RESEARCH ARTICLE

Relationship of phenotypic variation and genetic admixture in the Saltmarsh–Nelson's sparrow hybrid zone

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ABSTRACT

Hybridization is influential in shaping species' dynamics and has many evolutionary and conservation implications. Identification of hybrid individuals typically relies on morphological data, but the assumption that hybrids express intermediate traits is not always valid, because of complex patterns of introgression and selection. We characterized phenotypic and genotypic variation across a hybrid zone between 2 tidal-marsh birds, the Saltmarsh Sparrow (*Ammodramus caudacutus*) and Nelson's Sparrow (*A. nelsoni*) ($n = 290$), and we sought to identify morphological traits that could be used to classify admixed individuals. Sparrows were sampled from a total of 34 marshes, including 23 sympatric and 11 putatively allopatric marshes. Each individual was scored at 13 plumage traits, and standard morphometric data were collected. We used genotyping analysis at 24 microsatellite loci to categorize individuals into genotypic classes of pure, F_1–F_2, or backcrossed. Genetic data revealed that 52% of individuals sampled along the geographic transect were of mixed ancestry, and the majority of these were backcrossed. Traits related to the definition of plumage features (streaking, crown, and face) showed less overlap between genotypic classes than traits related to the amount or color of plumage features. Although morphological data performed well in distinguishing between the 2 taxa, pure and backcrossed individuals of each parental type could not be distinguished because of substantial overlap in plumage and morphology. We conclude that the discrimination of pure and hybrid individuals is not possible in the absence of genetic data. Our results have implications for conservation of pure populations, as extensive backcrossing throughout the hybrid zone may present challenges for monitoring pure species identified by morphology alone.

Keywords: *Ammodramus caudacutus*, *Ammodramus nelsoni*, hybridization, morphological variation, Nelson's Sparrow, plumage, Saltmarsh Sparrow

Relación de la variación fenotípica y la mezcla genética en la zona híbrida de *Ammodramus caudacutus* y *A. nelsoni*

RESUMEN

La hibridación influye la dinámica de modelado de las especies y tiene muchas implicancias evolutivas y para la conservación. La identificación de individuos híbridos se base típicamente en datos morfológicos; sin embargo, asumir que los híbridos presentan rasgos intermedios no es siempre válido debido a patrones complejos de introgresión y selección. Caracterizamos la variación fenotípica y genotípica a lo largo de una zona de hibridación entre dos aves de marea-pantano, *Ammodramus caudacutus* y *A. nelsoni* ($n = 290$), y buscamos identificar rasgos morfológicos que pueden ser usados para clasificar a los individuos mezclados. Los individuos fueron muestreados de un total de 34 pantanos, incluyendo 23 pantanos simpátricos y 11 pantanos supuestamente alopátricos. Cada individuo fue clasificado según 13 rasgos del plumaje y se colectaron los datos morfométricos estándar. Empleamos un análisis genotípico de 24 loci micro-satelitales para categorizar los individuos en las clases genotípicas de puro, F1/F2 o retro-cruza. Los datos genéticos revelaron que el 52% de los individuos muestreados a lo largo de la transecta geográfica tuvieron ancestros cruzados, y que la mayoría de estos fueron retro-cruzas. Los rasgos usados para definir las características del plumaje (raya, corona y rostro) mostraron menor superposición entre las clases genotípicas que los rasgos relacionados con la cantidad o el color de las características del plumaje. Mientras que los datos morfológicos resultaron adecuados para distinguir entre los dos taxa, no pudieron distinguirse los individuos puros y retro-cruzados de cada tipo de patrón debido a una superposición substancial en el plumaje y en la morfología. Concluimos que la distinción de los individuos puros e híbridos no es posible en ausencia de datos genéticos. Nuestros resultados tienen implicancias para la conservación de las poblaciones puras, ya que la ocurrencia frecuente de retro-cruza a lo largo de la zona de hibridación puede presentar desafíos para el monitoreo de especies puras identificadas solo en base a la morfología.

INTRODUCTION

Hybridization, or the crossing of genetically distinguishable groups of taxa (Mallet 2005), has long been a topic of interest for biologists. Hybridization occurs frequently in nature (Arnold 1997, Mallet 2005) and is particularly common in avian systems (documented in ~10% of bird species; Grant and Grant 1992, Mallet 2005, Randler 2006). Hybridization can result in genetic exchange between species and the introgression of foreign alleles into parental populations (Mallet 2005), a process that can introduce both variation and novelty into a system (Rheindt and Edwards 2011, Abbott et al. 2013). Rates of introgression are highly variable among loci, which indicates that the degree of reproductive isolation varies across the genome (Baack and Rieseberg 2007, Payseur 2010, Gompert et al. 2012). Depending on the selective forces at work, hybrid zones may be highly permeable to "neutral" genomic regions but act as strong filters for regions that play a role in reproductive isolation (Payseur 2010, Gompert et al. 2012, Baldassarre et al. 2014). Depending on the patterns of selection on phenotypic traits, differential introgression can lead to discordance between genetic markers and phenotype as indices of hybridization.

Detection of hybrids often relies on morphological characteristics (Allendorf et al. 2001, Mallet 2005). The use of phenotypic traits for hybrid identification broadly assumes that hybrids display intermediate characteristics in relation to parental individuals (Allendorf et al. 2001). Yet, as a result of differential rates of introgression, hybrids may express a mosaic of parental traits, be indistinguishable from parental forms (Allendorf et al. 2001), or display extreme phenotypes compared with parental forms (i.e. transgressive segregation; Seehausen 2004). Furthermore, extensive backcrossing can result in a continuous gradient of phenotypes across a hybrid zone, as opposed to a clear intermediate form (Gay et al. 2008). These processes pose challenges for the identification of hybrid individuals solely on the basis of morphology. In cases of morphologically similar sister species, intermediates may not be obvious, and identifying admixed individuals is best approached through a combination of multiple independent traits (Sattler and Braun 2000). To this end, neutral genetic markers offer an easily obtainable suite of traits to distinguish pure from admixed individuals. Comparing variation in neutral markers to that in phenotypic traits can help quantify the extent and direction of introgression and identify traits that are under selection and involved in reproductive isolation (Brumfield et al. 2001, Mettler and Spellman 2009, Baldassarre et al. 2014).

Saltmarsh Sparrows (*Ammodramus caudacutus*) and Nelson's Sparrows (*A. nelsoni*) are an example of hybridizing taxa in which hybrid identification has been challenging; consequently, the extent of genetic introgression has been difficult to quantify in this system. Both species breed in coastal marshes. A subspecies of Nelson's Sparrow (*A. n. subvirgatus*) breeds in marshes from coastal Québec, Canada, to northeastern Massachusetts, USA; and a subspecies of Saltmarsh Sparrow (*A. c. caudacutus*) breeds from Maine to New Jersey, USA (Greenlaw and Woolfenden 2007). Range overlap between what appear to be morphologically pure Nelson's and Saltmarsh sparrows occurs from the Weskeag River in Maine (44°04.60′N, 69°08.66′W) to the northeast shore of Massachusetts (42°77.42′N, 70°80.86′W; Rising and Avise 1993, Hodgman et al. 2002).

There are observable differences in plumage and morphology between the 2 species (Greenlaw 1993, Shriver et al. 2005). Saltmarsh Sparrows have more vibrant plumage color with more defined, dark chestnut streaking patterns on the breast and flanks, a bright orange face patch, and a dark chestnut back. They are also larger than Nelson's Sparrows and have a longer, thinner bill. Comparatively, Nelson's Sparrows are duller in color and have less defined, washed-out gray streaking on the breast and flanks. The face is dull yellow, with less definition between the supercilium and auriculars, and there is less color variation in the plumage overall. In addition to being smaller in size, Nelson's Sparrows have a shorter, thicker, blue-colored bill. Researchers have used plumage-score cutoffs based on this morphological variation to assign individuals to pure and admixed categories in the field. However, plumage differences are subtler within the overlap zone, and plumage intermediacies are not always apparent in sympatric populations (Walsh et al. 2011).

Previous investigation of hybridization in Saltmarsh–Nelson's sparrows is limited. Shriver et al. (2005) found a concordance between genotypic and phenotypic variation in hybrid sparrows from 3 sympatric marshes in the northern and middle portion of the overlap zone (Weskeag, Scarborough, and Webhannet, Maine) and suggested that hybrids occur wherever the 2 species are sympatric. Later work by Walsh et al. (2011) documented Nelson's-specific mitochondrial DNA in 8% of individuals identified morphologically as Saltmarsh Sparrows, with a relatively high proportion of introgressed individuals in the southern portion of the overlap zone and 1 introgressed individual as far south as Rhode Island, USA. The lack of a single intermediate phenotype in the individuals identified with Nelson's mitochondrial DNA suggests successful backcrossing, which appears to be more likely with pure Saltmarsh Sparrows (Shriver et al. 2005, Walsh et al. 2011, Kovach et al. 2015). Morphometric and plumage variation can reliably differentiate pure Saltmarsh and Nelson's sparrows (Shriver et al. 2005), but whether these phenotypic traits can provide a reliable approach for identifying hybrids remains unknown.
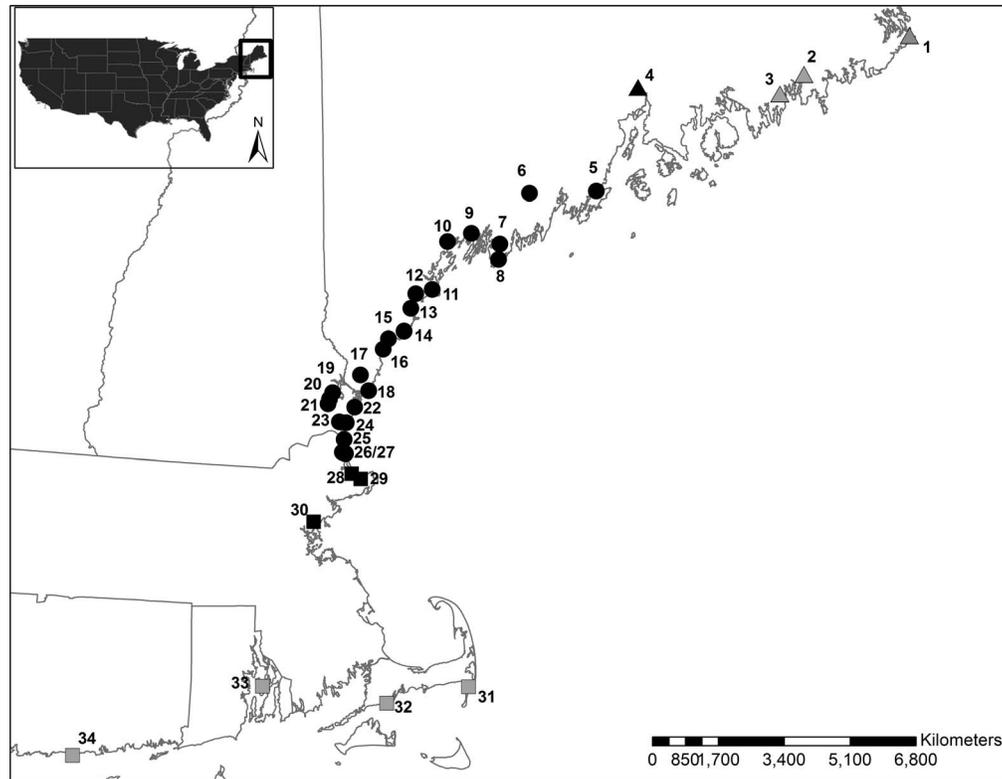
FIGURE 1. Location of 34 marshes (numbered from north to south) along the northeastern coast of the United States, where we sampled Saltmarsh and Nelson's sparrows. Triangles represent 4 putatively allopatric Nelson's populations, and squares represent 7 putatively allopatric Saltmarsh populations. For the purpose of defining "pure" individuals for admixture analyses, we used only individuals from sites in gray (28 Nelson's Sparrows and 32 Saltmarsh Sparrows from populations >100 km from the hybrid zone's edge). Circles represent 23 marshes sampled within the currently hypothesized overlap zone.

Depending on backcrossing rates and patterns of selection on morphology, the introgression of phenotypic traits across the Saltmarsh–Nelson's hybrid zone may or may not mirror genotypic patterns. The limited understanding of hybrid phenotypes may thus present challenges for effective monitoring of pure populations of both taxa. This warrants consideration, because both species are a high conservation priority in the northeastern United States (U.S. Fish and Wildlife Service 2008), and the Saltmarsh Sparrow is considered globally vulnerable to extinction (IUCN Red List criteria; BirdLife International 2004). As such, a clearer understanding of genetic and phenotypic variation in the hybrid zone may aid conservation management. To this end, our objectives in the present study were to (1) characterize patterns of phenotypic variation across the entirety of the Salt-marsh–Nelson's hybrid zone; (2) evaluate concordance between genotypic and phenotypic patterns; and (3) identify traits that are most useful in differentiating between the 2 species and assess how these traits can be used to identify pure and admixed individuals in the field.

METHODS

Sample Collection and Morphological Data

To capture the full extent of phenotypic and genotypic variation across the hybrid zone, we sampled Saltmarsh and Nelson's sparrows, and their hybrids, during the breeding seasons (June–August) of 2012 and 2013 from 34 marshes in the northeastern United States (Figure 1). We sampled sympatric marshes ($n = 23$) within the previously documented overlap zone (South Thomaston, Maine, to Newburyport, Massachusetts; Hodgman et al. 2002) and putative allopatric marshes to the north ($n = 4$) and south ($n = 7$) of the overlap zone (based on morphology, song, and previous surveys; Hodgman et al. 2002). We deployed three to six 12-m mist nets with 30-mm mesh to capture a target sample of 10 birds from each site. We scored each individual sparrow for 13 plumage traits developed for evaluating levels of phenotypic introgression (Shriver et al. 2005). Plumage traits include bill color (upper mandible ranging from yellow to blue), the color (ranging from orange to yellow) and definition (separation between supercilium, auriculars, and eye-stripe) of the face, and

the color of the back (ranging from chestnut to gray), the width and definition of the whisker line and crown, and the amount and definition of the streaking on the breast and flanks. All color scores were assessed visually in the field against written descriptions, based on the past success of this method in these species (Shriver et al. 2005). Plumage scores for each individual trait ranged from 1 to 5, with lower numbers representative of Nelson's Sparrows and higher numbers representative of Saltmarsh Sparrows. Thus, the final plumage score ranged from 13 (pure Nelson's Sparrow) to 65 (pure Saltmarsh Sparrow; Shriver et al. 2005). We used predefined cutoffs for the plumage scores (modified slightly from those used in Shriver et al. 2005) to classify individuals in the field as Nelson's Sparrows (scores of 13–31), hybrids (32–45), or Saltmarsh Sparrows (46–65). Although Shriver et al. (2005) placed the cutoff for pure Saltmarsh Sparrows at >55, we found this criterion to be narrow given the observed variation in the field, and our modified cutoffs provided a more even range of possible scores for each category. We used digital calipers to measure tarsus length, bill width, depth, and length (nares to tip; mm), a wing-chord ruler to measure unflattened wing chord (mm), and a digital scale to measure weight (to the nearest 0.1 g). Blood samples (10–20 μL) were drawn from the brachial vein and collected on Nobuto blood filter strips (Sterlitech, Kent, Washington, USA) and stored at room temperature for later genetic analysis.

Genotyping, Admixture Analysis, and Identification of Genotypic Classes

We extracted DNA from blood samples using a DNeasy blood kit (Qiagen, Valencia, California, USA) according to manufacturer protocol. DNA was amplified using 24 microsatellite loci: *Ammo*001, *Ammo*002, *Ammo*003, *Ammo*006, *Ammo*008, *Ammo*012, *Ammo*015, *Ammo*016, *Ammo*017, *Ammo*020, *Ammo*023, *Ammo*027, *Ammo*028, *Ammo*030, *Ammo*034, *Ammo*036 (Kovach et al. 2015), Escμ1 (Hanotte et al. 1994), Asμ15, Asμ18 (Bulgin et al. 2003), Aca01, Aca04, Aca05, Aca08, and Aca12 (Hill et al. 2008). Four 15- to 25-μL multiplexed polymerase chain reactions were performed containing 2 μL of eluted genomic DNA, 0.1–0.7 μM of each dye-labeled primer, 2.0 mM $MgCl_2$, 1X PCR buffer (Promega, Madison, Wisconsin, USA), 0.12 mM of deoxyribonucleotides, and 1 unit of Taq DNA polymerase (Promega). Cycling conditions for the Hanotte et al. (1994), Bulgin et al. (2003), and Hill et al. (2008) primers followed Walsh et al. (2012). Cycling conditions for the *Ammo* primers were as follows: 25 cycles of 94°C for 30 s, 56–60°C for 45 s, 72°C for 1 min, and a final extension step at 72°C for 5 min. Amplified products were electrophoresed on an automated DNA sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems, Foster City, California), and individual geno-

types were scored manually using PEAKSCANNER software (Applied Biosystems).

To quantify genetic admixture, we calculated hybrid index and interspecific heterozygosity in the R package 'introgress' (Gompert and Buerkle 2009, 2010). Calculating hybrid index requires a priori definition of pure individuals of each parental species. In doing so, we took a conservative approach to minimize the potential for including introgressed individuals in our parental samples; we defined pure individuals as those sampled from allopatric populations >100 km north and south of the currently recognized overlap zone. This included 28 Nelson's Sparrows from 3 sites (Narraguagus River and north) and 32 Saltmarsh Sparrows from 4 sites (Waquoit Bay and south; Figure 1).

For each sparrow sampled from the remaining 27 sites in our geographic transect, we calculated a hybrid index, defined as the proportion of alleles inherited from the Saltmarsh Sparrow (0 = pure Nelson's Sparrow, 1 = pure Saltmarsh Sparrow). We then estimated interspecific heterozygosity, defined as the proportion of genotypes that are heterozygous for the parental alleles (0 = all homozygous genotypes, 1 = all heterozygous genotypes). Using the combination of hybrid index and interspecific heterozygosity, we assigned sparrows to genotypic classes following the methods of Milne and Abbott (2008). Briefly, individuals with intermediate hybrid index (0.25–0.75) and high heterozygosity (>0.3) were considered recent-generation hybrids (F_1, F_2), and individuals with low hybrid index <0.25 or >0.75) and low heterozygosity (<0.3) were considered backcrossed. We considered individuals to be pure if they had a hybrid index of 0.00–0.05 (Nelson's Sparrow) or 0.95–1.00 (Saltmarsh Sparrow). This method is similar to the approach implemented in the software package NewHybrids (Anderson and Thompson 2002) but requires fewer assumptions (i.e. markers are unlinked and not subject to selection; Milne and Abbott 2008, Hamilton et al. 2013).

Correlating Phenotypic Variation with Genotype

To evaluate patterns of variation in admixed populations, we first compared the average and range of morphological traits between allopatric (only the 7 populations that were >100 km from the hybrid zone's edge) and sympatric groups (including the 4 populations within 100 km of the edge) separately. To explore the utility of each phenotypic trait for describing introgression patterns, we tested for differences between males and females of each species in individual structural measurements and plumage categories using 2-tailed student's *t*-tests. To evaluate significance, we applied a Bonferroni adjustment of $P = 0.0026$ for $\alpha = 0.05$ across 19 tests. Although there is not pronounced sexual dimorphism in either species (Greenlaw and Rising 1994, Shriver et al. 2011), we detected enough differences between
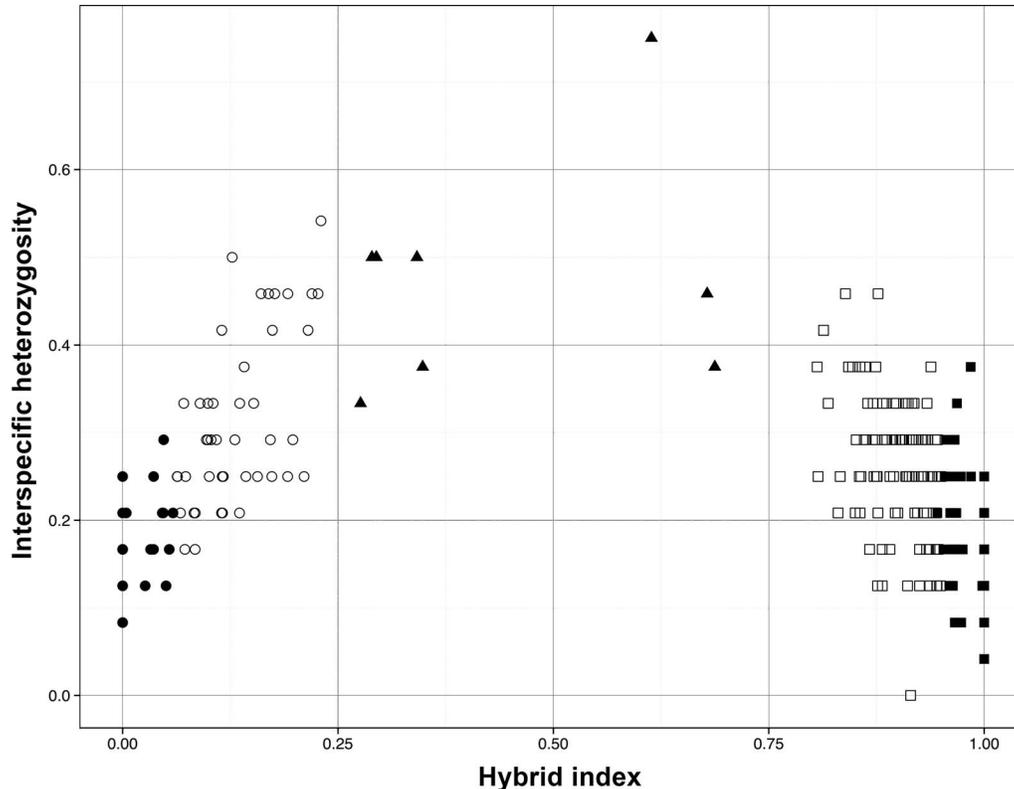
FIGURE 2. Interspecific heterozygosity plotted against hybrid index for 237 individuals sampled from sympatric populations (within the current hybrid zone) and 4 populations <100 km from the hybrid zone's edge. Symbols represent assigned genotypic classes: pure Nelson's Sparrows (filled circles), backcrossed Nelson's (open circles), recent generation hybrids (F_1–F_2; triangles), backcrossed Saltmarsh (open squares), and pure Saltmarsh Sparrows (filled squares).

males and females in both structural (significant differences in 3 of 6 measurements) and plumage (significant differences in 7 of 13 traits) measurements to warrant separating them in all further tests.

In addition to evaluating overall plumage score as a predictor of admixture, we aimed to assess the utility of individual traits. To accomplish this, we used linear regression to evaluate how well each individual plumage trait predicted individual genotypes (hybrid index) and to identify the traits that were most informative in differentiating among the groups. We used ANOVA and a Tukey's post hoc test to test for differences in univariate morphometric and overall plumage score among the 5 genotypic classes (significance testing was performed using a Bonferroni correction for multiple tests). The distribution of all morphometric measurements and the overall plumage score did not deviate from normality (based on visual assessment of histograms). We used a linear discriminant analysis (LDA) to evaluate the relationship between genotype and phenotype, and we assessed the accuracy of individual classification to the 5 genotypic classes based on plumage and morphometric measurements. We used a leave-one-out classification to validate

the accuracy of the resulting LDA functions. All statistical analyses were conducted in R (R Development Core Team 2014) using the 'MASS' package with α set to 0.05.

RESULTS

We collected blood samples from 290 individuals (99 females and 191 males) across the 34 study marshes and the full set of morphometric measurements and plumage scores from 254 individuals (89 females and 165 males) across 31 marshes (see Figure 1). All 290 individuals were genotyped at 24 microsatellite loci; 4 individuals (1.4%) had missing data for no more than 2 loci. Among the 290 individuals sampled, we identified 51 pure Nelson's (18%), 44 backcrossed Nelson's (15%), 8 F_1–F_2 hybrids (3%), 98 backcrossed Saltmarsh (34%), and 89 pure Saltmarsh individuals (30%; Figure 2). These distributions include the individuals we assigned to genotypic classes based on their hybrid index and intraspecific heterozygosity scores, as well as the 60 allopatric individuals we assumed to be genetically pure (see above). The F_1 and F_2 individuals were dropped from subsequent analyses because of the small sample size.
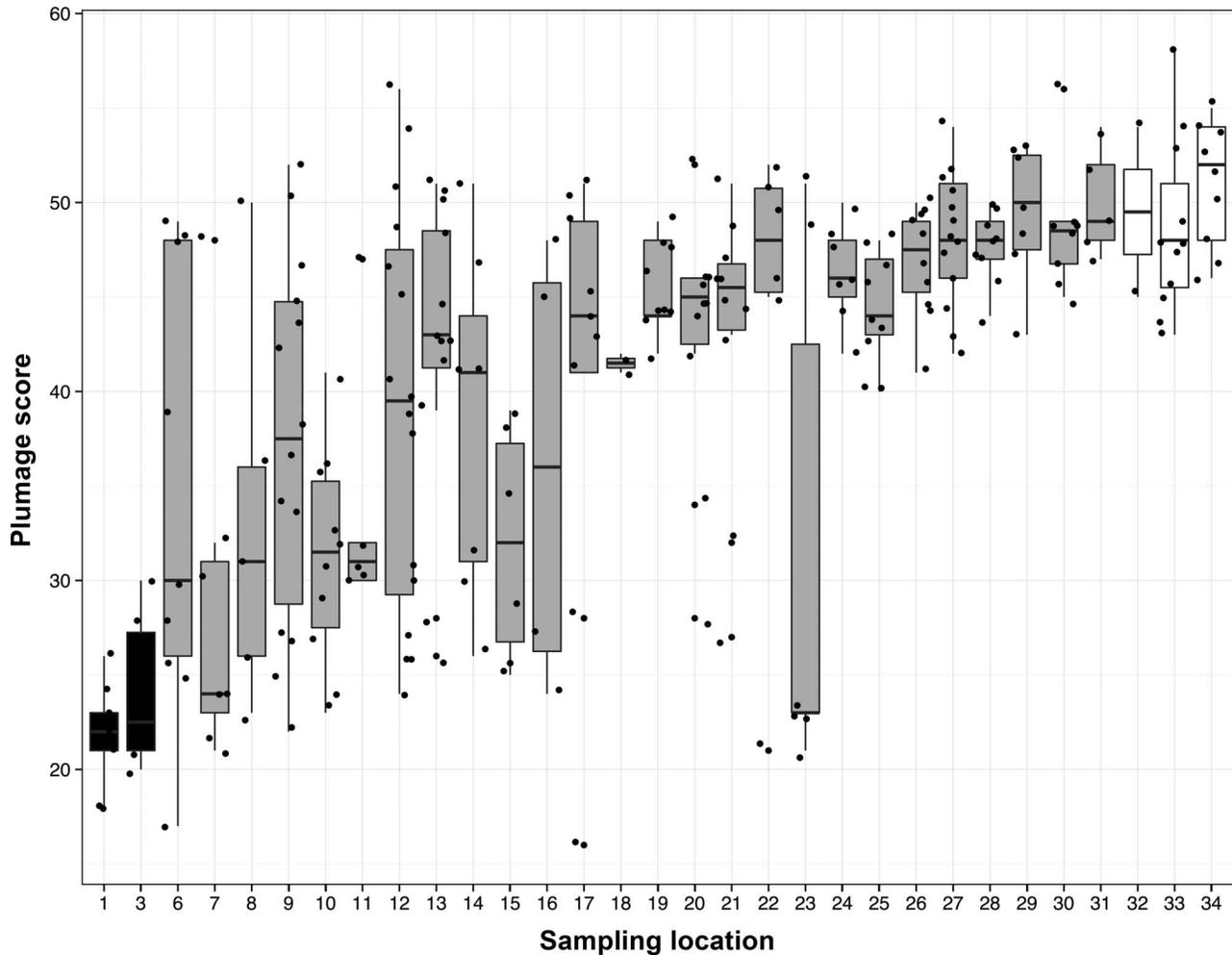
FIGURE 3. Boxplot of overall plumage scores for all sparrows sampled across the geographic transect (black = allopatric Nelson's Sparrows; gray = sympatric populations; white = allopatric Saltmarsh Sparrows).

Variation in plumage was greater within sympatric populations than within allopatric populations (Figure 3). Overall plumage score ranged from 16 to 41 (mean ± SD = 27.4 ± 4.9) in sympatric Nelson's Sparrows (genotypically pure and backcrossed individuals), compared with 18–30 (22.9 ± 3.3) in allopatric Nelson's Sparrows. In sympatric Saltmarsh Sparrow populations, overall plumage score ranged from 34 to 56 (46.5 ± 4.1), compared with 43–58 (50 ± 4) in allopatric populations. Structurally, females were smaller than males for all 4 genotypic classes, with significant differences in weight, wing chord, and tarsus ($t = 1.97$, $P < 0.001$). Female plumage scores were greater than male plumage scores for all 4 genotypic classes (2-tailed student's t-test: $t = 1.97$, $P < 0.001$). In allopatric Saltmarsh Sparrow populations, female measurements were smaller than males for wing chord ($t = 2.09$, $P < 0.001$), weight ($t = 2.11$, $P = 0.002$), and tarsus ($t = 2.09$, $P = 0.003$); we were unable to test for differences between males and females in allopatric Nelson's Sparrows

because of small female sample size ($n = 3$). In allopatric Saltmarsh Sparrow populations, female plumage scores were also significantly greater than male plumage scores (2-tailed student's t-test: $t = 2.08$, $P < 0.001$).

To assess the utility of individual traits in predicting genotype, we report results for the 4 genotypic classes (pure Nelson's, backcrossed Nelson's, backcrossed Saltmarsh, and pure Saltmarsh) across allopatric and sympatric sites. In males and females, most of the individual plumage traits associated with definition of plumage feature (malar, crown, breast, and flank streaking definition) consistently showed less overlap among the genotypic classes than other traits (largely related to amount and color of feature; e.g., crown and malar width, streaking amount, back color; Figure 4). More specifically, definition of streaking in the breast and flanks was more strongly correlated with genotype ($r = 0.67$ and $r = 0.66$, respectively) than was the amount of streaking ($r = 0.26$ for breast, $r = 0.33$ for flanks). Similarly, crown definition
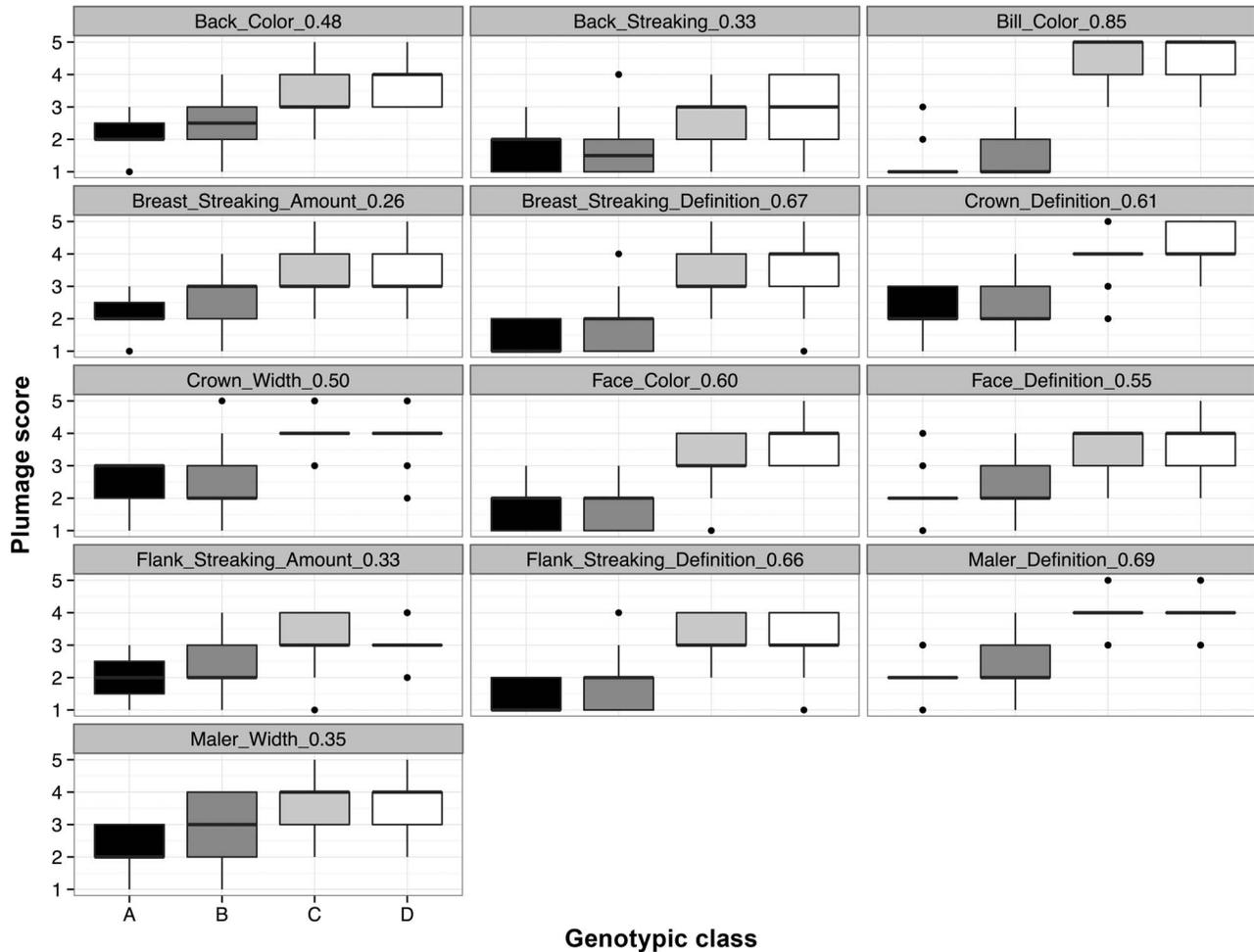
FIGURE 4. Boxplot of scores (range of values: 1–5) for the 13 individual plumage traits observed in 246 Saltmarsh, Nelson's, and hybrid individuals in this study, distributed across genotypic classes: pure Nelson's Sparrow (A), backcrossed in the direction of Nelson's (B), backcrossed in the direction of Saltmarsh (C), and pure Saltmarsh Sparrow (D). *R* values are provided above each plot indicating the strength of the correlation for each plumage trait when regressed against the genetic data (hybrid index).

showed a slightly stronger correlation with genotype ($r = 0.61$) than did crown width ($r = 0.50$), and malar definition was more strongly correlated with genotype ($r = 0.69$) than malar width ($r = 0.35$). Bill color ($r = 0.85$) and face color ($r = 0.60$) also showed a strong correlation with genotype (Figure 4). In the morphometric features, we found little difference between the 2 species in tarsus length, bill height, or bill width (bill width in pure Nelson's males was one exception to this; Table 1). However, we detected slight differences in wing chord (52–60 mm for pure Nelson's Sparrows; 53–61 mm for pure Saltmarsh Sparrows) and bill length (10.7–13.1 mm for pure Nelson's Sparrows; 11.0–14.1 mm for pure Saltmarsh Sparrows) between pure individuals (Table 1). In the structural measurements, male weight showed the least overlap between pure Nelson's (weight range: 14.9–19.2 g) and pure Saltmarsh (weight range: 18.9–23.9 g) individuals. Male plumage score

showed no overlap between pure Nelson's (plumage score range: 18–30) and Saltmarsh (plumage score range: 37–54) individuals (Table 1). Similarly, there was no overlap in female plumage scores (ranges: 20–32 for pure Nelson's; 43–58 for pure Saltmarsh), but we did detect slight overlap in female weights (ranges: 14.7–18.2 g for pure Nelson's; 16.0–21.8 g for pure Saltmarsh). For both sexes, backcrossed Nelson's were more similar to pure Nelson's Sparrows, and backcrossed Saltmarsh were more similar to pure Saltmarsh Sparrows, based on the 3 most informative structural measurements (bill length, wing chord, and weight) and plumage scores (Table 1). Pure and backcrossed individuals were very similar in morphometric traits; however, pure and backcrossed Nelson's groups differed in plumage score in both sexes, and pure and backcrossed Saltmarsh groups differed in plumage in males. This is consistent with the increased variation

TABLE 1. Mean (± SE) for morphometric and plumage features compared across 4 groups (pure Nelson's Sparrows, backcrossed Nelson's Sparrows, backcrossed Saltmarsh Sparrows, and pure Saltmarsh Sparrows). Individuals were assigned to groups based on genetic data. Values with different letters are significantly different, based on a Tukey's post hoc test.

Measures	Pure Nelson's		Backcrossed Nelson's		Backcrossed Saltmarsh		Pure Saltmarsh	
	Males (n = 21)	Females (n = 14)	Males (n = 27)	Females (n = 11)	Males (n = 60)	Females (n = 35)	Males (n = 51)	Females (n = 27)
Wing chord (mm)	57.66 (0.31) B	54.28 (0.31) B	57.60 (0.27) B	55.10 (0.35) B	58.84 (0.18) A	55.63 (0.20) A	59.10 (0.19) A	55.88 (0.23) A
Weight (g)	17.65 (0.21) B	16.63 (0.41) B	17.80 (0.18) B	17.35 (0.45) B	20.60 (0.12) A	18.98 (0.26) A	20.87 (0.14) A	19.02 (0.30) A
Tarsus (mm)	21.67 (0.18) A	21.05 (0.21) A	21.61 (0.16) A	21.51 (0.23) A	21.69 (0.12) A	21.06 (0.13) A	21.75 (0.12) A	20.80 (0.15) A
Culmen (mm)	11.60 (0.11) B	11.77 (0.14) B	11.85 (0.09) B	11.99 (0.16) AB	12.56 (0.06) A	12.41 (0.9) A	12.53 (0.07) A	12.46 (0.11) A
Bill width (mm)	4.30 (0.07) B	4.42 (0.08) A	4.54 (0.06) A	4.54 (0.08) A	4.59 (0.04) A	4.58 (0.05) A	4.59 (0.04) A	4.62 (0.05) A
Bill height (mm)	5.21 (0.07) A	5.13 (0.10) A	5.14 (0.07) A	5.06 (0.11) A	5.23 (0.04) A	5.17 (0.06) A	5.34 (0.05) A	5.19 (0.07) A
Plumage score	23.19 (0.84) D	26.64 (0.98) C	26.66 (0.74) C	31.36 (1.11) B	44.40 (0.50) B	48.57 (0.62) A	46.66 (0.54) A	50.74 (0.71) A

observed in plumage scores across sympatric populations, which may be driven by the increased range in plumage scores of backcrossed individuals.

The LDA separated pure and backcrossed Nelson's Sparrows from pure and backcrossed Saltmarsh Sparrows along the first linear discriminant (99% of the variation explained by axis 1, and 1% explained by axis 2) for both male and female groups but did not discriminate between pure and backcrossed individuals within the 2 species (i.e. pure Nelson's vs. backcrossed Nelson's or pure Saltmarsh vs. backcrossed Saltmarsh; Figure 5). Results from the LDA indicated that overall bill size, weight, and plumage score were most informative in differentiating among males, whereas wing chord, bill length, weight, and plumage score were informative for females (Table 2). Classification accuracy for the 4 genotypic classes ranged from 64% to 74% (males) and from 63% to 73% (females; Table 3). Among genetically pure individuals, 74% (males) and 69% (females) of Nelson's Sparrows and 67% (males) and 70% (females) of Saltmarsh Sparrows were classified correctly on the basis of morphology. Classification accuracy was similar for backcrossed individuals and ranged from 64% (males) to 73% (females) for backcrossed Nelson's and from 63% (females) to 64% (males) for backcrossed Saltmarsh. When misclassification occurred, in both males and females, pure Nelson's individuals were consistently misclassified as backcrossed Nelson's Sparrows or vice versa, and pure Saltmarsh individuals were consistently misclassified as backcrossed Saltmarsh Sparrows or vice versa (i.e. there was only 1 instance where a single backcrossed Nelson's female was classified as a backcrossed Saltmarsh). Despite a clear separation between Nelson's and Saltmarsh groups, even the most informative morphological variables performed poorly when classifying backcrossed individuals or distinguishing pure from backcrossed individuals of either parental species. Substantial overlap of canonical scores among pure and backcrossed individuals indicated that even the top variables identified by the LDA were poor predictors of genetic admixture (Figure 4).

DISCUSSION

Thorough sampling of the Nelson's–Saltmarsh sparrow hybrid zone revealed substantial variation in plumage within sympatric populations. This is consistent with current theory, which predicts that phenotypic variation will be greater in hybrid zones than in allopatric populations (Barton and Hewitt 1985, Seehausen 2004). This increased variation can arise when hybridization and introgression create novel recombinants between parental taxa (Buerkle and Lexer 2008) or complex mosaics of parental phenotypes (Allendorf et al. 2001). Based on our genetic findings, hybridization and backcrossing appear to
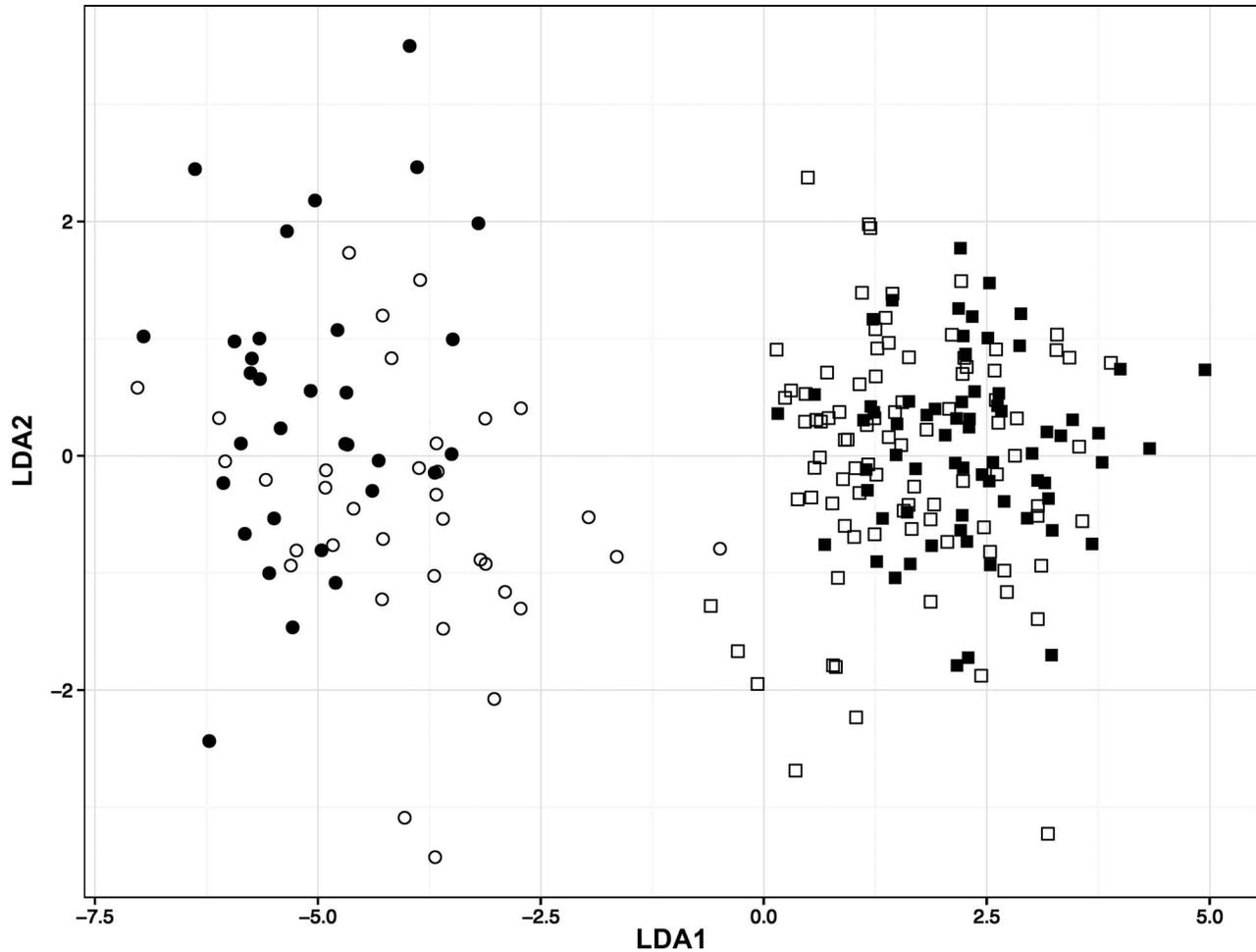
FIGURE 5. Linear discriminant analysis (LDA) of pure and admixed Nelson's and Saltmarsh sparrows based on morphometric measurements and plumage score. Males and females were pooled (based on consistency in LDA results for the 2 groups). Genotypic classes were identified using genetic data and are as follows: pure Nelson's Sparrow (filled circles), pure Saltmarsh Sparrow (filled squares), backcrossed Nelson's (open circles), and backcrossed Saltmarsh (open squares).

be frequent between Nelson's and Saltmarsh sparrows, which is congruent with the phenotypic variation observed in our study area. We documented a high proportion of admixed individuals (52%) among our sampled marshes

TABLE 2. Scoring coefficients calculated from linear discriminant analysis (LDA) of morphometric traits and plumage score collected from Nelson's and Saltmarsh sparrows across the overlap zone.

	Scoring coefficient (LDA1)	
Variable	Males	Females
Weight	0.546	0.136
Bill length	0.536	0.216
Wing chord	0.053	0.141
Tarsus	−0.194	−0.008
Bill width	0.430	−0.526
Bill height	0.346	−0.046
Plumage score	0.221	0.261

but an overall deficit of recent-generation (F_1–F_2) hybrids, with only 3% ($n = 8$) of sampled individuals assigned to the F_1–F_2 category. A low frequency of F_1–F_2 individuals indicates an advanced-generation hybrid zone characterized by high rates of recombination and limited reproductive isolation between 2 species (Culumber et al. 2010, Hamilton et al. 2013). Given the recent divergence between Nelson's and Saltmarsh sparrows (~600,000 yr; Rising and Avise 1993), coupled with the typically slow rate of evolution of postzygotic incompatibilities in birds (Price and Bouvier 2002), a finding of frequent backcrossing events in this system is not unexpected.

Although we observed greater phenotypic variation in sympatry than in allopatry, overall morphological similarities between pure and backcrossed parental groups posed a challenge for accurate hybrid identification in the field. We did not identify any clear intermediate phenotype for hybrids, and we found that backcrossed individuals were

TABLE 3. Pairwise comparisons of classification accuracy for pure and backcrossed categories of Saltmarsh and Nelson's sparrows, based on linear discriminant analysis (LDA; columns represent predicted genotypic class). All morphometric measurements were included in the LDA along with plumage score. Males (M) and females (F) are reported separately, and bold values along the diagonal indicate the percentage of individuals correctly classified for each genotypic class.

	Backcrossed Nelson's (M)	Backcrossed Nelson's (F)	Backcrossed Saltmarsh (M)	Backcrossed Saltmarsh (F)	Pure Nelson's (M)	Pure Nelson's (F)	Pure Saltmarsh (M)	Pure Saltmarsh (F)
Backcrossed Nelson's	**64.00%**	**73.00%**	0.00%	9.00%	36.00%	18.00%	0.00%	0.00%
Backcrossed Saltmarsh	0.00%	0.00%	**64.00%**	**63.00%**	0.00%	0.00%	36.00%	37.00%
Pure Nelson's	26.00%	31.00%	0.00%	0.00%	**74.00%**	**69.00%**	0.00%	0.00%
Pure Saltmarsh	0.00%	0.00%	33.00%	30.00%	0.00%	0.00%	**67.00%**	**70.00%**

typically indistinguishable from the more genetically similar parental species, on the basis of plumage and morphometrics alone. The use of linear discriminant analysis to assign individuals to genotypic classes using phenotypic traits resulted in only moderate classification accuracy for all individuals (average = 68%), and we found substantial overlap in canonical scores for pure and backcrossed individuals within the same taxonomic group (i.e. pure Nelson's compared with backcrossed Nelson's). Comparison of our plumage-based field ID protocol (scores <32 = Nelson's Sparrow, 32–45 = hybrid, >45 = Saltmarsh Sparrow) to the assigned genotypic classes revealed similarly low accuracy in hybrid identification. Fifty percent of genetically admixed (backcrossed) individuals were identified as "pure" Nelson's or Saltmarsh sparrows in the field. These results indicate that hybrid identification in the absence of genetic data will likely result in a substantial overestimation of the proportion of genetically "pure" individuals within a population. Of the 60 individuals we assumed to be genetically pure, 58 (28 Nelson's Sparrows and 30 Saltmarsh Sparrows) fell within the defined plumage cutoffs for morphologically pure individuals; the 2 other individuals fell within 2 points of the cutoff for pure Saltmarsh Sparrows. This confirms the finding of earlier work that plumage scores are reliable for differentiating the parental species (Shriver et al. 2005), at least in allopatric populations. Within sympatric populations, plumage scores were more reliable for pure than for backcrossed individuals, with fewer genotypically pure individuals (26% vs. 50%) misclassified as hybrids in the field. We suggest that high variation in phenotype of backcrossed individuals is leading to more frequent misclassification of admixed versus pure individuals. Although classification accuracy from linear discriminant analysis of morphological features was unable to differentiate between pure and backcrossed individuals within a group, it was consistently accurate in distinguishing individuals between the 2 groups (i.e. Nelson's or Nelson's-like hybrids were easily differentiated from Saltmarsh or Saltmarsh-like hybrids), which suggests that plumage and morphometrics are reliable for differentiating between the 2 groups in the field.

The strength of correlation with genotype varied among the phenotypic traits. In the 13 plumage features examined, the traits associated with plumage definition (darkness, uniformity, and clearness of the streaks found on the breast and flanks, along with the definition of the crown and face) consistently displayed a stronger correlation with genotype than traits associated with the amount of streaking on breast or flanks, the width of the whisker line and crown, or back color. Reduced introgression of certain morphological traits suggests that selection (premating or postmating) is acting within the Nelson's–Saltmarsh sparrow hybrid zone. We did not explicitly test hypotheses related to selective mechanisms in the present study, but variation in habitat, behavior, and mating strategy provide a means for both natural and sexual selection to maintain species boundaries within this system.

The darkness and definition of streaking patterns observed in Saltmarsh Sparrows may serve a convergent ecological function among tidal-marsh birds (Greenberg and Droege 1990, Grenier and Greenberg 2006). Higher levels of melanin have been documented in a range of tidal-marsh vertebrates (reptiles, mammals, and birds) in comparison to closely related upland and freshwater taxa (Grinnell 1913, Greenberg and Droege 1990, Grenier and Greenberg 2006, Olsen et al. 2010). Darker plumage has been suggested to reduce predation risk (Grenier and Greenberg 2006) while serving an important role in resisting both the mechanical (Roulin 2007) and bacterial degradation (Goldstein et al. 2004) of plumage, which may be more pronounced in salt-marsh environments (Peele et al. 2009). Although Saltmarsh and Nelson's sparrows, where sympatric, occupy the same habitats along the Atlantic coast, Nelson's Sparrows also inhabit less tidal, brackish marshes as well as upland habitats, including grasslands and hay fields (Nocera et al. 2007), whereas Saltmarsh Sparrows are restricted to salt marshes in all aspects of their life cycle (Greenlaw 1993). Habitat differences between Nelson's and Saltmarsh sparrows, coupled with the hypothesized benefits of increased melanin, may result in stronger selection for darker plumage in Saltmarsh Sparrows, thus explaining the observed patterns.

Conversely, the observed differences in structural measurements between Nelson's and Saltmarsh sparrows may be driven partially by sexual selection. Both species exhibit an unusual mating system among emberizines, characterized by nonterritoriality, lack of male parental care, and high levels of promiscuity (Greenlaw 1993, Hill et al. 2010). However, they differ in their mating tactics. Male Nelson's Sparrows spend substantial time on mate guarding and have a more distinctive song and flight display for attracting females (Greenlaw 1993, Shriver et al. 2007, 2010). Saltmarsh Sparrows are highly polygamous and exhibit a scramble-competition mating system whereby males search for and attempt to mate with multiple receptive females (Greenlaw and Rising 1994, Hill et al. 2010). These differences in mating strategy may drive size differences between male Saltmarsh and Nelson's sparrows. The scramble-competition mating system of the Saltmarsh Sparrow results in male–male competition, which should select for large body sizes (Greenlaw 1993, Andersson 1994, Fairbairn and Preziosi 1994, Székely 2004). By contrast, male Nelson's Sparrows perform frequent flight displays, which should select for a smaller, more acrobatic body size (Székely 2004, Byers et al. 2010). Given these differences, intermediately sized males would be at a disadvantage, both in aerial displays and in direct male–male competition; selection against intermediately sized males may thus act as a potential source of reproductive isolation between these 2 species.

Consistent phenotypic patterns provide useful information for identification of pure Nelson's and Saltmarsh sparrows in the field. Overlap in morphological features between admixed and pure individuals is too substantial, however, to distinguish between backcrossed and pure sparrows in the absence of genetic data. The inability to distinguish between pure and admixed individuals within sympatric populations may pose conservation challenges, because we are still unsure of the effects of extensive hybridization and introgression in this system. Hybridization and introgression can lead to harmful effects on the viability of a focal species, including hybrid swarms, reduced reproductive success, and outbreeding depression, and these events can be particularly problematic when one species is less abundant than the other (Rhymer and Simberloff 1996, Allendorf et al. 2001, Buggs 2007). Despite these potentially negative outcomes, introgression can also lead to adaptive gene combinations within admixed populations (Mallet 2005), resulting in the introduction of genetic novelty into a system, and may increase the adaptive potential of a population (Rheindt and Edwards 2011). Future studies of adaptive genetic variation, using current genome-sequencing technologies, may provide insight into the potential role of introgression in the adaptive capacity in this system.

Given the uncertain outcomes of hybridization, effective monitoring of hybridizing populations of Saltmarsh and Nelson's sparrows is important, because both species are a high conservation priority in the northeastern United States (U.S. Fish and Wildlife Service 2008) and the Saltmarsh Sparrow is considered globally vulnerable to extinction (IUCN Red List criteria; BirdLife International 2004). Our current knowledge indicates that the hybrid zone constitutes ~15% of the global Saltmarsh Sparrow range and may pose a greater threat to this species by limiting the range of genetically "pure" populations of Saltmarsh Sparrows. Furthermore, we found evidence for introgression beyond the boundaries of the currently hypothesized overlap zone, which suggests that the range of genetically "pure" Saltmarsh Sparrows may be smaller than is currently thought. Discriminating pure and admixed individuals is critical for monitoring hybrid-zone dynamics (i.e. whether the zone is stable, shifting, or expanding over time).

To aid in population monitoring, we recommend that future field studies within the hybrid zone include collection of blood or feathers for genetic identification of pure and admixed sparrows. When in the field, particularly in sympatric populations, closer observation of the darkness and definition of plumage traits (particularly on the flanks and breast) may aid in pure species identification. Weight and bill length are also informative for discriminating between pure Nelson's and Saltmarsh sparrows. Although these traits may not aid in hybrid identification, they may provide an easy way to confirm species identification in the field (i.e. discriminate Saltmarsh and backcrossed Saltmarsh from Nelson's and backcrossed Nelson's); this is particularly helpful in marshes near the center of the hybrid zone, where species identification can be challenging. Further, as a result of extensive backcrossing, admixture should be expected within the hybrid zone, regardless of morphology. Therefore, genetic analyses will be necessary when discrimination of pure and admixed individuals is an important goal. In such cases, we recommend a target of sampling 10–15 birds per marsh; in our experience, this can be accomplished within a single day and leads to a diverse sample of individuals per site. Lastly, careful plumage scoring and the collection of genetic data may be most informative outside of the hybrid zone, in populations that neighbor the northern and southern edges, where detecting unusual plumage patterns or recent-generation hybrids may aid in identifying hybrid-zone expansion.

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