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CHAPTER 13

ALASKA SONG SPARROWS (*MELOSPIZA MELODIA*) DEMONSTRATE THAT GENETIC MARKER AND METHOD OF ANALYSIS MATTER IN SUBSPECIES ASSESSMENTS

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ABSTRACT.—We examined genetic and morphological characteristics of the Song Sparrows (*Melospiza melodia*) of northwestern North America, which have a relatively large number of phenotypically described subspecies ($n = 6$ in this region). Mitochondrial DNA (mtDNA) sequences showed little information about these subspecies, with no reciprocal monophyly evident. However, differences in body mass and microsatellite allele frequencies supported continued recognition of subspecific units for taxonomy and conservation. Song Sparrow subspecies in this region are probably representative of many recently diverged populations that have not been isolated long enough for complete lineage sorting using mtDNA markers, yet which have evolved differences that are likely to be genetically based. We emphasize the importance of using multiple lines of evidence, genetic and morphological, in assessing subspecific status, lest we overlook important biological diversity that has accrued below the level of full species.

Key words: body mass, *Melospiza melodia*, microsatellite, mitochondrial DNA, population genetics, Song Sparrow.

Las Poblaciones de Alaska de *Melospiza melodia* Demuestran que los Marcadores Genéticos y los Métodos de Análisis Afectan la Evaluación de las Subespecies

RESUMEN.—Examinamos características genéticas y morfológicas en poblaciones del noroeste de Norteamérica de *Melospiza melodia*, una especie que tiene un número relativamente grande de subespecies descritas fenotípicamente ($n = 6$ en esta región). Las secuencias de ADN mitocondrial (ADNmt) mostraron poca información sobre estas subespecies, que no presentan monofilia recíproca evidente. Sin embargo, las diferencias en tamaño corporal y las frecuencias alélicas de microsatélites continúan apoyando el reconocimiento de unidades subspecíficas para taxonomía y conservación. Las subespecies de *M. melodia* en esta región probablemente representan varias poblaciones que se diferenciaron recientemente y que no han estado aisladas por suficiente tiempo para alcanzar la separación completa de linajes del ADNmt, pero que se han diferenciado evolutivamente en rasgos que probablemente tienen una base genética. Enfatizamos la importancia de usar líneas de evidencia múltiples, genéticas y morfológicas, al evaluar el estatus de las subespecies. De lo contrario, pasaremos por alto una diversidad biológica importante que se ha acumulado por debajo del nivel de las especies.

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THE USEFULNESS OF subspecies as units in conservation and taxonomy has been a subject of ongoing debate (Zink 2004, Remsen 2005, Phillimore and Owens 2006). Some researchers have stressed the continuing use of subspecies as valid taxonomic units (Patten and Unitt 2002, Phillimore and Owens 2006), whereas others have advocated the complete elimination of subspecies, elevating groups that are reciprocally monophyletic (assessed using mitochondrial DNA [mtDNA] sequences) to full species (Zink 2004). By using a single-criterion approach (even if using cladistic criteria on morphological characters), many readily identifiable subspecies that are based on phenotypic (morphological) characters but that do not show complete mitochondrial (or phenotypic) monophyly would be ignored in conservation decisions (Zink and Dittmann 1993a, Zink et al. 2000). The single-locus genetic approach advocated by Zink (2004), although often useful when assessing historical relationships (Avice 2000, Zink and Barrowclough 2008), can be problematic when examining the uniqueness of populations that have recently diverged, have large effective population sizes, or have rapidly evolved differences adapted to varying environments (Greenberg et al. 1998, Moritz 2002, Bulgin et al. 2003).

We present a case study of Song Sparrows (*Melospiza melodia*) found in northwestern North America, a group of populations that is relatively rich in phenotypically based subspecies (Patten and Pruett 2009) and has a recent colonization history over much of its distribution (Zink and Dittmann 1993a, Fry and Zink 1998). We examined eight breeding populations of Song Sparrows, representing six subspecies, using body-mass measurements, mtDNA cytochrome-*b* sequences, and eight microsatellite loci to determine the validity of a single-locus mtDNA approach for subspecies assessment. Regarding genetic differentiation between and among subspecies that are based on phenotype, we consider how the use of different genetic markers and different methods used to analyze these data might give different answers.

METHODS

Phenotype.—Song Sparrow subspecies ($n = 6$) in northwestern North America are described and recognizable on the basis of plumage and mensural characters (Patten and Pruett 2009); the most pronounced attribute of populations in this region is the increasing body size of individuals from east

to west in Alaska (Gabrielson and Lincoln 1951). Although intraspecific plumage variation may be adaptive (Zink and Remsen 1986, Mumme et al. 2006), minor variations among populations raise legitimate questions about just how different some described subspecies really are in an evolutionary, adaptive context. Dramatic changes in body size among populations arguably provide better evidence of localized adaptive variation within a species, and it is here that we focused our measure of phenotype in this species (although some body-size differences could be the result of developmental plasticity; West-Eberhard 2003). We measured the body mass of 268 male Song Sparrows collected during the breeding season (Appendix). We grouped birds into subspecies (Patten and Pruett 2009) on the basis of collection locality and plumage, and the mean and standard error of body mass were calculated for each group. We used one-tailed *t*-tests to determine whether there were differences in body mass between neighboring subspecies (Fig. 1). We recognize that body mass alone is not a sufficient character to separate these subspecies using the established threshold criterion of the 75% rule (Amadon 1949, Patten and Unitt 2002) but consider that differences among populations in such a fundamental attribute may have important biological significance.

Population genetics.—We extracted whole genomic DNA from the tissues of 205 Song Sparrows (Glenn 1997) from eight breeding populations that correspond to six named subspecies (Fig. 1 and Table 1). We collected birds during the breeding season. We amplified most of the mtDNA cytochrome-*b* gene (1,137 bp) and cycle-sequenced amplifications using four primer pairs per individual for a subset of the extracted tissues (Table 1). We used the following primers: L14851 (Kornegay et al. 1993), H16064 (Harshman 1996), L15350 (Klicka and Zink 1997), and H15424 (Hackett 1996). We sequenced amplified products in both directions using an ABI 373A or 3100 automated sequencer (Applied Biosystems, Foster City, California). We deposited all sequences in GenBank (Table 1). We amplified and genotyped eight microsatellite loci for all individuals (Table 1) as presented in Pruett and Winker (2005). We used this data set for microsatellite analyses.

We used maximum-likelihood (PAUP*, version 4.0b10; Swofford 2003) and Bayesian analyses (MRBAYES, version 3.1.2; Huelsenbeck and Ronquist 2001) to construct phylogenetic trees. We determined the most appropriate model and

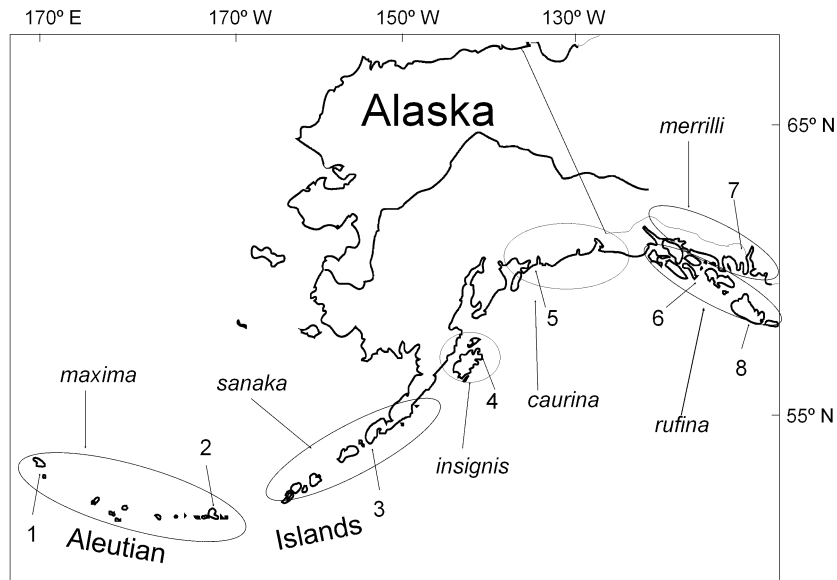


FIG. 1. Map of Song Sparrow (*Melospiza melodia*) subspecies and collection locations used in genetic data analyses. Numbers correspond to (1) Attu Island, (2) Adak Island, (3) Alaska Peninsula, (4) Kodiak Island, (5) Copper River Delta, (6) Alexander Archipelago, (7) Hyder, and (8) Queen Charlotte Islands, British Columbia.

parameter estimates for analyses using Akaike's information criterion in MODELTEST, version 3.06 (Posada and Crandall 1998) and PAUP*. We used the Hasegawa, Kishino, and Yano model (Hasegawa et al. 1985) with the shape of the gamma distribution accounting for substitution rate heterogeneity (HKY+G). We evaluated bootstrap support for the likelihood tree by resampling

the data matrix 100 times (Felsenstein 1985). For the Bayesian analysis, we used four independent runs with random starting trees to ensure that the Markov chain converged at optimal likelihood values. We sampled trees every 10,000 generations, and we ran each analysis for 8 million generations. We discarded trees sampled before the Markov chain reached a plateau as burn-in

TABLE 1. Sampling location, number of individuals sequenced, number of individuals genotyped, and GenBank accession numbers for Song Sparrows used in this study. Museum voucher numbers are provided on GenBank.

Location	Sequenced (<i>n</i>)	Genotyped (<i>n</i>)	GenBank accession numbers
Attu Island, Aleutian Islands, Alaska	10	30 ^a	AY156386–395
Adak Island, Aleutian Islands, Alaska	10	30	AY156396–405
Alaska Peninsula, Alaska	14	21 ^b	AY156406–411, 156162–165, 450608–611
Kodiak Island, Alaska	4	22	AY156166–169
Cordova, Copper River Delta, Alaska	10	30	AY156412–421
Alexander Archipelago, Alaska	5	30 ^c	AY156174–178
Hyder, Alaska	5	18	AY156161, 422–425
Queen Charlotte Islands, British Columbia	4	24	AY156170–173
Massachusetts	2	0	AY156179–180

^aIncludes individuals from Attu Island (27) and Shemya Island (3).

^bIncludes individuals from King Cove (10), Shumagin Islands (9), Unalaska Island (2), and Amak Island (3).

^cIncludes individuals from Prince of Wales Island (17), Gravina Island (8), Revillagigedo Island (2), Heceta Island (2), and Warren Island (1).

and used the remaining trees to approximate the posterior probability of the phylogeny (Huelsenbeck and Ronquist 2001). We imported trees into PAUP* and constructed a majority-rule consensus tree. We determined the posterior probabilities of clades as the percentage of occurrence of each clade among all sampled trees (Huelsenbeck and Ronquist 2001). We used Lincoln's Sparrow (*Melospiza lincolnii*; GenBank AY156181) as an outgroup in both phylogenetic analyses. We developed haplotype networks using NETWORK, version 4.5.10 (Fluxus Technology, Clare, United Kingdom; Bandelt et al. 1999) to compare with phylogenetic trees.

We performed tests for linkage disequilibrium and Hardy-Weinberg equilibrium using GDA (Lewis and Zaykin 2001). We used MICROCHECKER (van Oosterhout et al. 2004) to test for the presence of null alleles, stuttering, and large-allele drop-out. We estimated genetic distances based on the proportion of shared alleles between individuals (Bowcock et al. 1994) using MICROSAT (Minch et al. 1995). We constructed a neighbor-joining tree using individuals as operational taxonomic units (OTUs) by using the NEIGHBOR subroutine in PHYLIP, version 3.5 (Felsenstein 1993), and TREEVIEW, version 1.5 (Page 1996).

RESULTS

Body mass.—We found a pronounced east-to-west increase in individual body mass among subspecies from southeast Alaska and British Columbia to the western extreme of the species' distribution in the Near Islands of the Aleutian archipelago (Fig. 2). Going from west to east (Fig. 1), male *M. m. maxima* weighed, on average, 0.94–22.0 g ($\bar{x} [\pm \text{SE}] = 45.74 \pm 0.38$ g) more than any other subspecies (Fig. 2) but did not weigh significantly more than birds from their neighboring subspecies, *M. m. sanaka* ($P = 0.06$; $\bar{x} = 44.8 \pm 0.43$ g). *Melospiza m. sanaka* weighed, on average, 5.98 g more than the next-largest and neighboring subspecies, *M. m. insignis* ($P < 0.001$; $\bar{x} = 38.82 \pm 0.60$ g). Male *M. m. insignis* weighed, on average, 10.38 g more than male *M. m. caurina* ($P < 0.001$, $\bar{x} = 28.44 \pm 0.45$ g), and male *M. m. caurina* weighed, on average, 1.15 g more than male *M. m. rufina* ($P = 0.02$, $\bar{x} = 27.29 \pm 0.32$ g). Finally, male *rufina* weighed, on average, 3.55 g more than male *merrilli* ($P < 0.001$; $\bar{x} = 23.74 \pm 0.35$ g).

MtDNA analyses.—We found that maximum-likelihood bootstrap and Bayesian trees had similar topologies; in Figure 3, we present the maximum-likelihood phylogram, with individuals with identical haplotypes found on the same branch. We

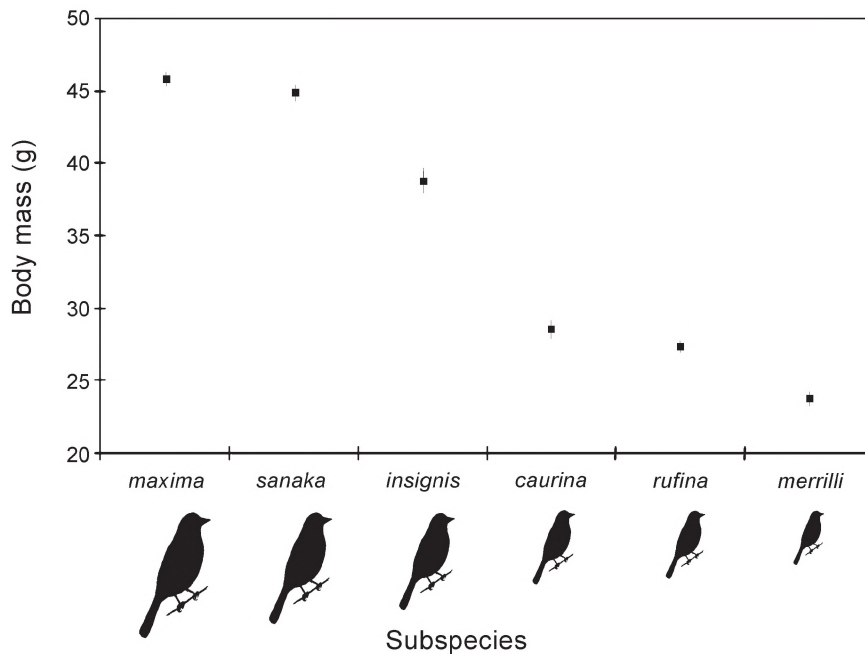


FIG. 2. Mean and standard error of body mass of each Song Sparrow subspecies.

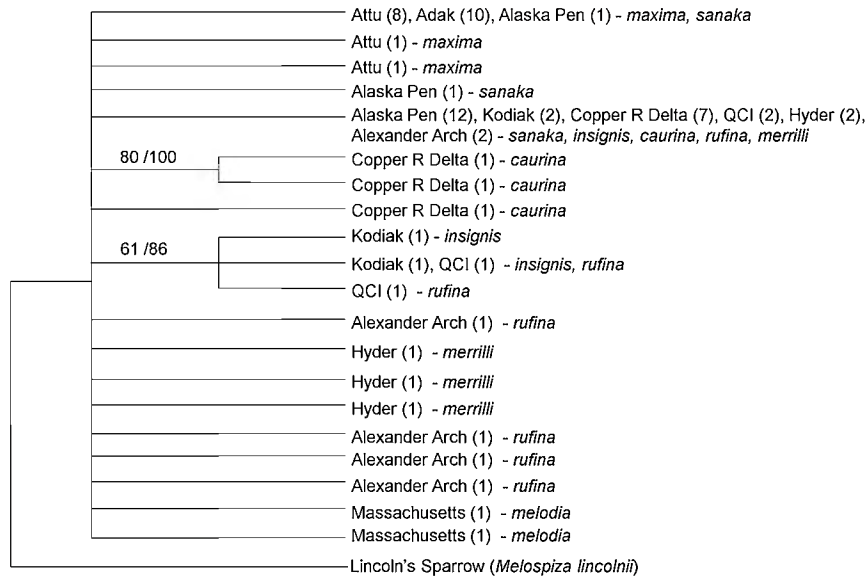


Fig. 3. Maximum-likelihood tree of cytochrome-*b* haplotypes of Song Sparrows. Bootstrap and posterior probability of nodes are presented above branches. Nodes with <50% bootstrap support were collapsed.

determined that bootstrap replicates and Bayesian posterior probabilities were similar for the branches that were moderately to strongly supported (>50% bootstrap and >90% posterior probability; Fig. 3) and that none of the subspecies or locations were reciprocally monophyletic (Fig. 3). We examined

the mtDNA haplotype network and found some structure among cytochrome-*b* sequences corresponding to at least one subspecies, *M. m. maxima*, but found that monophyly was not present (Fig. 4). We found that one individual *M. m. sanaka* had the most common haplotype for *maxima* and that the

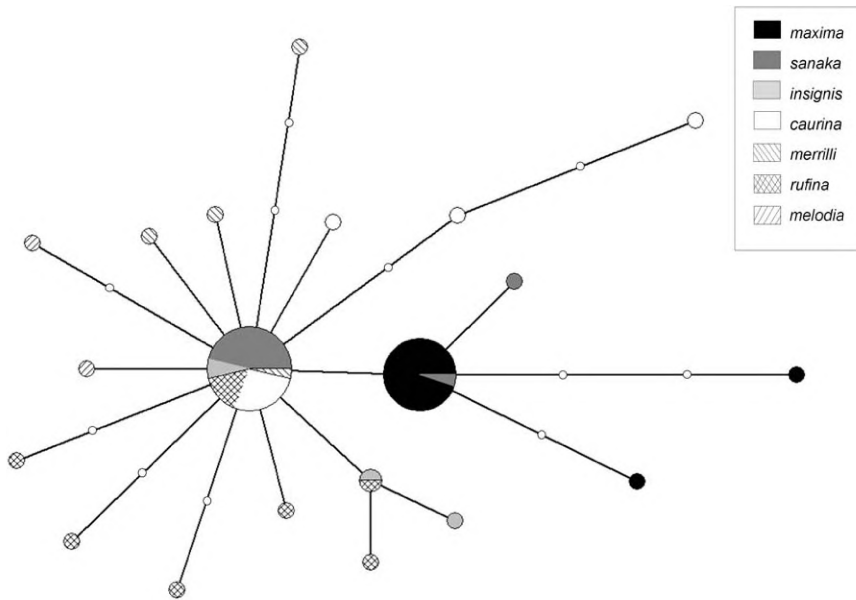


Fig. 4. Network of relationships among cytochrome-*b* haplotypes of Alaska Song Sparrows. Small white circles are missing haplotypes.

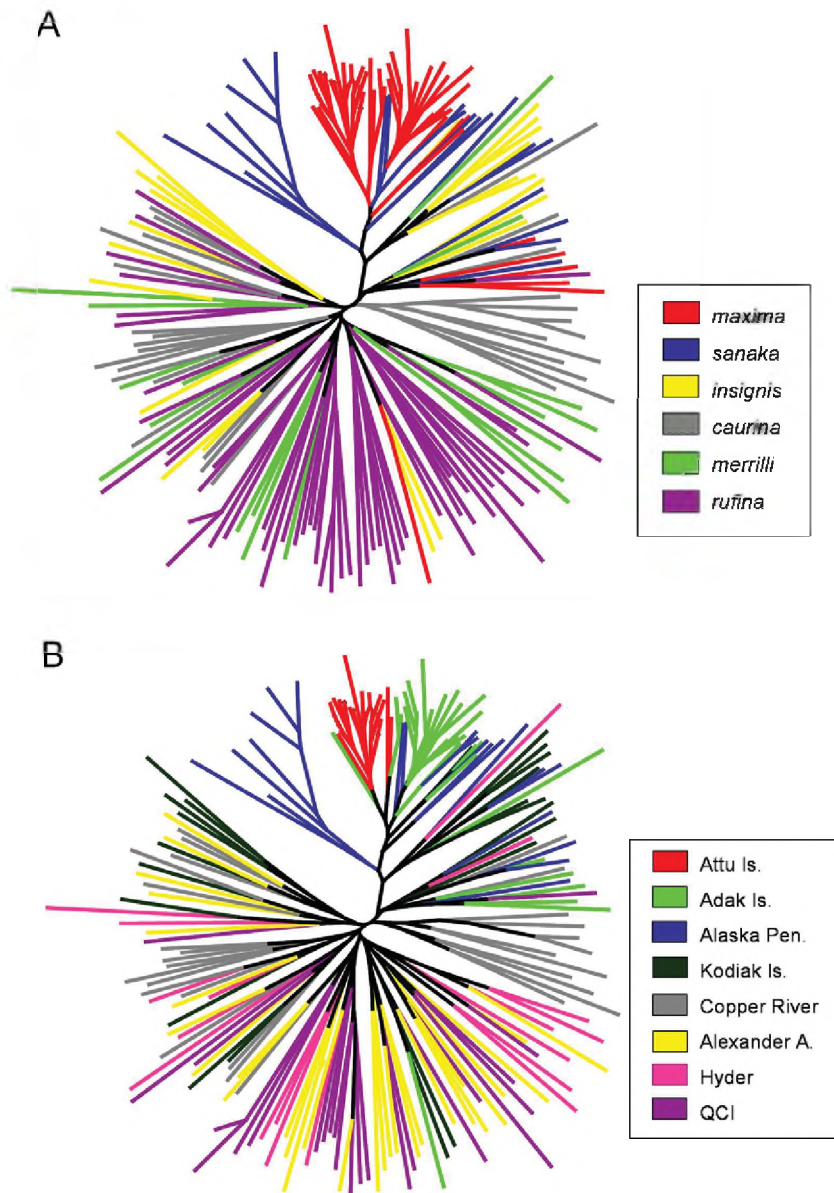


FIG. 5. Neighbor-joining trees based on the proportion of shared alleles among individual Song Sparrows genotyped at eight microsatellite loci, showing the relationships among (A) subspecies and (B) sampling locations.

most frequent haplotype in the entire data set was in individuals of all of the Alaska subspecies except *maxima* (Fig. 4).

Microsatellite analyses.—We determined that all loci were in linkage equilibrium, but two (Mme1 from Attu Island and Mme2 from Kodiak Island) were deficient in heterozygotes after adjustments for multiple comparisons (Pruett and Winker 2005). However, we found no evidence of genotyping

artifacts such as null alleles, stuttering, or large-allele drop-out.

We constructed a genetic distance tree based on the proportion of shared alleles among individuals and found clustering of locations and subspecies in several cases. Individuals from Attu Island within the subspecies *maxima* clustered together (Fig. 5). However, one grouping of the subspecies as a whole was not clearly defined (Fig. 5).

Individuals in the subspecies *caurina* were primarily in two clusters, and populations from the most western subspecies, *sanaka* and *maxima*, clustered together. Our findings match results based on genetic clustering analyses (STRUCTURE; Falush et al. 2003), wherein Pruett and Winker (2005) found nine genetic groups among northwestern Song Sparrow populations. These clusters represented each of the eight subspecies plus the population on Attu Island. We used microsatellite distance trees with individuals as OTUs to provide another way to visualize the relationships among individuals and found that there is gene flow among units but that population structure exists at several levels, even within one subspecies (e.g., *maxima*). It is noteworthy that these distance trees seem to provide substantially more information about populations and individuals than the mtDNA phylogenies.

DISCUSSION

On the basis of single-locus mtDNA criteria for diagnosing taxonomic units, Song Sparrows would be identified as a single species with no subspecies in northwestern North America, corroborating Zink and Dittmann (1993a) and Fry and Zink (1998). Yet body-mass data alone show that among-population differences are profound, representing an effective doubling of body mass from *merrilli* to *maxima* (Fig. 2). Although mass data do not enable separation of all of these subspecies using the 75% rule (Amadon 1949, Patten and Unitt 2002) and this character appears to form a step cline (Fig. 2), diagnosability using plumage characters achieves this threshold for each (not shown).

Despite subspecies-level phenotypic variation (e.g., Fig. 2), we found, on the basis of mtDNA cytochrome-*b* sequence data, a lack of reciprocal monophyly and remarkably little structure for the subspecies or populations in this region. For example, birds from the eastern portion of the United States (Massachusetts; subspecies *M. m. melodia*) are not separable from those found in the Aleutian Islands by mtDNA phylogenetic tree-building methods and are separated only by a single mutation from the most common Alaskan haplotype (Fig. 4). It is important to note that these birds are separated by >3,000 km, differ by 20–25 g in body mass, and have distinctive differences in plumage coloration (Patten and Pruett 2009). These facts alone should eliminate the possibility

that there is ongoing gene flow between these locations; thus, incomplete lineage sorting and the retention of ancestral haplotypes would appear to be the best explanation for the sharing of these haplotypes across such geographic space.

Although these findings support previous conclusions about the phylogeography of Song Sparrows (Hare and Shields 1992, Zink and Dittmann 1993a, Fry and Zink 1998), with a likely recent range expansion throughout much of North America, they reveal very little about the current subspecific status of Song Sparrows in Alaska. If we used haplotype frequency differences to diagnose subspecies, only the subspecies *maxima* differed from the remaining five subspecies (Fig. 4). In addition, researchers who used the mtDNA control region (an area thought to have the highest mutation rate within the mitochondrial genome; Baker and Marshall 1997) and mtDNA restriction fragment-length polymorphisms (RFLPs) did not find monophyly or frequency differences between Alaskan birds and those in the rest of North America (Hare and Shields 1992, Zink and Dittmann 1993a, Fry and Zink 1998). Thus, the mutation rate of mtDNA appears to be too slow to provide a useful measure of current isolation (if we consider only complete or near-complete lineage sorting). In this case, this locus provides little or no information about adaptive variation among these populations, despite small current population sizes and evidence of historical population reductions (Pruett and Winker 2005).

Studies that have used rapidly mutating genetic markers (i.e., microsatellites; Goldstein and Schlötterer 1999) have found accurate assessments of recent isolation and current gene flow (Cornuet et al. 1999, Johnson et al. 2003, Berry et al. 2004, Paetkau et al. 2004, Underwood et al. 2007) that might provide a means of inferring population history (Sun et al. 2009). Although the fixation of neutral nuclear alleles through drift is not likely in a short period (Zink and Barrowclough 2008), higher mutation rates in microsatellites lead to greater allelic variation, enabling shifts in allele frequencies to be readily observable and, thus, informative in understanding whether groups are diagnosably different. Genetic analyses based on microsatellites show very limited or nonexistent gene flow among many of these populations and subspecies (Pruett et al. 2008a, b), supporting the idea that differences in plumage color, body size, and mass are found in isolated populations that are on independent evolutionary paths. In addition,

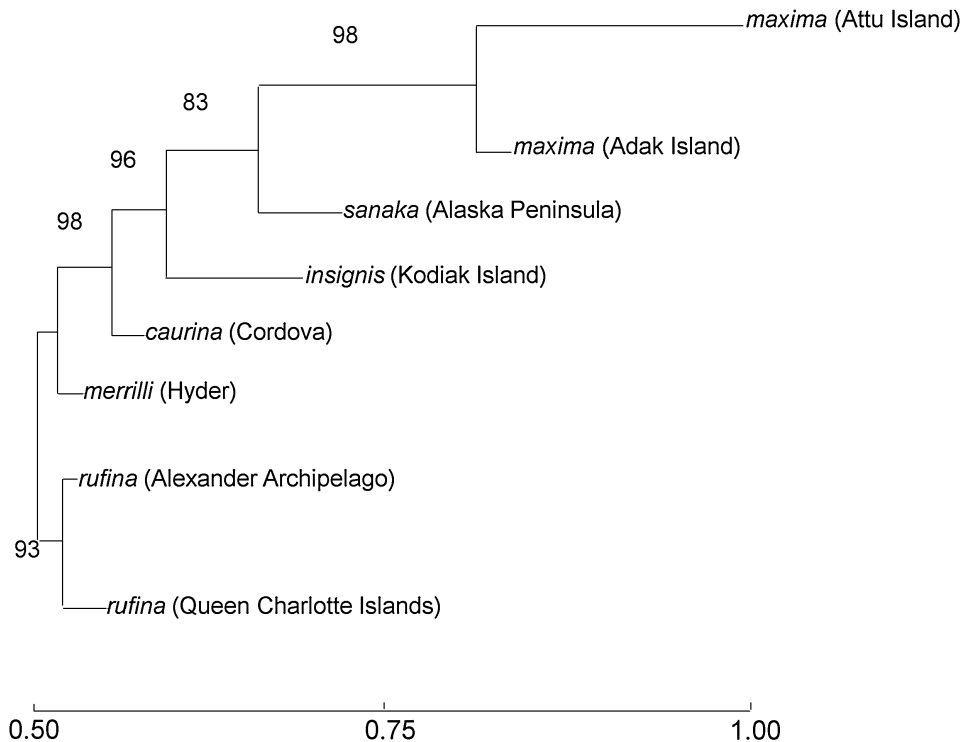


FIG. 6. Neighbor-joining tree based on population allele frequencies, based on tree presented by Pruett and Winker (2005).

a genetic distance tree based on population-level differences in allele frequencies shows grouping of populations that are the same subspecies, with these nodes having high bootstrap support (Fig. 6). These relationships are also revealed using clustering analyses (Pruett and Winker 2005, Pruett et al. 2008a) such as STRUCTURE (Falush et al. 2003) and, to some extent, in the individual-based microsatellite genotype distance trees (Fig. 5). These findings support the traditional subspecies assessed using phenotypic differences that are readily observable in this group (e.g., Fig. 2; Patten and Pruett 2009). These results indicate that many Song Sparrow subspecies likely represent units on different evolutionary trajectories. Yet, in part because gene flow is ongoing (albeit at very low levels in some cases; Pruett 2002; Pruett and Winker 2005; Pruett et al. 2008a, b), these units are not full species, but rather quintessential subspecies (Fig. 5).

The northwestern Song Sparrow subspecies we examined are morphologically and genetically distinctive, just not diagnosable (e.g., reciprocally monophyletic) using mtDNA sequences.

Should wildlife managers and conservation biologists ignore such clear and striking differences because of the recent timing of isolation? If so, then we likely put too much emphasis (1) on the timing of population-level divergences and (2) on the stochastic aspects of genetic drift in relation to effective population size occurring during divergence, rather than taking into account the effects of current isolation and ongoing adaptive evolution (Moritz 2002). For these reasons and others (such as sampling error, neutral or near-neutral aspects of contemporary genetic data, and the importance of gene flow), we conclude that mtDNA data cannot be considered definitive (whether one is using monophyly or distance thresholds) for biodiversity assessment (Winker 2009).

The problem of using a single genetic measure to identify units, as shown clearly here with Song Sparrows, is likely to occur in other morphologically diverse taxa found in temperate and high-latitude areas. Many novel environments have opened for colonization within the past 10,000 years (Pielou 1991), and it is not surprising that birds moved into these areas and evolved unique

adaptations to new environments. These populations, often identified as subspecies, represent a large amount of the biodiversity found in non-tropical areas and should be appropriately managed and conserved. For example, Song Sparrows found on islands contain a large proportion of the unique alleles found in this group, and these island populations are also differentiated morphologically and genetically (Wilson et al. 2009). They also exhibit differences in behavior, in that some populations are at least partially migratory and others are sedentary (Pruett et al. 2008b). Thus, several northwestern Song Sparrow populations (e.g., Attu Island birds) should be managed as unique evolutionary units (e.g., Pruett et al. 2008a).

Phylogeography and its workhorse mtDNA sequence data do not provide a complete view of evolutionary genetics, nor does this approach provide the complete toolkit required to understand the distribution of genetic variation in time and space. Microsatellites are also imperfect, but they can provide important indicators of evolutionary divergence on shorter time-scales. And because adaptive evolution can occur fairly rapidly in some populations (e.g., Clegg et al. 2008),

reliance on a single-locus approach or a single analytic methodology risks overlooking or inadvertently minimizing important population-level differentiation. We advocate using multiple genetic markers in addition to phenotypic characters to determine taxonomic and management units. (For a case in which a Song Sparrow subspecies was found to be invalid using these criteria, see Pruett et al. 2004; for a case in which morphology alone was insufficient to describe unique populations, see Pruett et al. 2008a). Without examining additional genetic markers in groups identified by phenotypic characters, we would overlook much of the important biodiversity found in Song Sparrows.

ACKNOWLEDGMENTS

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APPENDIX. Song Sparrows (*Melospiza melodia*) in the collections of the University of Alaska Museum, Fairbanks (UAM), used for body-mass measurements in this study.

Subspecies	Collection location	UAM catalogue numbers
<i>M. m. maxima</i>	Alaska, Aleutian Is., Adak Is.	8461, 10040, 10041, 10165, 10170, 10179, 10185, 10186, 10188, 10942, 10946, 10948, 11175–11178, 11229, 11267, 11268, 11280, 11511, 11557–11562, 11827, 12143, 13059, 13186, 13289, 13290, 14611, 15298, 15305, 15307, 15312, 15315, 15319, 15321, 15326, 15339–15341, 15344, 15353, 15366, 15367, 15369
	Alaska, Aleutian Is., Amlia Is.	11289–11297, 13160
	Alaska, Aleutian Is., Attu Is.	7223–7225, 7228, 7650, 7651, 8091, 8092, 8097, 8099, 8100, 8102, 8107, 8127, 8128, 8308, 8416, 8418, 8462, 8463, 8609, 8611, 8612, 8774, 8775, 9302–9305, 10072, 10073, 11173, 11174, 11224–11228, 11242, 11270, 11277, 11556, 11790, 11828, 11846–11849, 12094, 12141, 13056, 13140, 14165, 15115–15117, 15299, 15301, 15302, 15304, 15313, 15320, 15325, 15330, 15331, 15342, 15343, 15346, 15352, 15354, 15356, 15358, 15363, 15378, 19345, 19346, 19351, 20518,
	Alaska, Aleutian Is., Buldir Is.	8781
	Alaska, Aleutian Is., Igitkin Is.	15323
	Alaska, Aleutian Is., Kanaga Is.	8779
	Alaska, Aleutian Is., Kasatochi Is.	13173
	Alaska, Aleutian Is., Semisopchnoi Is.	9602
	Alaska, Aleutian Is., Shemya Is.	9418, 10944, 11171, 11243, 11244, 11363, 11825, 13128, 19048
	Alaska, Aleutian Is., Tanaga Is.	15318
<i>M. m. sanaka</i>	Alaska, Alaska Peninsula, King Cove	10091, 11362, 11365, 11366, 11381, 11389, 11823
	Alaska, Aleutian Is., Bogoslof Is.	11236–11238
	Alaska, Aleutian Is., Ugamak Is.	15334, 15335, 15384, 18514
	Alaska, Aleutian Is., Samalga Is.	15374, 15377
<i>M. m. insignis</i>	Alaska, Shumagin Is., Popof Is.	10171, 10187, 11276, 11379, 11390, 12142
	Alaska, Kodiak Archipelago, Kodiak Is.	7522, 8776, 8777, 8807, 11871, 12139, 14001–14003, 14009, 14010
<i>M. m. caurina</i>	Alaska, Copper River Delta	8922, 10652, 11046, 11101, 11103, 11104, 11141, 11180, 11182, 11210, 11223, 11231, 11234, 11272, 11361, 11382–11384
<i>M. m. rufina</i>	Alaska, Alexander Archipelago, Gravina Is.	13438, 13886, 13887, 13941, 13944, 15426, 15433, 17575
	Alaska, Alexander Archipelago, Heceta Is.	13241, 13288
	Alaska, Alexander Archipelago, Prince of Wales Is.	11712, 11824, 12455, 13463, 13912, 13913, 13915, 13916, 13936–13938, 13943, 13945, 13952, 13953, 14658, 14714, 14942–14944, 15086, 15087, 15193
	Alaska, Alexander Archipelago, Revillagigedo Is.	11516, 11517
	British Columbia, Queen Charlotte Is., Graham Is.	11179, 11544, 11546–11550, 11552, 11553, 13079, 13080, 13908–13910, 13919, 13920
<i>M. m. merrilli</i>	Alaska, Hyder	7341–7343, 7346, 8115, 8379, 8447, 8449, 8607, 10159, 13921, 15338, 18103