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MITOCHONDRIAL DNA VARIATION, SPECIES LIMITS, AND RAPID EVOLUTION OF PLUMAGE COLORATION AND SIZE IN THE SAVANNAH SPARROW

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Abstract. We compared sequences from two mitochondrial DNA (mtDNA) genes (ND2, ND3) in Savannah Sparrows (*Passerculus sandwichensis*; $n = 112$) sampled from Baja California (five sites), coastal Sonora and the continental range (eight sites). Populations from Baja California, San Diego and Sonora formed a clade within which there was no phylogeographic structure; this clade merits species status (*Passerculus rostratus*). The other clade, consisting of phenotypically ''typical'' savannah sparrows, should be classified as *P. sandwichensis*. Among the typical sparrows, there was no phylogeographic structure, although two major clades were discovered. Representatives of each of the two main clades occurred at most sampling localities, excluding Suisan Bay, California and Sable Island, Nova Scotia, Canada. Haplotypes found on Sable Island, representing the ''Ipswich Sparrow,'' were not distinctive, thereby failing to support species status for this taxon. On Isla San Benito, a single haplotype was found, which also occurred in other Mexican localities. The results for Sable Island and Isla San Benito show that size and plumage coloration can evolve rapidly.

Key words: islands, mitochondrial DNA, phylogeography, species limits, subspecies.

Variación en ADN Mitocondrial, Límites entre Especies y Evolución Rápida de la Coloración del Plumaje y el Tamaño en *Passerculus sandwichensis*

Resumen. En este estudio comparamos secuencias de dos genes mitocondriales (ND2 y ND3) entre individuos de la especie *Passerculus sandwichensis* ($n = 112$) muestreados en Baja California (5 sitios), la costa de Sonora y el rango de distribución continental (8 sitios). Las poblaciones de Baja California, San Diego y Sonora formaron un clado, al interior del cual no existió estructura filogeográfica; este clado merece estatus de especie (*Passerculus rostratus*). El otro clado, conformado por individuos fenotípicamente ''típicos'', debe clasificarse como *P. sandwichensis*. Entre los individuos típicos no existió estructura filogeográfica, aunque se descubrieron dos clados principales. Individuos representativos de cada uno de estos dos clados se encontraron en la mayoría de las localidades, excepto Suisan Bay, California y Sable Island, Nova Scotia. Los haplotipos encontrados en Sable Island, correspondientes al ''gorrión de Ipswich'' no fueron distintivos, lo que no apoya el estatus de especie para este taxón. En Isla San Benito se encontró un solo halpotipo, el cual también se encontraba en otras localidades mexicanas. Los resultados de Sable Island e Isla San Benito muestran que el tamaño y la coloración del plumaje pueden evolucionar rápidamente.

INTRODUCTION

The Savannah Sparrow (*Passerculus sandwichensis*) is one of the more phenotypically variable birds in North America (AOU 1957). The causes of this variation appear to be related to adaptation to local environments. Most of the phenotypic variation can be characterized as gradually clinal, with the exception of the population on Sable Island, Nova Scotia, Canada (*P. s. princeps*), and populations living in salt marshes in Baja California and along the coast of Sonora and Sinaloa, Mexico (Rising 2001). In the latter instances, variation is stepped in nature. The question arises as to whether there are genetic patterns of variation that are congruent with the geographic variation in morphology. In some continentally distributed species that exhibit considerable morphological variation, such as the Song Sparrow (*Melospiza melodia*), there is little differentiation in mitochondrial DNA

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TABLE 1. Sample sites and genetic characteristics of Savannah Sparrows (n = 112; *Passerculus sandwichensis*). Two wintering individuals are excluded from table.

Locality	Subspecies	n	Number of haplotypes	π[a]
Newfoundland	*labradorius*	6	6	0.0150
Nova Scotia	*savanna*	7	6	0.0120
Sable Island	*princeps*	21	6	0.0007
Ontario[b]	*oblitus*	15	10	0.0130
Alaska[c]	*anthinus*	8	6	0.0140
Washington[d]	*brooksi*	6	5	0.0110
Suisan Bay, California	*alaudinus*	6	4	0.0014
San Elijo Lagoon, California	*beldingi*	1	1	—
Puerto Peñasco	*rostratus*	5	5	0.0010
Bahía San Quintín	*beldingi*	7	2	0.0002
Isla San Benito	*sanctorum*	14	1	0.0
Guerrero Negro	*anulus*	10	5	0.0018
Puerto López Mateos	*magdalenae*	4	4	0.0017

[a] Nucleotide diversity (π) is the average number of nucleotide differences per site between two pairs of sequences in a sample.
[b] Ontario samples were from Toronto, Moosonee, and Attawapiskat.
[c] Alaska samples were collected from Lake Louise, Paxson, and the North Slope near Barrow.
[d] Washington samples include individuals from Kitsap Co. and Klickitat.

(mtDNA; Fry and Zink 1998). In other species, distinct mtDNA breaks occur (Rising and Avise 1993).

In this study, we assessed broad patterns of mtDNA variation in Savannah Sparrows from Baja California and coastal Sonora, where ''Belding's'' Savannah Sparrows (*P. s. beldingi*) and ''Thick-billed'' Savannah Sparrows (*P. s. rostratus*) occur, and from much of continental United States and Canada where ''typical'' Savannah Sparrows occur. Although our sampling of the continental population is not extensive, we sampled populations in California, Alaska, Washington, Ontario, and mainland Nova Scotia. In addition, we examined samples from three islands, Isla San Benito, Sable Island, and Newfoundland, to explore genetic-phenotypic evolution in an island context. We performed tests to determine whether sequences were evolving in a selectively neutral manner.

METHODS

SAMPLING

Specimens (n = 112) were obtained from 13 breeding localities (Table 1, Fig. 1). Of the three individuals from southern California, one was a breeding individual (identified as ''*beldingi*'') from San Elijo Lagoon (San Diego County) and two (identified as ''*nevadensis*'') were migrants from Murrieta Hot Springs (Riverside County). With the exception of the birds from Nova Scotia, DNA was isolated from frozen tissue. Voucher specimens are housed in the Royal Ontario Museum (Toronto, Ontario, Canada), the Burke Museum (University of Washington, Seattle, WA) and the San Diego Natural History Museum (San Diego, CA). For samples from Sable Island and mainland Nova Scotia, DNA was extracted from blood taken from live birds, and voucher specimens were not saved (birds from Sable Island are phenotypically distinct and, except during migration, virtually all individuals from Sable Island are ''Ipswich'' Sparrows). Baird's Sparrow (*Ammodramus bairdii*) and Le Conte's Sparrow (*A. leconteii*) were used as outgroups based on phylogenetic results of J. Klicka (unpubl. data).

MOLECULAR LAB METHODS

DNA isolation and purification was performed using the phenol-chloroform protocol of Hillis et al. (1996), or a DNeasy Tissue Kit (QIAGEN, Valencia, California). Two mitochondrial gene regions were amplified by Polymerase Chain Reaction (PCR; Saiki et al. 1988) using Perkin-Elmer PCR reagents. Primers L5215 (Hackett 1996) and H1064 (CTTTGAAGGCCTTCGGTTTA, designed by S. V. Drovetski) were used to amplify the complete ND2 gene; primers L10702 and H11289 (Groth, pers. comm.) were used to obtain 48 base pairs (bp) of $tRNA^{Gly}$ and 351 bp of ND3. PCR reaction volumes of 50 μL

FIGURE 1. Map showing general sample localities for Savannah Sparrows (n = 112 birds total) across Canada, U.S., and Mexico.

were used, including 1.0 μL of *Thermus flavus* polymerase, 3.0 μL of 10 mM solution of each primer, 4.0 μL of 25 mM MgCl2 solution, 2.5 μL of 20× reaction buffer, 1.0 μL of 10 mM dNTP mixture, and 3.0–4.0 μL DNA template. The PCR protocol was as follows: 2.5 min at 95°C, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and a final extension step of 10 min at 72°C. PCR amplifications were cleaned using a QIAquick PCR Purification Kit (QIAGEN Inc.). For sequencing, either standard Sephadex columns were used to clean sequencing reactions, which were sequenced on an ABI 310 automated sequencer using the Dideoxy Terminator Kit Protocol, or cleaned PCR fragments were directly sequenced on ABI 3700 automated sequencer using BigDye chemistry (Applied Biosystems). Amplification primers and primers L347 (CCATTCCACTTCTGATTCCC, designed by S. V. Drovetski) and H5578 (Hackett 1996) were used for sequencing of 1041 bp of ND2. For ND3, PCR primers were used for sequencing.

DATA ANALYSIS

Sequences were aligned and edited in Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, Michigan). Sequence data have been deposited in Genbank under accession numbers (AY584869–AY585096). Mitochondrial origin of sequenced DNA fragments was supported by the absence of stop-codons in the ND2 and ND3 sequences and the existence of a large number of haplotypes, both of which are inconsistent with nuclear copies (Zhang and Hewitt 1996).

We used PAUP* (Swofford 2000) to generate maximum parsimony trees from equally weighted characters and a strict consensus trees was constructed. Software Mega2 (Kumar et al. 2001) was used to construct a neighbor-joining tree based on *p*-values. Arlequin (Schneider et al. 2000) was used to compute the number of haplotypes in each population (with 0% allowed missing data per site), nucleotide diversity (π), haplotype diversity (h), Φ_{ST}, τ, θ_0, θ_1, and mismatch distributions.

Nucleotide diversity (π) is the average number of nucleotide differences per site between two pairs of sequences in the sample (Tajima 1983). Tau ($\tau = 2\mu t$) is a relative measure of time since population expansion measured in number of generations (t). Theta is a measure of variability; $\theta_0 = 2\mu N_0$ and $\theta_1 = 2\mu N_1$, where μ is the mutation rate, N_0 and N_1 are effective population sizes before and after expansion.

Φ_{ST} is a standardized measure of the genetic variation among populations, showing the extent of population subdivision and taking into account the degree of haplotype differentiation. The mismatch distribution is the distribution of pairwise base-pair differences among all individuals in a breeding population (Rogers and Harpending 1992). Assuming neutral evolution, past demographic expansions can be inferred from unimodal mismatch distributions (Rogers and Harpending 1992). We used the approach (Schneider and Excoffier 1999) implemented in

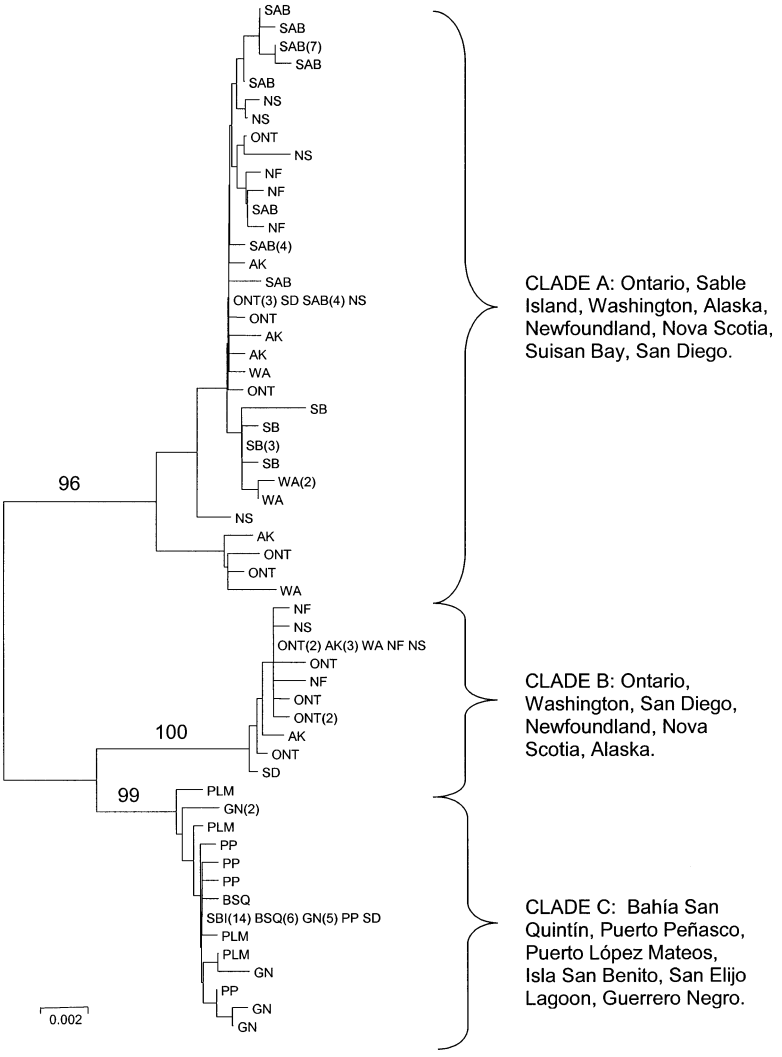

FIGURE 2. Neighbor-joining tree of haplotypes (based on pairwise distances) showing three major groups (the same topology was recovered in maximum parsimony analyses). Numbers above branches are percentage of bootstrap replicates supporting that node. No other nodes received greater than 60% support. Sampling locations are identified as follows: SAB = Sable Island, NS = Nova Scotia, ONT = Ontario, NF = Newfoundland, AK = Alaska, SD = San Diego, WA = Washington, SB = Suisan Bay, PLM = Puerto López Mateo, GN = Guerrero Negro, PP = Puerto Peñasco, BSQ = Bahía San Quintín, and SBI = Isla San Benito. Numbers in parentheses indicate numbers of individuals with that haplotype.

Arlequin to test the empirical distribution against a model of sudden expansion.

We used McDonald-Kreitman (M-K) tests implemented in DnaSP (Rozas et al. 2003) to test for the effects of natural selection on the combined ND2 and ND3 sequences between pairs of population samples and between clades. This test assumes that the ratio of synonymous: nonsynonomous polymorphic sites should be the same as the ratio of synonymous:nonsynonomous fixed replacement substitutions between groups.

RESULTS

A total of 57 haplotypes was resolved. Phylogenetic analysis (Fig. 2) revealed three main clades of haplotypes, corresponding to two continentally distributed (including Sable Island and

Newfoundland) clades of ''typical'' Savannah Sparrows (clade A and clade B), and one consisting of individuals from Baja California, Baja California Sur, San Diego, and Sonora (Clade C). Clade B was sister to clade C. This result was confirmed in an analysis of ND2 only using all relevant related taxa (J. Klicka, unpubl. data). No geographic structure was apparent in any of the three clades; this suggests that although there are large unsampled gaps in the continental range, there is unlikely to be geographic structure. Haplotypes from clades A and B were mostly co-distributed, although the less common clade (B) was not detected at Suisan Bay (where there were only 6 specimens) or Sable Island. One migrant from San Diego exhibited a clade A haplotype and the other a clade B haplotype. Considering all localities, analysis of molecular variance (AMOVA) indicated that 57% of variation existed among localities.

Continental samples (excluding Suisan Bay) tend to show an order of magnitude more variation (mean $\pi = 0.013$) than those from Baja California and Sonora (mean $\pi = 0.0009$). Island samples exhibited low diversity, with π ranging from 0.0 for Isla San Benito to 0.0007 for Sable Island. The higher diversity in continental samples of typical Savannah Sparrows reflects the fact that representatives of both major haplotype clades occurred at most locations (excluding Sable Island and Suisan Bay). Localities with both clades show mismatch distributions (not shown) that are inconsistent with population growth; whereas unimodal mismatch distributions (not shown) suggest that populations have grown for the Baja, Sonora, and Sable Island populations. The sample from Suisan Bay was too small to make a definitive conclusion on the growth of this population.

Samples were pooled into salt marsh (Baja California peninsula, San Diego [excluding migrants] and Sonora) and typical groups, yielding a Φ_{ST} of 0.63 ($P < 0.001$). The mismatch distribution for all individuals (not shown) was clearly bimodal, reflecting the two groups, although the bootstrap test implemented in Arlequin failed to reject a model of population expansion ($P = 0.07$). For the 41 Baja individuals the mismatch distribution (Fig. 3) did not differ statistically from that expected for recent population growth.

Clades A and B were analyzed separately. Clade B had lower values of π and τ (0.0009 and 1.6, respectively) than those from clade A (0.003 and 2.3, respectively). Mismatch distributions (Fig. 3) for the two typical clades suggest that each has had a different history, with greater and more recent growth in clade B ($\theta_1 = 3415$) than in clade A ($\theta_1 = 43$).

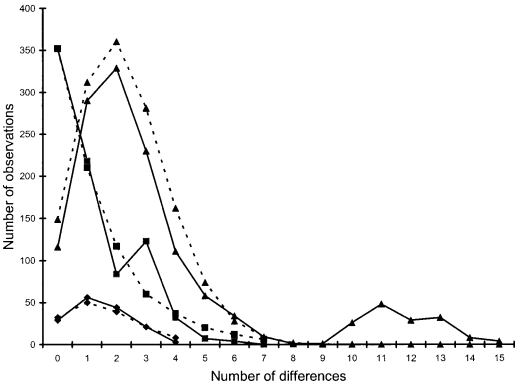

FIGURE 3. Mismatch distributions for clade A (triangles) showing a unimodal pattern consistent with population growth, clade B (diamonds) showing a unimodal pattern consistent with population growth, and Baja samples (clade C, squares) combined showing a unimodal pattern consistent with population growth. For each distribution, the expectation for a population that has recently expanded is shown with a dashed line.

M-K tests were not significant except for Sable Island versus the continental Clade B ($P = 0.01$) and Baja ($P = 0.03$). Significance was a result of an excess of polymorphic replacement substitutions. For example, comparing Sable Island and Clade B, there were 23 synonymous and 5 nonsynonymous replacement substitutions, but 9 synonymous and 11 nonsynonymous polymorphic sites (Fisher's Exact test, $P = 0.01$).

DISCUSSION

SPECIES LIMITS

The mtDNA tree (see also Zink et al. 1991) shows that the populations in Baja California and Sonora (and presumably Sinaloa) are genetically distinct from continental ones. Uncorrected sequence divergence averages 2%. Therefore, on the basis of these molecular data as well as morphological data (Rising, 2001), variation in plumage color and pattern (Rising, unpubl. data) and differences in vocalizations (Bradley 1977, Wheelwright and Rising 1993), we suggest that ''typical'' Savannah Sparrows and the saltmarsh populations of Savannah Sparrow from coastal

Baja California, San Diego, and Sonora be treated as separate species. Further sampling along the California coast between San Diego and Suisan Bay is required to locate the contact zone between the two major groups.

POPULATION HISTORY

A striking feature of the data is the sister-group relationship between clades B and C. Although this relationship does not have bootstrap support, it occurs in both methods of tree reconstruction. Thus, one interpretation is that two groups of typical Savannah Sparrows and one of saltmarsh Savannah Sparrows existed at one time, with the saltmarsh birds plus the smaller typical group (clade B) being sisters. Subsequently, clades A and B were intermixed as a result of post-glacial range expansion, whereas clade C remained isolated.

An alternative explanation comes from coalescence theory (Hudson 1990), which predicts the existence, on average, of two major clades of haplotypes in a single breeding population. In most surveys of passerine birds, only a single major clade is discovered within single populations (e.g., Merila et al. 1997, Zink et al. 2000), whereas in nonpasserines, often two major clades exist in single populations (e.g., Barrowclough et al. 1999). The Savannah Sparrow is unusual among surveyed passerines in having two deep haplotype clades (A, B) that are each relatively widespread. Thus, another scenario to explain the haplotype tree is that the saltmarsh populations (Clade C) were founded by individuals carrying, by chance, haplotypes from clade B. If clades A and B were not previously isolated, but were part of the same ancestral population, they should show the same genetic signatures. If they were once isolated, but are today in secondary contact, each might have different genetic signatures reflecting independent histories.

Comparison of clades A and B suggests that they have had different histories. Clade B shows a signature of recent growth whereas clade A has a higher value of π and a mismatch distribution that suggests an older expansion event. Hence, we suggest that there were once three isolated groups of sparrows, and that the clades A and B have recently become admixed. Either habitat barriers or distance has maintained the isolation of clade C.

SELECTION

Use of DNA sequences to infer population history and relationships carries the assumption that natural selection does not influence the pattern of base substitution. Some recent studies (Ballard and Whitlock 2004) have suggested that selection might interfere with phylogeographic inference, although the generality of this concern is unclear (Zink 2005). We found that most M-K tests yielded nonsignificant results, suggesting basic conformity to neutral expectations. However, two comparisons involving Sable Island showed an excess of replacement polymorphisms within samples. The significance of these comparisons was due to the absence of Clade B haplotypes from Sable Island. Because this absence could be a chance event, we do not think that the significant M-K tests have biological importance. Overall conformance with neutral expectation allows demographic and evolutionary interpretations of the sequence data.

EVOLUTION ON ISLANDS

Island populations of Savannah Sparrows are known for their phenotypic distinctiveness. For example, the Ipswich Sparrow (*P. s. princeps*) is a morphologically and behaviorally distinct form that breeds almost exclusively on Sable Island, a 40-km long, less than 2.5-km wide sand island 250 km off the coast of Nova Scotia. This sparrow's large size and pale coloration make it readily separable from continental forms (Rising 2001). Although once classified as a species, some taxonomists considered it a subspecies owing to hybridization with individuals bearing the continental phenotype.

Our analysis of 21 individuals from Sable Island does not support a long history of isolation. We found little mtDNA variation among the Sable Island birds, and the phylogenetic tree of all haplotypes did not show that haplotypes from the island were reciprocally monophyletic. Other than its large size and pallid coloration it resembles other Savannah Sparrows (Wheelwright and Rising 1993), and large-bodied Savannah Sparrows occur on other islands (the Aleutians and Isla San Benitos [Rising 2001]). Thus, our results imply that the large size and pale coloration evolved rapidly on Sable Island, and that these characteristics are not markers of a historically isolated species.

The population on Isla San Benitos (*P. s. sanctorum*) is distinctive in both morphology and breeding phenology. They are large-bodied and large-billed (bill size similar to that of the large-billed birds from coastal Sonora, *P. s. rostratus*). However, they are not found in salt marshes, and at least in late April 1999, were not in breeding condition while Savannah Sparrows (*P. s. anulus*), in the salt marshes along the coast of adjacent mainland Baja California, were in the early stages of nesting (chasing, carrying nesting material, etc., Rising, pers. obs.).

We observed no variation among the 14 individuals on Isla San Benitos. Furthermore, the haplotype present on the island is present in mainland saltmarsh samples. Thus, the distinctive morphology and different breeding schedule appear to have been modified very recently, more recently than it takes for haplotypes to undergo mutation and become reciprocally monophyletic on islands. Because all other populations in the clade breed in salt marshes, it is possible that a shift back to non-saltmarsh habitat also occurred recently.

The small sample from Newfoundland did not show signatures of isolation or bottlenecks. The close proximity of Newfoundland to the mainland likely facilitates gene flow.

SUBSPECIES

As the preceding discussion reveals, the mtDNA tree does not support the evolutionary distinctiveness of the subspecies of Savannah Sparrows that we analyzed. This has often been reported for birds (Ball and Avise 1992, Zink 2004). However, in most species there have been no detailed, quantitative morphological assessments of subspecies limits. The mtDNA results reported here parallel a detailed analysis of morphology (Rising 2001) for the non-saltmarsh birds. Rising (2001) concluded that the subspecies in Baja California were more distinctive, but this is not revealed in the mtDNA data. The implication of the mtDNA data is that there is gene flow throughout the range of the salt marsh birds, or that isolation was in the recent past, too recent for the evolution of reciprocal monophyly in mtDNA gene trees. High gene flow in continental populations should lead to gradual clinal variation in morphology, which was the pattern observed by Rising (2001) in a variety of characteristics.

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