

# Geographic population structure and subspecific boundaries in a tidal marsh sparrow

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**Abstract** Subspecific genetic diversity is a source for ongoing evolutionary processes, can be predictive of a population's ability to respond to conservation challenges, and may represent the raw material for incipient speciation. As such, diagnosable subspecies are increasingly recognized as an important unit for conservation. Differentiating among subspecies can be particularly difficult in ecosystems characterized by recent phenotypic divergence, such as the tidal marshes of North America. These systems require approaches that can capture selective changes which occurred over only a few millennia as species adapted to new habitats following the Holocene glacial retreat. Here we test for genetic differentiation in morphologically distinct tidal-marsh-endemic subspecies of the swamp sparrow (*Melospiza georgiana*) using nuclear microsatellites. This case study serves as a test approach for the quantification of conservation units for tidal marsh ecosystems. Though prior surveys of mtDNA variation

showed no detectable phylogeographic structure, we found evidence of genetic differentiation in seven microsatellite loci between two *M. georgiana* subspecies. The most likely model of population structure suggested two clusters in western Maryland/Pennsylvania and Delaware/Chesapeake Bays, with a zone of uncertain population assignment in New Jersey. The microsatellite intergrade zone is broader than the known area of morphological intergrades. We show that microsatellites can be used to support a subspecific status for tidal-marsh taxa such as the swamp sparrow, where changing post-glacial environments likely selected for locally adapted traits while neutral genetic structure is weak. This approach thus allows for the identification and conservation of hot spots that foster ongoing evolutionary change.

**Keywords** Swamp sparrow · *Melospiza georgiana* · Microsatellite DNA · Local adaptation · Subspecies · Tidal marsh · Endemism

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## Introduction

Growing threats from human alteration of landscapes and climate make it imperative to identify and preserve biodiversity at multiple biological scales. Intraspecific variation, which is commonly a lower or an unstated priority for conservation, is an index of ongoing evolutionary processes that may protect species from extinction or represent emergent species (Endler 1977; Lewis 2012; Mayr and Ashlock 1991; Winker 2010). Accordingly, conservation planners are becoming more interested in explicitly preserving such evolutionary processes through the protection of intraspecific genetic diversity and population genetic structure (Cronin et al. 2015; Degner et al. 2007; Funk

et al. 2007; Haig et al. 2006; Phair et al. 2015). Identifying important evolutionary processes for conservation, however, is not simple. Methods of assessing subspecific diversity (or alternatives such as Evolutionarily Significant Units, Ryder 1986) use various phenotypic and genotypic approaches (Avice 2000; Avice and Ball 1990; Crandall et al. 2000; Moritz 1999), although morphological differences may (Miller-Butterworth et al. 2003; Polly 2001) or may not parallel molecular differences (Greenberg et al. 1998; Roca et al. 2001; Talbot and Shields 1996; Waits et al. 1997).

The choice of a particular molecular method to measure intraspecific diversity can be consequential for conservation actions and outcomes. Coarser measures of neutral genetic structure (e.g., mtDNA, Avice 2000) prioritize deeper evolutionary divergence at the risk of missing recent evolutionary events that can predict population risk under current landscape change (Schiffers et al. 2012). Conversely, an over-emphasis on phenotypic differences may place an unrealistic burden on conservation efforts and result in the expenditure of resources to preserve non-heritable components of diversity. The use of more rapidly evolving or higher resolution neutral markers, such as microsatellites or Single Nucleotide Polymorphisms (SNPs), should allow for the identification of more recent evolutionary processes (e.g., rapid adaptation to recently deglaciated landscapes), while preventing the unnecessary preservation of non-heritable components of biodiversity.

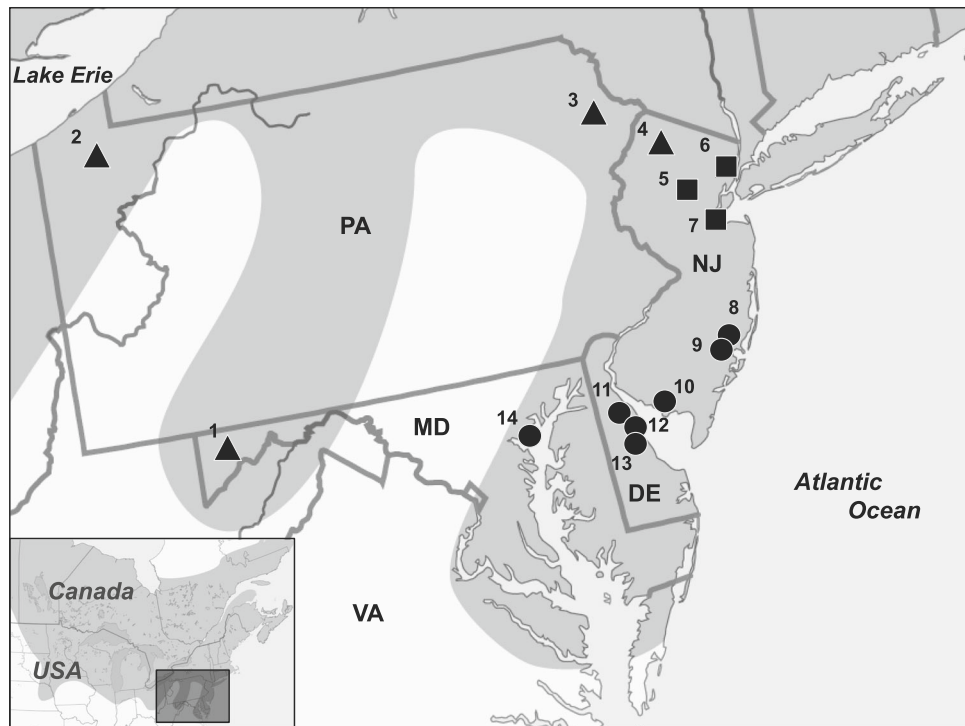
One ecosystem where rapid evolutionary changes have occurred since the last glacial maximum is the tidal salt marsh of North America (Greenberg et al. 2006a). Understanding and characterizing the existing biodiversity in these coastal marshes is particularly important given the multitude of human-driven threats (e.g., sea-level rise, coastal development, water quality degradation) currently facing these ecosystems (Bromberg Gedan et al. 2009). Many vertebrate species that colonized coastal marshes after their rapid expansion 5000–7000 years ago (Bratton et al. 2003; Malamud-Roam et al. 2006) now possess morphologically distinct populations or subspecies, although few species are constrained completely to tidal marshes (for a complete list of terrestrial vertebrate taxa endemic to tidal marshes, see Table 2 in Greenberg et al. 2006a). A lack of neutral genetic structure in many of these species suggests that ecological selection, even in the face of ongoing gene flow, may have been important in producing the distinct morphological features that currently exist in these populations (Greenberg et al. 1998; 2006a; Greenberg and Droege 1990), although phenotypic plasticity has not been eliminated as a possible cause for many taxa (but see Ballentine and Greenberg 2010).

Among tidal marsh birds, local adaptation is suggested by convergence across taxa in coloration (Luttrell et al.

2015; Greenberg and Droege 1990), bill size (Greenberg and Olsen 2010; Grenier and Greenberg 2005), nest construction (Gjerdrum et al. 2005; Humphreys et al. 2007; Reinert 2006; Shriver et al. 2007), life history strategies (Greenberg and Droege 1990; Olsen et al. 2008), and social systems (Greenberg and Olsen 2010; Hays and Lidicker 2000; McDonald and Greenberg 2006). However, analyses of mtDNA may not offer the resolution necessary to identify “hot spots” of rapid adaptive evolution and differentiate among subspecies in this ecosystem (Cardoni et al. 2013; Greenberg et al. 1998). There is a demonstrated risk of losing subspecific diversity in this ecosystem if too coarse of a genetic marker is used to set conservation priorities. Two recognized coastal endemic subspecies have already gone extinct in North America since European colonization, the dusky seaside sparrow (*Ammodramus maritimus nigrescens*) and the heath hen (*Tympanuchus cupido cupido*).

In this paper, we use microsatellite loci to study genetic divergence in one vertebrate species with a morphologically distinct tidal marsh population, the swamp sparrow (*Melospiza georgiana*). Coastal plain swamp sparrows (*M. g. nigrescens*; Bond and Stewart 1951) breed in brackish tidal marshes of the Mid-Atlantic U.S. states (Greenberg and Droege 1990; Watts et al. 2008). Southern swamp sparrows (*M. g. georgiana*) breed in freshwater wetlands within formerly glaciated areas of eastern North America and occur only sporadically south of the glacial line. Except for a few scattered populations, a gap separates the two subspecies over much of the piedmont and coastal plain of Maryland and Pennsylvania, forming a semi-ring distribution from the Allegheny Highlands of West Virginia and western Maryland to the tidal wetlands of the Mid-Atlantic U.S. states. Northern New Jersey thus forms the most likely contact zone between the coastal plain and southern forms of the species (Fig. 1; Greenberg and Droege 1990). Coastal plain swamp sparrows are morphologically and behaviorally divergent from southern swamp sparrows in ways that are similar to many other endemic tidal-marsh taxa (Greenberg and Droege 1990; Greenberg et al. 2006b; Liu et al. 2008; Olsen et al. 2010, 2013). Breeding origin is differentiable using plumage and bill characteristics with 98 % accuracy (Greenberg and Droege 1990), and a common-garden experiment showed similar accuracy in the identification of captive-reared offspring (Ballentine and Greenberg 2010), suggesting a genetic basis to these subspecific morphological differences. However, a previous study of mtDNA sequences (5' end of control region, COII/t-lys/ATPase8, and ND2) found low levels of genetic variation and no evidence of geographic structure between the two subspecies (Greenberg et al. 1998).

In October of 2014, a group of biologists and stakeholders met to develop a Conservation Business Plan to protect the tidal-marsh bird populations of the Atlantic and



**Fig. 1** Location of sampling sites in Maryland (MD), Pennsylvania (PA), Delaware (DE), and New Jersey (NJ). The populations representing *M. g. georgiana* (indicated by triangles) are 1 Garrett (N = 20), 2 Erie (N = 10), 3 Hawley (N = 11), and 4 Newton (N = 18). The morphological intergrade populations (indicated by squares) are found in 5 Great Swamp National Wildlife Refuge (N = 15), 6 Meadowlands (N = 6), and 7 Cheesequake (N = 5). The

*M. g. nigrescens* populations (indicated by circles) are 8 Bass River (N = 9), 9 Mullica River (N = 15), 10 Dividing Creek (N = 8), 11 Woodland Beach (N = 53), 12 Bombay Hook (N = 31), 13 Port Mahon (N = 15), and 14 Black Marsh (N = 14). The rectangle in the inset shows where sampling occurred within the broader swamp sparrow range (shaded in both the inset and the main figure)

Gulf coast U.S. states (Saltmarsh Habitat & Avian Research Program 2015). The coastal plain swamp sparrow was identified as one of five taxa with the highest priority for conservation: “those that are potential candidates for ESA [U.S. Endangered Species Act] listing”. This subspecies is also currently listed as a ‘Species of Greatest Conservation Need’ in the U.S. states of Delaware (Tier 1: Delaware Division of Fish and Wildlife 2006) and Maryland (Maryland Department of Natural Resources 2005). It receives no special attention for conservation, however, in the states of New Jersey and Virginia, where declines have been reported (Watts 2014), or in Pennsylvania, where it is largely extirpated [observed in the late eighteenth century by both Alexander Wilson (Baird et al. 1905) and William Bartram (Coues 1875)]. This geographic heterogeneity in conservation priorities is common among tidal marsh vertebrates as a whole, where there is either a lack of knowledge concerning genetic structure or a lack of structure in mtDNA markers between tidal and non-tidal populations (Chan et al. 2006). To address this uncertainty in subspecific classification for swamp sparrows in particular and the variation this uncertainty causes for conservation efforts of

tidal marsh fauna in general, we sampled swamp sparrows along the southeastern range of *M. g. georgiana* and most of the known range of *M. g. nigrescens* to analyze geographic structure in microsatellite DNA in relation to both geographic distance and subspecies identity.

## Methods

### Sampling protocol

Swamp sparrows were mist netted during the breeding season from 2001 to 2005 by placing a small number of nets in known territories or by attracting birds to a net with a recording of a male song. A small (20–50 µl) sample of blood was collected in a glass capillary tube and the sample was transferred to a small plastic vial and preserved with lysis buffer (20 % DMSO, 0.25 M EDTA, 100 mM Tris, pH 7.5, saturated with NaCl; Amos et al. 1992). After collection, samples were stored in a –70 °C freezer at the Smithsonian Center for Conservation and Evolutionary Genetics at the National Zoological Park.

The total sample size was 230 birds collected from 14 sites (5–53 per site, Fig. 1). All birds were adults ( $N = 216$ ) or independent juveniles born that breeding season ( $N = 14$ ), so that collection of material from family groups was unlikely. The species is migratory, but philopatry is high in both the inland and coastal subspecies based on simple band returns (Olsen et al. 2008). The field sampling methods resulted in a male-biased sample; of the adult birds for which sex was recorded in the field based on morphology, 126 were male and 64 were female.

DNA was extracted from blood samples using Qiagen DNeasy Tissue Kits according to manufacturer's instructions. Individuals were genotyped at seven microsatellite loci: Mme2, Mme3, Mme7, Mme8, Mme12 (Jeffery et al. 2001), Mg2we (F primer: 5'-CCCCTGTCAGCAATGT TAGG-3', R primer: 5'-GAACCACACAGTGCCAAC-3', annealing temperature: 60 °C) and Esc $\mu$ 1 (Hanotte et al. 1994). The microsatellite PCR profiles and reaction conditions are provided in Olsen (2007), with the exception of Esc $\mu$ 1, which is detailed in Chan and Arcese (2002). For two sex-linked microsatellites (Mme3 and Mme7), individuals of unknown sex were genotyped as male ( $N = 26$ ) if either locus contained two allele peaks (heterozygous). If both loci contained a single allele peak ( $N = 14$ ), genotypes for the two sex-linked loci in individuals of unknown sex were coded as missing data, as it was not possible to distinguish between a homozygous male with two copies of the same allele and a female with a single copy of the allele.

## Analyses

To evaluate the extent to which subspecific grouping reflected geographic genetic structure we took two approaches. First, we applied standard distribution-based statistical approaches to examine the relative contribution of putative subspecies affiliation, geographic distance, and population assignment to the variation in microsatellite frequencies. Using quantified patterns of morphological divergence (Greenberg and Droege 1990), sites along the Maryland, Delaware, and New Jersey coasts (excluding the Hudson River estuary) were assigned to the *M. g. nigrescens* population; sites in interior Maryland, Pennsylvania and the New Jersey highlands were assigned to the *M. g. georgiana* population; and three sites in the vicinity of the Hudson River estuary were classified as possibly "intergrade." Intergrade populations were morphologically intermediate in both coloration and bill size (Greenberg and Droege 1990). For our second approach we used a Bayesian estimation program (STRUCTURE) to determine if genetic substructure existed among the sampling sites.

We first tested for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using

ARLEQUIN v3.5.1.3 (Excoffier and Lischer 2010). Tests were run for each individual sampling site as well as each subspecies (grouping sites by their predominant morphology as *M. g. georgiana*, *M. g. nigrescens*, or intergrade) to reflect our current uncertainty of the genetic structure in this system. Studies have demonstrated fine-scale genetic structure among sampling sites of other tidal-marsh emberizid sparrows (Walsh et al. 2012), but we acknowledge that movement of individuals among sites in close geographic proximity is possible. For loci that deviated from HWE, we tested for the presence of null alleles using MICRO-CHECKER v2.2.3 (van Oosterhout et al. 2004) and for neutrality using LOSITAN (Antao et al. 2008; Beaumont and Nichols 1996) run with 50,000 simulations, a stepwise mutation model, and a forced 'neutral' mean  $F_{ST}$ . We calculated allelic diversity corrected for differences in sample size using rarefaction implemented in HP-RARE v1.1 (Kalinowski 2004; 2005). All analyses were corrected for multiple comparisons using the Benjamini–Yekutieli false discovery rate procedure (Benjamini and Yekutieli 2001), implemented in R v3.1.3 (R Core Team 2015).

Pairwise  $F_{ST}$  was estimated using Weir and Cockerham's (1984) theta to account for differences in sample size.  $F_{ST}$  was calculated using the diversity package (v1.9.89, Keenan et al. 2013) in R; significance was evaluated with confidence intervals and an alpha level of 0.001 calculated using 1000 bootstraps carried out over individuals within samples. We conducted an analysis of molecular variance (AMOVA) with 1000 permutations using ARLEQUIN v3.5.1.3 (Excoffier and Lischer 2010) to test for population and subspecific differentiation.

We used partial Mantel tests in IBDWS v3.15 (Jensen et al. 2005) to examine the influence of geographic distance and subspecies affiliation on genetic structure. Genetic distance was estimated using Rousset's (1997) distance,  $F_{ST}/(1 - F_{ST})$ . Geographic distance between sampling locations was calculated using the geosphere package (Hijmans 2015) in R as the shortest distance between two points according to the law of cosines; geographic distances were log-transformed for analysis. Comparisons between designated subspecies and intergrade populations were coded as '1', comparisons between populations of different subspecies were coded as '2', and comparisons within the same subspecies or between intergrade sites were coded as '0'. Statistical significance was calculated based on 20,000 permutations.

We implemented the Bayesian clustering program STRUCTURE v2.3.3 (Pritchard et al. 2000) to estimate the number of genetic clusters (K) in our samples collected across 14 localities. We used the admixture model with correlated allele frequencies and ran the analyses with and without sampling locality as a prior. We ran five replicates of the STRUCTURE analyses and tested K values ranging



from 1 to 3, representing the three morphological variants observed in swamp sparrows. Each STRUCTURE run consisted of an initial burn-in of  $10^5$ , with  $5 \times 10^6$  iterations. STRUCTURE HARVESTER Web v0.6.94 (Earl and vonHoldt 2012) was used to process the STRUCTURE results files, and CLUMMP v1.1.2 (Jakobsson and Rosenberg 2007) and DISTRICT v1.1 (Rosenberg 2004) were used to summarize across replicates and visualize the results.

## Results

Two microsatellite loci were found to significantly deviate from HWE (following correction for multiple tests) in a subset of the sampling sites (Escu1: Great Swamp National Wildlife Refuge and Bombay Hook; Mme8: Mullica River, Woodland Beach, and Bombay Hook; Table S1). When sampling sites were grouped based on morphology, Escu1 and Mme8 were also found to deviate from HWE in three and two of the morphologically defined sample groups, respectively (Table S1). There was significant evidence for null alleles at both of these loci (Table S2). All analyses were therefore run with and without Escu1 and Mme8; because little difference was observed between runs, we primarily report only the results from analyses with all seven loci. There was little evidence of significant LD among any of the loci (following correction for multiple tests, Table S3), with the one exception of Mme3 and Mme7, which are both located on the Z chromosome (Dawson et al. 2006). No loci showed evidence of non-neutrality. There was no significant difference in allelic diversity between subspecies following correction for multiple tests (Table S4). All supplemental data tables are given in Online Resource 1.

Multiple analyses suggested that genetic differences exist between *M. g. georgiana* and *M. g. nigrescens*. While the AMOVA analyses showed that the majority of genetic variation occurred within sampling locations (97.83 %,  $F_{ST} = 0.022$ ,  $P < 0.00001$ ), a small but significant portion of the variation could also be explained by variation among subspecies (including “intergrade” as a subspecies) ( $F_{CT} = 0.013$ ,  $P < 0.001$ ). Little genetic variation occurred among locations sampled within subspecies ( $F_{SC} = 0.009$ ,  $P = 0.071$ ), though this proportion was significant when Escu1 and Mme8 were removed from the analyses ( $F_{SC} = 0.014$ ,  $P < 0.001$ ). In addition, we observed a higher proportion of significant pairwise  $F_{ST}$  values in comparisons of sampling locations from different subspecies (7 out of 28 pairwise comparisons) than comparisons within subspecies (3 out of 30 pairwise comparisons) or between subspecies and intergrade sampling locations (1 out of 33 pairwise comparisons) (Table 1). Finally, the partial Mantel tests indicated there was a

significant correlation between genetic distance and log-transformed geographic distance ( $r = 0.27$ ,  $P < 0.05$ ), as well as between genetic distance and subspecific membership ( $r = 0.29$ ,  $P < 0.01$ ) (Fig. 2). The effect of subspecies remained significant when controlling for geography ( $r = 0.29$ ,  $P < 0.01$ ), but the effect of geographic distance was no longer significant when controlling for subspecies ( $r = 0.05$ ,  $P = 0.39$ ).

The STRUCTURE cluster analyses identified the model with the highest likelihood (LnP(K)) as  $K = 2$  when sampling locality was used as a prior (Table 2), though the support for multiple genetic populations was not significant without the use of priors. These results remained robust to the removal of Escu1 and Mme8, the two loci that showed deviations from HWE in some sampling sites. The lack of significant structure without the use of sampling locality as a prior suggests that the level of divergence between clusters is relatively low (Hubisz et al. 2009); however, this structure appears biologically significant as the clusters correspond closely with the recently diverged subspecies. Under the  $K = 2$  model, all individuals in populations from *M. g. georgiana* were assigned with high probability to the first cluster (Fig. 3, black), while the majority of individuals in *M. g. nigrescens* were assigned with high probability to the second cluster (Fig. 3, gray). The majority of individuals in populations in northern New Jersey (4–6) were assigned with high probability to the first cluster, though these populations included individuals that were morphologically intergrade. Many of the individuals in populations in central New Jersey (7–9) exhibited a hybrid signature of mixed cluster assignment, though populations 8 and 9 were assigned on the basis of morphology to the *M. g. nigrescens* subspecies. If we assume that the populations at the southern edge of the swamp sparrow range in the mid-Atlantic form an incomplete ring, and gene flow across the gap between the Appalachians and the coast is much less frequent than among adjacent populations along the known continuous distribution, then the resulting STRUCTURE diagram shows a cline between the populations along the Delaware and Chesapeake Bays and populations to the North and West.

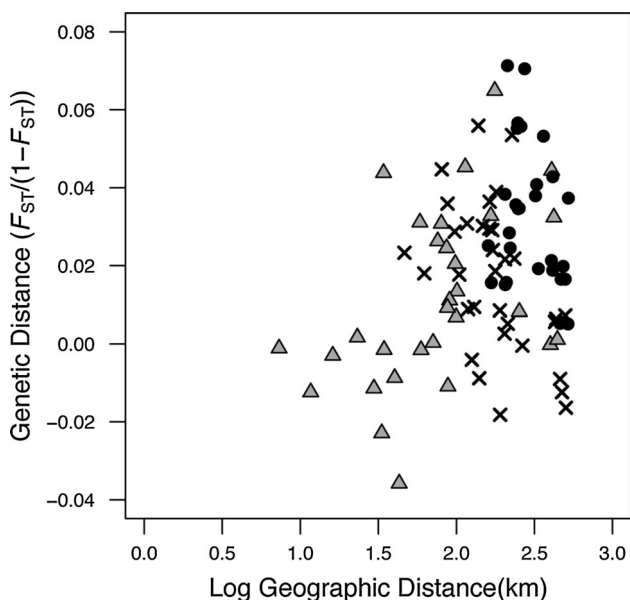
## Discussion

Which molecular markers provide the most useful information on stochastic processes, population history, ongoing gene flow, and heritable phenotypic variation is a matter of continued debate. The methodological uncertainty adds controversy and ambiguity to conservation policy (Fallon 2007). Here, however, we show that microsatellite markers can detect structure in natural populations of recently diverged tidal-marsh taxa, which are supported by

**Table 1** Pairwise genetic differentiation ( $F_{ST}$ ) between sampled populations of *M. g. georgiana* (1–4), a morphological intergrade zone (5–7) and *M. g. nigrescens* (8–14)

Subspecies	<i>M. g. georgiana</i>				Intergrade			<i>M. g. nigrescens</i>						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Garrett	–													
2. Erie	0.007	–												
3. Hawley	0.040	0.019	–											
4. Newton	0.028	0	0.014	–										
5. Great Swamp	0.010	0	0.011	0.019	–									
6. Meadowlands	0.001	0	0	0.010	0	–								
7. Cheesequake	0.019	0.002	0.046	0.036	0.035	–0.038	–							
8. Bass River	<b>0.055</b>	0.042	0.053	0.028	0.029	0.016	0.030	–						
9. Mullica River	0.032	0.021	0.021	0.018	0	0	0.026	0	–					
10. Dividing Creek	<b>0.068</b>	0.031	0.041	<b>0.038</b>	0.030	0	0.020	0.001	0	–				
11. Woodland Beach	<b>0.039</b>	0.011	0.026	0.013	0.018	0.003	0.025	0.015	0.014	0	–			
12. Bombay Hook	<b>0.037</b>	0.011	0.036	0.012	<b>0.033</b>	0.011	0.032	0.023	0.014	0.010	0.001	–		
13. Port Mahon	0.022	0.015	0.038	0.020	0.009	0.008	0.027	0.012	0	0.010	0.004	0.004	–	
14. Black Marsh	<b>0.047</b>	0.029	<b>0.054</b>	0.035	0.025	0.003	0.050	<b>0.060</b>	0.032	0.043	<b>0.029</b>	<b>0.033</b>	0.032	–

Bold values are significantly greater than zero as evaluated using 1000 bootstrap iterations ( $\alpha = 0.001$ ) for each pairwise comparison

**Fig. 2** Relationship between genetic distance and log-transformed geographic distance among pairs of swamp sparrow sampling sites. Pairs include comparisons within subspecies (gray triangles), between a subspecies and morphological intergrade sampling location (cross marks), and between subspecies (black circles)

experimental evidence of heritable differentiation (Baltentine and Greenberg 2010). We detected differentiation in microsatellite genotypes that corresponds to the known distribution of the morphologically defined *M. g. georgiana* and *M. g. nigrescens* and expanded our understanding of

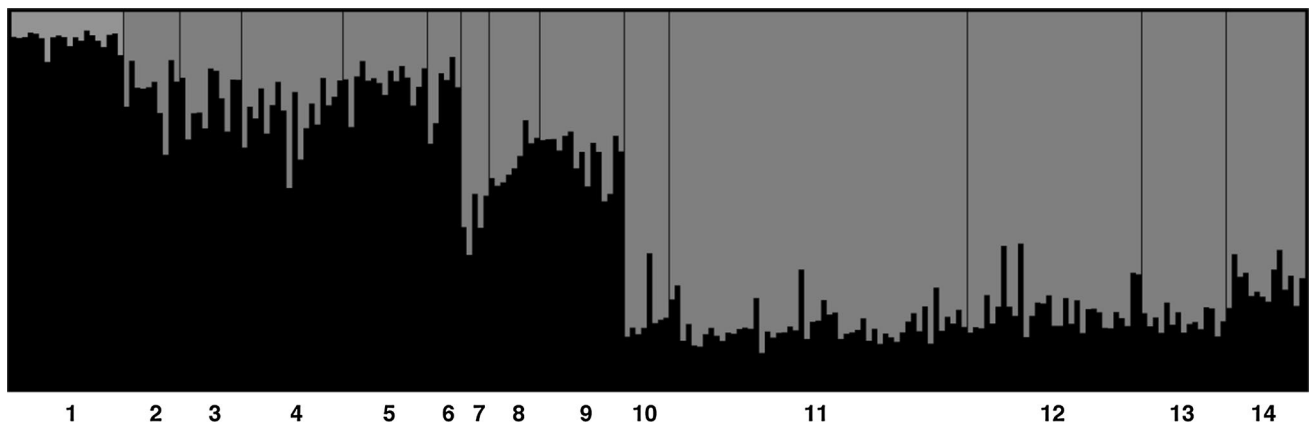
**Table 2** Log likelihood values for each K averaged over five runs of STRUCTURE (using sampling locality as a prior) indicating the statistical support for one to three population clusters

K	Reps	Mean LnP(K)	Stdev LnP(K)
1	5	–5505.92	0.08
2	5	<b>–5475.20</b>	<b>5.45</b>
3	5	–5532.76	37.35

Bold values indicate the model with the highest likelihood (LnP(K)) using cluster analyses.

the intergrade zone between these recently diverged subspecies. As the phenotypic differences between these subspecies are also seen in a suite of other vertebrate species that have colonized tidal marshes (Greenberg et al. 2006a), and as colonization of and adaptation to tidal marshes is likely a geologically recent event in most taxa (Malamud-Roam et al. 2006), our approach contributes to a broader understanding of evolutionarily meaningful units of conservation across the tidal marsh ecosystem.

A previous analysis of swamp sparrow mtDNA from a subset of our study sites (Garrett County and Black Marsh, Maryland; Dividing Creek, New Jersey;  $N = 24$ ) was unable to detect structure among morphologically distinct subspecies of swamp sparrow (Greenberg et al. 1998). However, though mtDNA has proven broadly effective in detecting genetic structure among populations with long histories of isolation, more rapidly evolving microsatellite DNA may be better suited for describing population structure that has developed over recent time frames



**Fig. 3** Results from STRUCTURE cluster analysis with populations (codes and spatial arrangement as in Fig. 1) arranged around a semi-ring distribution (rassenkreis). A model of  $K = 2$  was most

supported. Each column represents an individual, and the membership of that individual in each cluster (black or gray) is shown on the y-axis

(Goldstein and Schlötterer 1999; Sunnucks 2000; Wan et al. 2004; but see Zink 2010). In this study we evaluated a larger sample set of swamp sparrows ( $N = 230$ ) from 14 populations at the interface between the two subspecies (Fig. 1). Despite finding over 95 % of the variation in microsatellite frequency within populations, the AMOVA provided evidence of limited, but significant, genetic structure at the level of the two described subspecies. The Mantel test showed that subspecies designation predicted pairwise genetic differentiation better than geographic distance between populations. The STRUCTURE analysis, using sampling location as a prior, showed two clusters of microsatellite genotypes, one cluster associated with sites along the Delaware and Chesapeake Bays and the second cluster associated with sites in western Maryland, Pennsylvania, and central and northern New Jersey (Fig. 3). Our results contextualized within Greenberg et al.'s (1998) prior findings suggest that segregation between the coastal and inland populations occurred recently, presumably during post-Pleistocene expansion.

The discrepancy between structure identified using mtDNA and microsatellite loci is not surprising. We acknowledge that our larger sample sizes and the higher geographic resolution of our sampling may partially contribute to the differences observed between these two methods. However, many other studies have shown similar differences between the two methods in migratory birds (e.g., bluethroat *Luscinia svecica*, Johnsen et al. 2006; Zink et al. 2003). In particular, a similar relationship between morphologically described subspecies, unstructured mtDNA haplotypes, and structured microsatellite loci has been documented across subspecies of the song sparrow (*Melospiza melodia*), a congener of the swamp sparrow. Song sparrows are widely distributed throughout North America and well known for their ecotypic variation

(Aldrich 1984) with up to 28 recognized subspecies (Patten and Pruett 2009). Little of this subspecific variation in plumage and size is associated with variation in mtDNA (Fry and Zink 1998; Zink and Dittmann 1993), however, leading to speculation that the species expanded rapidly into a diversity of habitats where relatively rapid selection then shaped phenotypic variation (Fry and Zink 1998; Pruett et al. 2008b). Multiple studies using microsatellite markers (Pruett et al. 2008a, b; Pruett and Winker 2010) and SNPs (Srivastava et al. 2012) have concluded that geographic variation in these finer-scale neutral markers often, but not always, corresponds to subspecific designations in this species.

Further support that the weak neutral structure in microsatellite markers is produced by recent, rapid selection is provided by variation in the apparent zone of genotypic and phenotypic introgression. Based on morphological analysis of museum study skins, the coastal plain subspecies of the swamp sparrow breeds along the New Jersey coast north to the Hudson River estuary, with some indication of morphological intergradation at the very northern edge of the New Jersey range (Greenberg and Droege 1990). The zone of apparent microsatellite introgression, however, is much broader, encompassing northern and central New Jersey. Variable introgression across the genome, which can generate variation in cline width among genetic markers and morphological traits, has also been described in other birds, including another tidal-marsh sparrow (Walsh 2015), at the species- and subspecies-level (Parchman et al. 2013; Baldassarre et al. 2014). This difference for swamp sparrows we report here is consistent with the hypothesis that two distinct morphotypes are maintained by selection, and the larger cline in neutral markers suggests disjunct morphological characters in the face of ongoing gene flow (Gockel et al. 2001).

The divergence in microsatellite DNA in the Delmarva populations (Delaware and Chesapeake bays) of swamp sparrows may be a result of historical isolation, reduced gene flow, or both. Female *nigrescens* have been shown to prefer male *nigrescens* over male *georgiana* based on differences in song structure between the two subspecies (Ballentine et al. 2013), which is one likely behavioral mechanism for reduced gene flow between subspecies. Interestingly, Patten et al. (2004) documented female song choice as being important in maintaining reduced gene flow in the species ring of southern California song sparrows.

As used in ornithology (AOU 2015), subspecies remain a unit of geographically based phenotypic variation. Most subspecies were described in the late 19th and early 20th centuries and, in the case of birds, assignment was almost always based on plumage coloration or size. Thus the designation of a portion of a species as a subspecies essentially established a hypothesis of evolutionarily important local adaptation for further research. Structure in neutral or nearly neutral genetic markers can provide further information to infer history or process. Although it has been proposed that the evolutionary importance of distinct morphologies resides only with those portions of a species that show reciprocal monophyly in mtDNA or other evidence of long-term evolutionary cohesion (Zink 2004), distinct local morphological adaptations in the absence of genealogical distinctiveness (or even strong population structure) are still of conservation interest. In this case, we know that a number of taxa that occupy coastal marshes, including the coastal plain swamp sparrow, are morphologically and physiologically distinct with evidence for recent colonization of the ecosystem (Greenberg et al. 2006a). These convergent phenomena should alert us to ecosystems that may be hotspots of recent or current evolutionary divergence or, possibly, ongoing ecological speciation.

As one of the most thoroughly studied tidal-marsh subspecies in North America, the coastal plain swamp sparrow is a prime example of the convergent patterns of phenotypic divergence observed across other tidal-marsh vertebrates. Our findings of genetic structure in this species therefore suggest that wider ecosystem patterns of phenotypic divergence may also be due to genotypic divergence under recent selection. To preserve these ongoing evolutionary processes will require an understanding of the spatial arrangement of intraspecific diversity upon the landscape. Microsatellites appear adequate for documenting such processes in swamp sparrows, and similar neutral markers or emerging high-throughput SNP-based techniques may allow for informed conservation in other tidal-marsh taxa.

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