus*, which is a member of a South American sister clade (Arnaiz-Villena et al. 2007).

To estimate time of divergence among lineages we used *Beast in the Beast package ver. 1.7.4 (Drummond et al. 2012) using an HKY + G model of evolution and a strict clock model given the low rate heterogeneity among lineages. We set an average mutation rate of 0.022 substitutions per site per lineage (4.4% my^{-1}) for ND2 gene sequences (Norman et al. 2007, Lerner et al. 2011), with a normal distribution for the rate prior, and rates for ATPase genes and introns were estimated by the program. Both analyses were run for 200 M steps with sampling of the chains every 20 000 steps. Convergence and mixing of the chains, and effective sample sizes (ESS) were checked with Tracer ver. 1.5.

Because intraspecific phylogenies are often based on closely related haplotypes, representing them as the nodes of a network rather than at the tips of a bifurcating tree can be a better way of representing the fact that ancestral and derived haplotypes can be found in the same gene pool (Posada and Crandall 2001). Therefore, we generated haplotype networks for each individual marker and for the two concatenated mtDNA genes using the median-joining algorithm with the software Network ver. 4.6.1.0 (fluxus-engineering.com).

Genetic diversity and demographic history analysis

We estimated indices of haplotype (h) and nucleotide (π) diversity for each population, and generated genetic distances between and within populations using Arlequin ver. 3.5 (Excoffier and Lischer 2010). To assess recent changes in population size we used Fu's test of neutrality (Fu 1997), which is specifically designed to detect events of sudden population growth as inferred from the presence of an excess of young mutations, and we computed values of F_s in Arlequin ver. 3.5. We also explored the correlation between latitude and nucleotide diversity with a simple linear regression analysis in R ver. 2.15.1 (R Core Team).

Relationship between phenotype and sex in *S. p. perplexus*

In order to investigate potential plumage color dimorphism related to sex in Guatemalan *S. p. perplexus*, we sexed individuals using a molecular PCR test with primers P2 and P8 (Griffiths et al. 1998). PCR products were generated with an initial denaturation step of 4 min 30 s at 94°C followed by 40 cycles of 30 s at 94°C, 45 s at annealing temperature 49°C and an extension of 45 s at 72°C, with a final extension of 5 min at 72°C. The amplification results were visually checked in 2% agarose gels, to determine whether individuals were female (two amplified bands), or male (a single band).

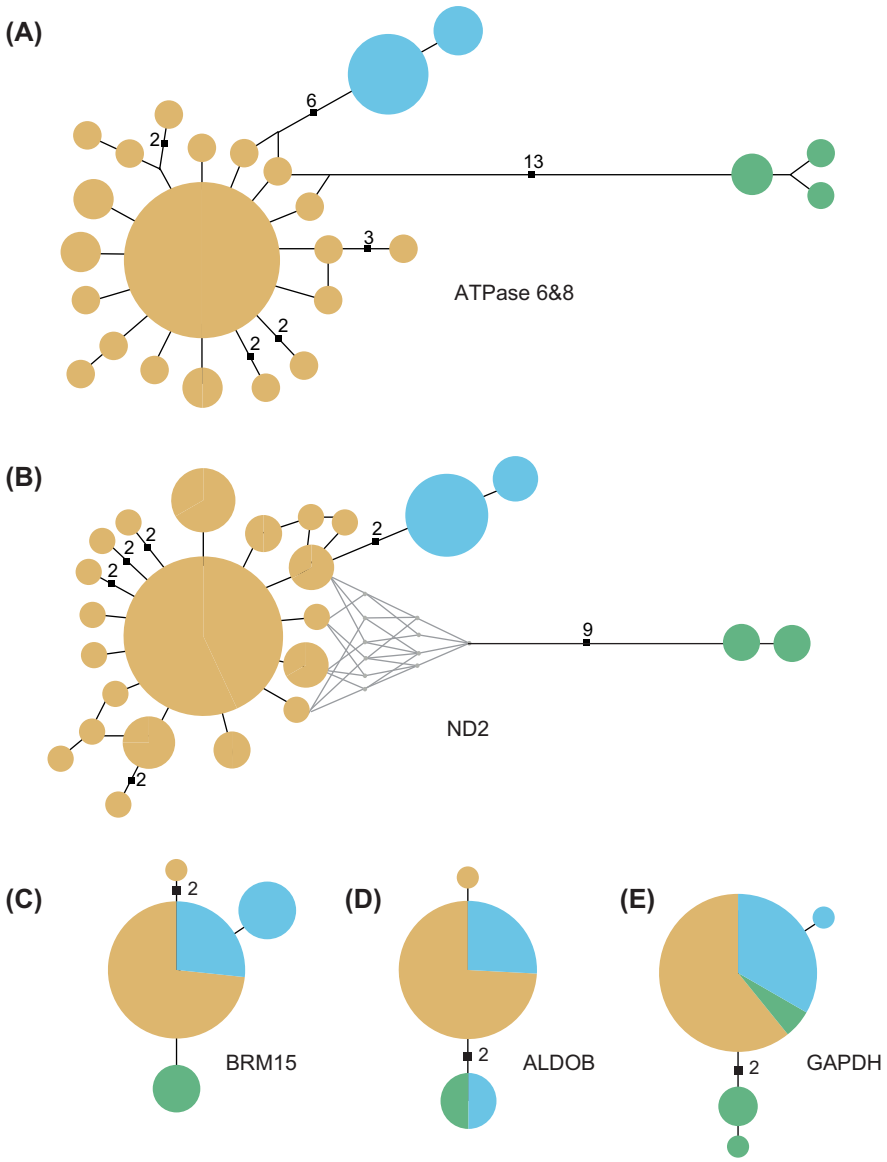

Figure 2. Haplotype networks for the different DNA markers. Shown are the networks for ATPase 6&8 (A) and ND2 (B) mtDNA genes, and nuclear introns BRM15 (C), ALDOB (D), and GAPDH (E). Orange haplotypes correspond to *S. p. pinus* (Canada and USA) and *S. p. macropterus* (Mexico), blue to *S. p. perplexus,* and green to *S. atriceps*. Branches correspond to a single nucleotide change, and figures next to black squares indicate additional changes.

Positive and negative controls for each sex were included in all PCR reactions.

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.dd3qs> (Alvarez et al. 2015).

Results

Genetic structure and lineage divergence

Sequence data revealed the existence of three main geographically structured and phenotypically distinct genetic lineages in the *pinus/atriceps* complex, which correspond respectively to 1) green *S. atriceps* individuals from Guatemala, 2) gray-breasted *S. p. perplexus* birds from Guatemala (including also some streaked birds from Chiapas), and 3) streaked pine siskins found north of the Isthmus of Tehuantepec (*S. p. pinus* and *S. p. macropterus*) (Fig. 1B). No further structure was evident within the latter lineage, and extensive haplotype sharing was seen across the continent (Fig. 1A). The three lineages were best discriminated by the mtDNA genes (ND2 and ATPase 6&8), both of which showed a similar pattern of variation and similar topology, although ATPase genes showed a few additional changes along the main branches (Fig. 2A, B). As expected, the three nuclear introns showed lower levels of variation (Fig. 2C, D, E), and only BRM15 showed separate alleles for *S. atriceps* and *S. pinus*. The three pine siskin subspecies shared most intron alleles, although both BRM15 and GAPDH also showed private alleles for *perplexus*. ALDOB was the least variable intron, with alleles shared between *pinus* and *perplexus*, and between *perplexus* and *atriceps*.

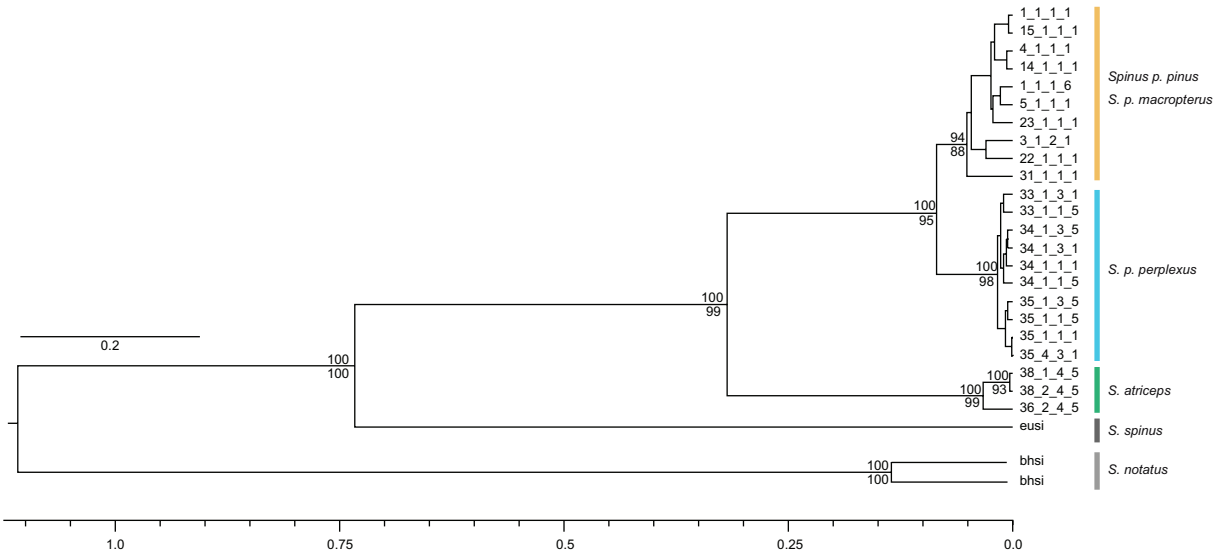

Figure 3. Phylogenetic relationships of pine and black-capped siskin (*S. pinus* and *S. atriceps*) based on concatenated mtDNA and nDNA markers. Terminal labels correspond to haplotype designations in Table 1. Node support above 80% for BI and ML analyses is indicated above and below the branches, respectively. European siskin *S. spinus* and black-headed siskin *S. notatus* are used as outgroups. Time scale along the bottom of the tree in million years.

The three-lineage structure was also recovered in the ML and BI phylogenetic analyses, which show three well-supported clades corresponding to the three main mitochondrial lineages (Fig. 3). Pine siskins, including *perplexus*, form a monophyletic clade that is sister to black-capped siskins, and coalescence analysis revealed that all three lineages originated within the late Pleistocene: *S. pinus* and *S. atriceps* shared a common ancestor about 0.30 million yr ago (MA) (95% HPD: 0.19–0.42 MA), and the two main *S. pinus* lineages (*S. p. pinus*/*macropterus* and *S. p. perplexus*) diverged around 0.11 MA (95% HPD: 0.06–0.17 MA) (Fig. 3).

The short mtDNA fragments sequenced from old museum specimens of *S. p. perplexus* from Chiapas showed three mutations (one mutation at position 5709 in the 93-bp fragment of the ND2 gene, and two mutations at positions 9524 and 9536, respectively, within the 41-bp fragment of the ATPase 6 gene) that are shared among birds in Guatemala and found to be absent or much less common in North American haplotypes, suggesting a closer relationship of these Chiapas individuals with Guatemalan *perplexus* than with *macropterus* populations (Supplementary material Appendix 1, Fig. A4). The two individuals from Jalisco and Durango had identical sequence to that of several *pinus*/*macropterus* haplotypes from North America, as expected.

Genetic diversity and demographic history

The mtDNA haplotype networks for North American *S. p. pinus* and *S. p. macropterus* revealed a star-shaped phylogeny characterized by a single high-frequency and widespread haplotype surrounded by numerous low-frequency, closely related haplotypes (Fig. 1B, 2A, B). This pattern suggests a recent population expansion, as corroborated by results from Fu's test of neutrality, which yielded significant negative values across all mitochondrial markers (Table 2).

Within the concatenated mitochondrial dataset, nucleotide diversity varied between 0.0005–0.0015. In comparison, haplotype diversity values were high and varied between 0.71–0.94 (Table 2). The lowest values in both diversity indices were mostly found in *S. p. perplexus,* a population likely to have suffered long periods of small population size. We found no correlation between nucleotide diversity indices and latitude for *S. pinus* across North and Central America ($R^2 = 0.0179$, $p = 0.736$).

The nuclear introns had generally low diversity values that ranged between 0.0000–0.6667 for *h* and 0.0000–0.0040 for π. Genetic distances were also low, with the strongest differentiation observed between populations of *S. pinus* and *S. atriceps* from Guatemala and Mexico in mtDNA markers (Table 3).

With respect to sexual dimorphism in *S. p. perplexus*, the genetic tests of sex determination of 13 individuals from Guatemala confirmed six of them as female and seven as male. The amount of black on the crown in *perplexus* appears to be associated with sex, with males showing the darkest and females the lightest crowns (Supplementary material Appendix 1, Fig. A5). Of the six females, two had a dark wash on the crown, and six of the seven males had blackish crowns and one had a dusky crown similar to the females.

Discussion

Phylogeography and lineage divergence in the *Spinus pinus/atriceps* complex

Our results revealed the existence of three well-supported phylogenetic lineages which correspond to the main phenotypic groups in the pine/black-capped siskin complex. These include a widespread North American clade that includes

Table 2. Genetic distances between pine siskin *S. pinus* and black-capped siskin *S. atriceps* lineages. Above the diagonal: uncorrected values of mean pairwise differences between populations. Below the diagonal: corrected values of percent mean pairwise differences between populations. Along the diagonal (in italics): percent within-population differences. Lengths of sequenced fragments are as follows: 906 bp for ND2, 890 bp for ATPase, 1798 bp for concatenated mtDNA, 306 bp for GAPDH, 350 bp for BRM15, 1017 bp for ALDOB.

	pinus US	*macropterus* MX	*perplexus* GT	*atriceps*
All mtDNA				
S. pinus US	*0.2*	2.4	12.5	30.2
S. pinus MX	0.0	*0.1*	12.4	31.3
S. pinus GT	0.6	0.6	*0.0*	32.0
S. atriceps	1.5	1.7	1.7	*0.1*
ND2				
S. pinus US	*0.2*	1.2	3.9	13.9
S. pinus MX	0.0	*0.1*	3.6	13.8
S. pinus GT	0.3	0.3	*0.0*	14.7
S. atriceps	1.4	1.4	1.6	*0.1*
ATPase				
S. pinus US	*0.1*	1.2	8.6	16.4
S. pinus MX	0.0	*0.2*	8.9	16.6
S. pinus GT	0.9	0.9	*0.0*	17.3
S. atriceps	1.7	1.7	1.8	*0.2*
GAPDH				
S. pinus US	*0.0*	0.0	0.0	1.6
S. pinus MX	0.0	*0.0*	0.0	1.6
S. pinus GT	0.0	0.0	*0.0*	1.7
S. atriceps	0.4	0.4	0.4	*0.5*
BRM15				
S. pinus US	*0.0*	0.2	0.6	1.0
S. pinus MX	0.0	*0.1*	0.8	1.2
S. pinus GT	0.1	0.1	*0.2*	1.6
S. atriceps	0.3	0.3	0.4	*0.0*
ALDOB				
S. pinus US	*0.0*	0.0	0.8	2.0
S. pinus MX	0.0	*0.0*	0.8	2.0
S. pinus GT	0.0	0.0	*0.1*	1.2
S. atriceps	0.2	0.2	0.1	*0.0*

all streaked birds north of the Isthmus of Tehuantepec, and two more geographically restricted clades in the highlands of Chiapas and Guatemala, one corresponding to the gray-breasted birds in the subspecies *perplexus* (Chiapas and Guatemala), and the other to the green birds in *S. atriceps* (Guatemala).

Marked genetic divergence among pine siskin mitochondrial DNA lineages occurs on either side of the Isthmus of Tehuantepec, a common barrier for numerous avian taxa (Cortés-Rodríguez et al. 2008, Barber and Klicka 2010, Barrera-Guzmán et al. 2012, Rodríguez-Gómez et al. 2013), and molecular dating suggests that gene flow among them has been interrupted for about 200 000 yr, a period of time that corresponds approximately to two full glacial cycles in the late Pleistocene. Demographic histories of populations on either side of the isthmus are markedly different as well. Patterns of genetic diversity and haplotype networks are consistent with a recent population expansion across the North American continent, a pattern that has been observed in a number of North American passerine and non-passerine species (Zink 1996, Ruegg and Smith 2002, Milá et al. 2006, 2007b, Spellman and Klicka 2006, Malpica and Ornelas 2014). In contrast, and despite limited sample sizes, populations in the highlands of Guatemala showed evidence of long-term small effective population sizes. The pattern of intraspecific phylogeography in pine siskins is thus consistent with a scenario of parallel processes of latitudinal range expansions and altitudinal isolation that have resulted in the evolution of independent lineages in temperate regions and high-elevation areas of subtropical regions, respectively.

A potential role for irruptive vagrancy in preventing genetic structure

In contrast to previously studied species, genetic variation in pine siskins north of the isthmus of Tehuantepec reveals a complete lack of structure, even between high latitude populations in the temperate and boreal zones (subspecies *pinus*) and those in the Mexican highlands (subspecies *macropterus*). Studies on other avian species have revealed varying degrees of divergence between northern and southern populations, from low levels of divergence involving differences in haplotype frequencies (Milá et al. 2006, 2007a, b), to the existence of highly divergent intraspecific lineages (Spellman and Klicka 2006, 2007, Milá et al. 2007b, Manthey et al. 2011, Walstrom et al. 2012, van Els et al. 2014).

The lack of structure and the relatively uniform plumage coloration among *S. p. pinus* and *S. p. macropterus* individuals across North America may be explained by irruptive vagrancy, a flexible foraging behavior that in years of food shortage results in individuals dispersing towards localities where food supply is more abundant. Irruptive movements driven by years of high spatio-temporal variation in food resource availability (Koenig and Knops 2001), can potentially have an impact on population mixing and gene flow, leading to low intraspecific structure and weak phenotypic differentiation. The pine siskin is a widespread and regular irruptive migrant in forests across the species range, and groups of individuals are known to move across hundreds of kilometers in search of explosive food resources (Dawson 1997). Also consistent with our genetic results is that across the species range there seems to be a latitudinal pattern in annual migratory behavior, with northern breeding populations moving south in winter, and southern populations in Mexico and Guatemala showing more sedentary behavior and limited altitudinal movements (Howell and Webb 1995). Of course it would be necessary to sample a large number of markers from throughout the genome before drawing definitive conclusions about a lack of genetic structure between the northern subspecies.

Divergence and potential introgression in the highlands of Guatemala

Sedentary *S. p. perplexus* from the Guatemalan and Chiapas highlands have differentiated from *S. p. pinus*/*macropterus* through long-term isolation in high-elevation pine forests, where the relatively low levels of genetic diversity we observed may be due to past population bottlenecks in this small 'island' of suitable siskin habitat. Our results also confirm that the black-capped siskin *S. atriceps* represents a well differentiated sister lineage to *S. pinus* that diverged from the latter around 0.60 MA. The marked divergence of the unique *perplexus* lineage relative to both *S. atriceps* and

Table 3. Indices of genetic diversity and population expansion of pine siskin *S. pinus* and black-capped siskin *S. atriceps* populations. Significance values from Fu's test are marked by asterisks: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

DNA marker	Taxa	n	No. haps.	*H*	π	F_s
All mtDNA						
(1798 bp)	*S. pinus* US	29	21	0.9409 ± 0.0348	0.0015 ± 0.0009	−19.58***
	S. pinus MX	25	14	0.8133 ± 0.0804	0.0011 ± 0.0007	−9.49***
	S. pinus GT	11	3	0.7091 ± 0.0827	0.0005 ± 0.0004	0.32
	S. atriceps	4	3	0.8333 ± 0.2224	0.0013 ± 0.0011	0.36
ND2						
(906 bp)	*S. pinus* US	36	15	0.8159 ± 0.0610	0.0017 ± 0.0012	−11.39***
	S. pinus MX	33	12	0.6004 ± 0.1014	0.0010 ± 0.0008	−10.95***
	S. pinus GT	13	2	0.3846 ± 0.1321	0.0004 ± 0.0005	0.69
	S. atriceps	4	2	0.6667 ± 0.2041	0.0007 ± 0.0008	0.54
ATPase						
(890 bp)	*S. pinus* US	30	11	0.6437 ± 0.1004	0.0012 ± 0.0009	−8.34***
	S. pinus MX	25	11	0.6500 ± 0.1110	0.0015 ± 0.0011	−7.42***
	S. pinus GT	11	2	0.4364 ± 0.1333	0.0005 ± 0.0005	0.78
	S. atriceps	4	3	0.8333 ± 0.2224	0.0019 ± 0.0016	−0.13
GAPDH						
(306 bp)	*S. pinus* US	10	1	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
	S. pinus MX	10	1	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
	S. pinus GT	12	2	0.1667 ± 0.0809	0.0005 ± 0.0009	−0.48
	S. atriceps	7	3	0.6667 ± 0.1598	0.0040 ± 0.0033	0.41
BRM15						
(350 bp)	*S. pinus* US	10	1	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
	S. pinus MX	10	2	0.2000 ± 0.1541	0.0011 ± 0.0013	0.59
	S. pinus GT	16	2	0.5250 ± 0.0546	0.0015 ± 0.0015	1.33
	S. atriceps	4	1	0.0000 ± 0.0000	0.0000 ± 0.0000	0.39
ALDOB						
(1017 bp)	*S. pinus* US	8	1	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.
	S. pinus MX	10	2	0.2000 ± 0.1541	0.0000 ± 0.0000	n.a.
	S. pinus GT	10	2	0.5333 ± 0.0947	0.0011 ± 0.0009	2.00
	S. atriceps	4	1	0.0000 ± 0.0000	0.0000 ± 0.0000	n.a.

S. p. pinus suggests that *perplexus* is not of hybrid origin (if it were, one would expect individuals to carry mtDNA haplotypes from either one of the parental taxa). Diversity of intron BRM15 was congruent with mtDNA in showing separation between *pinus* and *atriceps*, whereas GAPDH and ALDOB showed a pattern of allele sharing between the two lineages. This could be due to incomplete lineage sorting or to partial male-biased introgression. We cannot rule out some degree of ongoing introgression from *S. atriceps* into *S. p. perplexus*, which is also implied by some phenotypic characters shared between the two, particularly the presence of a blackish cap and a green wash on the upperparts of some individuals (Supplementary material Appendix 1, Fig. A1). An alternative explanation is that the sexual dimorphism we have detected in our *S. p. perplexus* sample evolved independently in this taxon, and traits shared with *S. atriceps* are not due to recent introgression. Additional genome-wide data will be needed to definitively assess the degree of introgression, if any, between taxa.

Streaked individuals, some similar to *S. p. macropterus,* have been observed more often in Chiapas than in Guatemala (Vallely et al. 2014), so it is worth asking whether *S. p. perplexus* in Chiapas share a closer relationship to *S. pinus/macropterus*. Guatemalan populations cluster with populations from Chiapas in various species examined to date, such as *Picoides villosus*, (Klicka et al. 2011), *Certhia americana* (Manthey et al. 2011) or *Ergaticus versicolor* (Barrera-Guzmán et al. 2012). Our results from the short sequences obtained from historical museum samples from Chiapas suggest that these individuals belong to the *perplexus* mtDNA lineage found in Guatemala, rather than to the *pinus/macropterus* lineage found north of the Isthmus of Tehuantepec, a pattern generally consistent with phenotype. However, because of its matrilineal inheritance, mtDNA provides information only about females, and with the present dataset we cannot rule out incidental introgression from *macropterus* males, which may explain the presence of more streaking in the plumage of Chiapas birds. Although there are no eBird records (<www.ebird.org>) for *S. pinus/macropterus* in Chiapas, vagrants of *S. pinus* are known to cross large distances, and sporadic vagrants from northern populations could occasionally occur there. Additionally, some species show genetic divergence between Chiapas and Guatemalan highland populations (McCormack et al. 2010, Rodríguez-Gómez and Ornelas 2014, Milá et al. 2015). Further multilocus analysis of samples from this region will be necessary to assess fine-scale patterns of gene flow and divergence.

The phenotypic and genetic divergence of *Spinus p. perplexus* underscores the importance of the Guatemalan highlands as a phylogeographic hotspot where congruent patterns of lineage divergence across species coincide in a small geographic area. In addition to *S. p. perplexus*, independent evolutionary lineages in this area have been documented in broad-tailed hummingbirds *Selasphorus*

platycercus (Malpica and Ornelas 2014), yellow-rumped warblers *Setophaga coronata* (Milá et al. 2007b, 2011), and yellow-eyed juncos *Junco phaeonotus* (Milá et al. 2007a). Given the restricted size of this geographic region, the human pressures on its remaining natural habitats, and the increased extinction risk of high elevation taxa (Oliveras de Ita et al. 2012, White and Bennett 2015), the Guatemalan highlands and adjacent high-elevation areas represent an important evolutionary refugium that should be the target of increased research and conservation.

Taxonomic implications

Our results contribute two major findings with taxonomic implications. First, they confirm that *S. atriceps* and *S. p. perplexus* are two different taxa showing considerable genetic and phenotypic divergence. Thus, gray birds do not represent a different gender or age category as proposed previously (Salvin 1863, Ridgway 1884), a conclusion recently reached as well by Vallely et al. (2014) following their thorough examination of available museum specimens. Second, our results reveal a pattern of marked phenotypic divergence and reciprocal monophyly in mtDNA genes between *S. pinus* lineages on either side of the Isthmus of Tehuantepec, so that if a lack of significant interbreeding is confirmed with *S. p. pinus* and *S. atriceps* using additional genome-wide markers, *S. p. perplexus* will likely constitute a separate taxonomic species. Our findings are in line with several examples of recent speciation events reported in fringillid finches, some of which show congruent divergence in phenotype and mtDNA (Töpfer et al. 2011), with others showing no genetic divergence in the face of marked divergence in plumage characters (Marthinsen et al. 2008, Drovetski et al. 2009). Increased access to genomic resources and analyses will help reveal the evolutionary histories of these young systems, where detecting divergence in a few genes under selection may be critical in order to establish proper species limits (Mason and Taylor 2015).

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Supplementary material (Appendix JAV-00814 at <www.avianbiology.org/appendix/jav-00814>). Appendix 1–2.

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