Population differentiation and conservation of song sparrows (*Melospiza melodia*) in the San Francisco Bay region inferred by morphological and microsatellite loci analysis

By

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ABSTRACT

In this study I examined geographic variation in morphology and genetic population structure in five putative subspecies of song sparrows (*Melospiza melodia*) in the San Francisco Bay region (*M. m. samuelis, M. m. maxillaris, M. m. pusillula, M. m. gouldii,* and *M. m. heermanni*). My first goal was to describe genetic population structure at microsatellite loci to assist with conservation and management strategies for song sparrow populations in the San Francisco Bay Area. I sampled nine populations from five putative subspecies and found low estimates of differentiation between populations within subspecies (Fst analog: $\Phi sc = 0.0122$, $p<0.0001$, Rst analog $\Phi sc = 0.00433$, $p=0.05963$) and between subspecies (Fst analog: $\Phi ct = 0.0137$, $p = 0.04985$, Rst analog $\Phi ct = 0.0174$, $p=0.09873$) at microsatellite loci. Despite low estimates of divergence, genetic structure at the subspecies level was indicated by the larger amount of variance accounted for by subspecies than populations. I propose a Management Unit (MU) consisting of the range of *M. m. pusillula* be prioritized for conservation efforts based on the larger extent of genetic divergence shown by Cavalli-Sforza and Edward's chord distance and topology of the unweighted pair group cluster analysis which displayed 100% support of bootstrap replicates across loci. Additionally, I propose the ranges of *M. m. samuelis* and *M. m. maxillaris* be designated an MU despite low differentiation from *M. m. heermanni*, because it remains possible that adaptive differences between these types were not identified with neutral loci. The second goal of this study was to compare morphological and genetic estimates of divergence in order to evaluate previous hypotheses proposed for differentiation. Fourteen populations were included in a multivariate analysis of morphological traits and compared with the genetic differentiation derived from microsatellite loci analysis in Chapter 1. In contrast to the low genetic differentiation at microsatellite loci, morphological differentiation was high between song sparrow subspecies. Due to the lack of concordance between estimates of morphological and genetic divergence, selection or phenotypic plasticity in morphology are implicated as causes for morphological differentiation among song sparrow subspecies. It is probable that song sparrow subspecies in the San Francisco Bay region are recently diverged or have high current gene flow and, therefore, that the rate of evolution at morphological traits (assuming a heritable basis for those traits) is faster than at neutral loci.

Keywords: *Melospiza melodia*, song sparrows, conservation genetics, geographic variation, population differentiation, microsatellites, AMOVA, analysis of molecular variance.

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CHAPTER 1

Analysis of variation at microsatellite loci in song sparrows (*Melospiza melodia*) of the San Francisco Bay region: Implications for conservation

1.1 INTRODUCTION

The deterioration, fragmentation, and loss of natural environments has led conservationists to protect and restore areas that remain relatively intact and harbour high biodiversity. Although the importance of ecological and demographic factors in conserving biodiversity have been emphasized (Caughley 1994; Lande 1988), genetic factors also play a role. Surveys of genetic variation allow quantification of the extent and distribution of genetic variability crucial to recognizing and maintaining biodiversity (Moritz 1994a; Moritz et al. 1996). Genetic surveys also provide insight into the evolutionary processes that generate biodiversity (Smith and Wayne 1996) and aid in identifying unique populations with distinct evolutionary potential (Moritz 1994b). Genetic studies may also complement ecological and demographic studies related to population viability. Loss of genetic variability has been linked to declines in fitness (Bouzat et al. 1998), and the preservation of genetic variability may help species adapt to novel conditions (Lande and Shannon 1996).

I examined genetic variation and population structure within and between five subspecies of song sparrow (*Melospiza melodia*) in the San Francisco Bay region. This region, 70 by 100 kilometres in size, has several phenotypically distinct year-round resident subspecies of song sparrow described. Three endemic subspecies are found in tidal salt marshes, each restricted to

one of three sub-bays of the greater San Francisco Bay (*M. m. samuelis* referred to as *samuelis*; *M. m. maxillaris*, *maxillaris*; *M. m. pusillula*, *pusillula*). The Marin song sparrow (*M. m. gouldii; gouldii*) occupies the uplands surrounding these bays (Grinnell and Miller 1944) and the Modesto song sparrow (*M. m. heermanni, heermanni*) occupies riparian habitats adjacent to the east (Figure 1.1). Due to diking, landfilling, and conversion to salt evaporation ponds (Walton 1978), less than 15% of the original tidal salt marsh in San Francisco Bay remains (Marshall and Dedrick 1994) and what is left is highly fragmented (Walton 1978). This dramatic decrease in habitat size may threaten the long-term persistence of the tidal marsh song sparrow populations. Although the song sparrows species as a whole is widespread and common, *samuelis*, *maxillaris*, and *pusillula* have been recognized in the California Natural Diversity Database (CNDDB) as endangered subspecies; ones in which survival and reproduction are in immediate jeopardy. In addition, these three subspecies are federally and state listed as Special Concern Species, in reference to the possibility of declining populations levels, limited range, and other threats that may make them vulnerable to extinction.

Previous research on song sparrows has found marked patterns of geographic structure in plumage and morphology but encountered difficulty detecting structured variation in other traits. Marshall (1948b) studied over 2,000 study skins of song sparrows from the San Francisco Bay region and described well-ordered geographic structure in plumage and morphology. However, Mulligan (1963) studied song variation in tidal marsh song sparrows and found that each bird had a unique repertoire of songs and that variation among birds was so great that differences between subspecies could not be resolved (Mulligan 1963). Ferrell (1966) used variation in erythrocyte antigen frequencies and also found variation among populations to be more than

twice that observed among subspecies. Variation between subspecies was explained largely by variation between populations (Ferrell 1966). In addition, studies on song sparrows across North America using molecular methods have had difficulty finding concordance between genetic variation and subspecific designations based mainly on morphology. In particular, extensive studies using variation in mitochondrial DNA (mtDNA) revealed no clear patterns between variation in haplotypes and morphology (Hare and Shields 1992; Zink and Dittman 1993; Zink and Blackwell 1996; Fry and Zink 1998).

In this study I used hypervariable tandem repeat nuclear loci (microsatellites) to study differentiation in song sparrows under the assumption that their high mutation rates and large numbers of alleles (Goldstein et al. 1995) might provide more sensitive estimates of divergence than mtDNA. Microsatellites have been found to be more appropriate for detecting fine scale structuring than other molecular markers in polar bears (*Ursus maritimus*; Paetkau et al. 1995), brown trout (*Salmo trutta*; Estoup et al. 1998), and introduced fire ants (*Solenopsis invicta*; Ross et al. 1999). In birds, hypervariable markers have been shown to be effective for detecting fine scale structure in red grouse (*Lagopus lagopus scoticus;* Piertney et al. 1998) and savannah sparrows (*Passerculus sandwichensis;* Freeman-Gallant 1996).

Clarifying the amount of differentiation and the degree of genetic structure among song sparrow populations and subspecies would aid in determining conservation units and appropriate management actions. In particular, since the upland subspecies are not threatened and the tidal marsh subspecies are, I was interested in genetic differentiation between populations in the two habitats. This study was designed such that two populations within each tidal marsh subspecies

were sampled and compared to an upland population nearby (Figure 1.1). First, I asked whether the upland song sparrow populations (*gouldii* and *heermanni*) were different in microsatellite allele frequencies from tidal marsh song sparrow populations (*samuelis, maxillaris,* and *pusillula*). Second, I asked if there were differences in allele frequency between tidal marsh populations. Also, to clarify the existing subspecific designations on which current conservation status is based, I examined the differentiation and extent of genetic structure that could be accounted for by subspecific designations. I did this by partitioning the genetic variance among subspecies, among populations within subspecies, and within populations. Finally, I examined genetic relationships between populations to identify populations that are genetically divergent and of unique conservation value, and to identify groups of populations that may be considered conservation units.

1.2 MATERIALS AND METHODS

Birds were sampled March-May, 1999, during the breeding season from tidal salt marshes and surrounding freshwater riparian areas in the San Francisco Bay region (Table 1.1, Figure 1.1). Adults were captured in mist nets, measured, sampled for blood, and released. Twelve blood samples were also taken from nestlings (one per nest) at Petaluma Marsh (PM). I sampled a total of 215 birds from nine different populations, two populations from each of the three tidal marsh subspecies, and three from the upland subspecies.

The tidal marsh subspecies sampled were the Samuel's song sparrow (*samuelis)* found in San Pablo Bay, the Suisun song sparrow (*maxillaris)* in Suisun Bay, and the Alameda song sparrow

(*pusillula*) in South San Francisco Bay. The corresponding freshwater upland populations sampled for each tidal marsh subspecies were the Marin song sparrow (*gouldii*) from the uplands surrounding San Francisco Bay and the Modesto Song Sparrow (*M. m. heermanni*; *heermanni*) whose range borders *maxillaris* to the east and inhabits freshwater riparian areas of northern California's Central Valley (Grinnell and Miller 1944; Marshall 1948b) (Figure 1.1).

DNA extraction and microsatellite amplification

Blood was collected from the brachial vein in heparinized capillary tubes after puncturing with a 27G ½ needle. Blood was then transferred to 1 ml of 1X lysis buffer (Applied Biosystems Division of Perkin Elmer, Inc., Foster City, CA; ABI) and stored at 4° C. DNA was extracted using standard phenol/chloroform extraction methods. Twenty-five microlitres of blood was added to 233 ul of extraction buffer (1X TNE, 1M Tris-HCl, Proteinase K, 25% SDS) and incubated overnight at 37° C. The next day, 150 ul of 6M NaCl was added and the solution was centrifuged at 3000 rpm. The supernatant was collected and remaining pellet discarded. This was followed by a phenol/chloroform (1:1) extraction and spin at 3000 rpm and 4° C. The second extraction consisted of just chloroform followed by a spin at 3000 rpm and 4° C. The DNA was then precipitated using 700 ul of 100% ethanol, the DNA pellet was washed with –20° C 70% ethanol, dried, and resuspended in 50ul of 1X TE buffer (10 mM Tris, 1mM EDTA, pH 7.4).

Nine microsatellite loci were amplified with the primers listed in Table 1.2. MME 1, MME 3, MME 7, MME 12, ESCU 1, GF 2.35, and PSAP 335 were amplified and sized at the University of Wisconsin, Madison. PCR reactions were carried out separately for each locus and consisted

of approximately 25 ng of template DNA combined with 1X PCR reaction buffer (500mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0 % Triton X-100), 0.2 mM dNTPs, 1.5-2.0 mM $MgCl_2$, 0.2 uM forward and reverse primer, 0.75-1.5 units of Promega *Taq* DNA polymerase, and water to a total reaction volume of 25-50 ul. Thermocycling conditions were 94 ^{0}C for 1 minute, annealing temperature for 1 minute, 72 ^{0}C for 1 minute, repeated 35 times. Annealing temperatures and magnesium concentrations for each locus are listed in Table 1.2. The forward primers were fluoro-labelled at the 5' end with various fluorescent labels (HEX, 6-FAM, or TAMRA) depending on the loci. PCR products were analyzed on an Applied Biosystems model 373A automated sequencer. PCR products from multiple loci were combined in each lane with an internal sized ROX standard (ABI) and resolved on a 6% polyacrylamide gel, 0.4 mm thick. Up to 4 Loci (total of 2 ul PCR products) were combined in each lane so and PCR products separated based on the colour and size. Results were analyzed using the GENESCAN (version 1.1) software and viewed using GENOTYPER (version 2.0; ABI). The ROX standard was used to accurately size the length of the alleles therefore the genotype of the two alleles reflects the size in base pairs.

MME 2 was amplified and sized in the Genetic Data Centre, Department of Forest Sciences, University of British Columbia. PCR reactions consisted of approximately 20 ng of template DNA combined with 1X PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, pH 8.3), 0.2 mM dNTP's, 0.05 uM forward and reverse primer, 0.03 uM M13 dye-labelled primer, and 1.0 units of Roche *Taq* DNA polymerase. The forward primer was synthesized with an M13 tail for product labelling during the PCR reaction with the M13 dye-labelled primer. Thermocycling conditions for MME 2 consisted of touchdown PCR with five cycles each of an

annealing temperature of 58 ^{0}C, 57 ^{0}C, 56 ^{0}C, and 20 cycles of 53 ^{0}C. PCR products were run on a Licor 4200 DNA analyzer, 7% polyacrylamide gel, 0.4 mm thick. Known alleles that were sized and run in Madison were combined into an "allelic ladder" which was run every ten sample lanes to facilitate sizing of alleles on the Licor. Therefore, allele length represents the number of base pairs comparable to the Madison genotypes.

MME 3 and MME 7 are Z-linked, therefore females are hemizygous and appear as homozygotes. In order to include MME 3 and MME 7 in the analyses, females were coded as "missing data" for the second allele and included with the remaining loci in all subsequent analyses. The remaining loci are inherited in Mendelian fashion (Jeffery et al. 2000).

Hardy-Weinberg Equilibrium and Linkage Disequilibrium

Nei's (1978) unbiased estimate for expected heterozygosity and observed heterozygosity were calculated using BIOSYS-2 (Swofford et al. 1997), step VARIAB. The program GENEPOP Version 3.1d (updated from version 1.2; Raymond and Rousset 1995b) was used to estimate allele frequencies, test for departures from Hardy-Weinberg equilibrium (HWE), and test for linkage disequilibrium. Testing for HWE in microsatellite data sets aids in detecting nonamplifying alleles (Paetkau et al. 1997) and internal genetic structure, which would result in a Wahlund effect (Hartl and Clark 1997). In a previous study Jeffery et al (2000) observed allelic dropout at MME 2 and MME 12 caused by three phenomena: 1) dropout related to DNA concentration; 2) dropout related to DNA extraction; 3) dropout due to unknown reasons. GENEPOP Version 3.1d employs a Markov chain method to estimate p-values for departure from HWE using the method of Guo and Thompson (1992) and Fisher's exact test to test the null

hypothesis that genotypes at two different loci were independent of one another (Raymond and Rousset 1995b). Exact tests are most appropriate for hypervariable markers such as microsatellite loci because they are appropriate even when large numbers of rare alleles are present (see Rousset and Raymond 1995 and reference therein). Significance of multiple P-values were combined using Fisher's method.

Genetic population and subspecific structure

Heterogeneity of allele frequencies between population pairs was examined using the Fisher exact test as described by Raymond and Rousset (1995a; GENEPOP Version 3.1d) with significance of multiple p-values adjusted by a sequential Bonferroni correction (Rice 1989). The null hypothesis tested is that the distribution of alleles across populations is homogeneous. The degree of population differentiation between subspecies and between populations was also examined by partitioning of genetic variance in an analysis of variance framework (Weir and Cockerham 1984) using Arlequin version 2.000 (Schneider et al. 2000). Two sets of statistics were calculated, Φ - statistics that employed differences in allele frequencies only (analogous to F-statistics) and Φ - statistics (analogous to R-statistics; Slatkin 1995) which used an analysis of molecular variance (AMOVA) and accounted for variance in size between pairs of alleles (Excoffier et al. 1992). Estimation of overall population differentiation was calculated as Fst and Rst. A hierarchical model was then used to partition variation into three components: "within populations" (Φ_{ST}), "among populations/within groups" (Φ_{SC}), and "among groups" (Φ_{CT}) (Excoffier et al. 1992). A permutation approach is used to test the significance of the variance components and Φ - statistics (Excoffier et al. 1992). In this study, groups represented the

putative subspecies (localities in parentheses, abbreviations listed in Table 1.1); *gouldii* (MM, LG), *pusillula* (DM, PB), *samuelis* (PM, SC), and *pusillula* (DM, PB).

Because of their high polymorphism, microsatellites can give a downward-biased estimate of Fst (Hedrick 1999). Measures that are not biased by polymorphism include the rare alleles method of estimating Nm (Barton and Slatkin 1986) and genetic assignment tests (Cornuet et al. 1999). Therefore, differentiation between subspecies was also estimated by calculating the effective number of migrants (Nm) by the private alleles method (GENEPOP version 3.1d; Barton and Slatkin 1986). Additionally, an assignment test using GENECLASS (Piry and Cornuet 1999) was used to assign individuals to the subspecies where their genotype is most likely to occur following the likelihood approach of Paetkau et al. (1995).

Genetic divergence

Genetic divergence among populations was estimated using Dce, Cavalli-Sforza and Edward's chord distance (1967). Dce was calculated using the GENEDIST program in PHYLIP version 3.57c (Felsenstein 1995). Takezaki and Nei (1996) advised that correct tree topology was more likely to be obtained using a distance measure independent of mutation models. In addition, Dce has a lower sampling error and makes no assumptions about constant population size or mutation rates among loci (Takezaki and Nei 1996). The magnitude of this distance is not proportional to evolutionary time, however, it has been found to resolve close relationships more accurately (Angers and Bernatchez 1998; Paetkau et al. 1997; Takezaki and Nei 1996). Unweighted pair group cluster analysis (UPGMA; program NEIGHBOR) was used to construct a tree of relationships between populations for comparison to morphological relationships. Loci were

bootstrapped using BIOSYS-2 (Swofford et al. 1997) and consensus tree of 100 distance matrices were combined using NEIGHBOR and CONSENSE in PHYLIP version 3.57c (Felsenstein 1995). The bootstrapped consensus tree was rooted with the Pacific Northwest subspecies *M. m. morphna* (site Burn's Bog (BB), British Columbia, Canada) and only used 8 loci (i.e. it did not include Psap 335). As an alternative way to visualize the relationships between the populations, I performed a principal components analysis on the allele frequencies using PCA-GEN version 1.2 (Goudet 1999). The principal components analysis used a subset of the genetic data used in the previous analyses (e.g. analyses did not include MME 3 or MME 7) due to the computer programs' inability to incorporate individuals with missing data.

1.3 RESULTS

San Francisco Bay populations of song sparrows showed substantial variation at all microsatellite loci with the number of alleles per locus ranging from 10 (MME 3) to 27 (GF 235), and an average of 17 alleles per locus (Table 1.3). Average heterozygosity was high and ranged from 0.762-0.846 (Table 1.3). Allelic drop-out was observed in a previous study at MME 2 and MME 12. In the present study, significant deviations from HWE were detected at GF 235 (combined probability over all populations using Fisher's method = 0.0032, Chi-square = 38.6 18 df). This deviation was the result of a significant heterozygote deficiency ($p<0.0001$, S.D.= 0.0000, score test). The cause of this heterozygote deficiency is unknown. However, GF 235 showed some differential amplification of alleles, with larger alleles amplifying less well, and this could have caused some mis-scoring of heterozygotes as homozygotes. Nevertheless, the decision to include GF 235 had little effect on the significance of the analyses. Two populations

also showed a significant deficiency of heterozygotes across all loci, RR (p=0.0233, S.D. 0.0061) and SC (p=0.0276, S.D. 0.0064; Fisher's exact test). Significant linkage disequilibrium was detected in 3 out of 36 possible pairwise loci comparisons, which is about as expected by chance.

Genetic subspecific and population structure

Heterogeneity in allele frequencies across all loci was detected among all but five population comparisons after sequential Bonferroni correction. Population comparisons not significantly differentiated from each other included pairwise comparisons of populations of *samuelis, maxillaris,* and *heermanni,* which comprise collectively Northern San Francisco Bay and CO from California's central valley (e.g. CO & PM, CO & SC, GS & RR, PM & SC, RR & SC).

Differentiation was weak between populations based on estimates of population variance to the total variance, but statistically significant (Fst = 0.02288, $p<0.0001$; Rst = 0.02757, $p<0.0001$), as was differentiation among subspecies (Table 1.4). Additionally, differences between subspecies accounted for a larger percentage of the total variance in allele frequency than differences between populations among subspecies, especially for the Rst analog statistics (Rst: among subspecies = 1.88%, p = 0.0987, among populations within subspecies = 0.28 %, p = 0.000; Fst: among subspecies = 1.38%, p = 0.0499, among populations within subspecies = 1.18 %, p = 0.0596). Arlequin calculates Φ-statistics, analogous to Cockerham's F-statistics (Cockerham 1969; Cockerham 1973). Values for Φ_{CT} (the between subspecies measurement of differentiation) were 0.0137 for the Fst analog (significant at $p<0.05$), and 0.0174 for the Rst analog. Φ_{SC} (the between populations among subspecies measurement of differentiation) was 0.0122 for the Fst analog and 0.00433 for the Rst analog. Importantly, the amount of variance

accounted for by subspecies compared with the total variation was greater than 50% (Φct / (Φct + Φsc) = 0.53).

I also calculated two estimates of differentiation that are not biased by high polymorphism, the private alleles estimate of Nm and a genetic assignment test. These estimates indicated high gene flow despite evidence of genetic structure. The private alleles method, corrected for population size, gave an average estimate of 7.78 immigrants per generation between populations. However, the assignment test correctly assigned individuals to their presumptive subspecies 60.19% of the time (comparing 5 groups, Table 1.5). Notably, individuals from *pusillula* were assigned to the correct population 83.9% of the time.

Genetic divergence

Pairwise comparisons in divergence based on Dce showed relatively higher genetic distances for pairwise comparisons including *pusillula* than for other putative subspecies. The greater divergence of *pusillula* was emphasized by the topology of the population UPGMA tree calculated from the pairwise Dce matrix which showed two major groups, a *pusillula* group, and one that contained all the others (Table 1.6, Figure 1.2). The branch separating *pusillula* from the other populations was supported by 100% of bootstrap replicates. The distinctness of *pusillula* was also confirmed by a principal components analysis of allele frequencies and emphasized by its differentiation from the closest upland population (LG), which grouped with the remaining upland subspecies.

1.4 DISCUSSION

This study corroborates mtDNA studies that have failed to detect marked genetic differentiation between morphologically distinct subspecies of song sparrow (Hare and Shields 1992; Zink and Dittman 1993; Zink and Blackwell 1996; Fry and Zink 1998). My analysis of genetic variation at hypervariable loci in song sparrows also indicated low differentiation between populations in the San Francisco Bay region. However, low fixation indices can be expected with highly polymorphic loci that have large numbers of alleles and high heterozygosities (Hedrick 1999). High polymorphism can result in downward-biased estimates of Fst given the same amount of divergence (Charlesworth 1998) by as much as an order of magnitude assessed by less variable markers (Hedrick 1999). Despite this expectation, another method of estimating differentiation not biased by high polymorphism, the private alleles method of estimate Nm, also indicated low differentiation with gene flow of seven migrants per generation. Slatkin (1987) estimated that only for values of $Nm < 1$ will genetic drift result in fixation.

Birds generally display low estimates of divergence compared with other vertebrate classes (Barrowclough 1983). Barrowclough (1983) found the mean Fst among populations within species for birds to be 0.02 (using allozyme loci), in comparison to other non-avian vertebrate classes wherein Fst ranged from 0.11-0.38. The extent of differentiation found within song sparrows in the San Francisco Bay region ($Fst = 0.02288$, $p<0.0001$; $Rst = 0.02757$, $p<0.0001$) was therefore similar to other estimates for birds. In addition, the level of differentiation that I found is of the same order of magnitude as those found within some species of marine fish (mean $Fst = 0.062$; Waples 1998). However, the level of differentiation found in this study is lower

than in red grouse (*Lagopus lagopus scoticus*, Rst = 0.157; Piertney et al. 1998), another study that considered a similar geographic scale and used microsatellites.

Despite the low level of genetic differentiation, I obtained important information concerning delineation of conservation units. First, I wanted to determine if upland populations were differentiated from tidal marsh populations. Microsatellite allele frequencies were significantly different between the upland populations of *gouldii* and the tidal marsh populations. Second, I examined differentiation among the tidal marsh populations and found significant differences in allele frequencies between *pusillula* and the northern bay tidal marsh populations (*maxillaris* and *samuelis*). Third, I estimated the amount of genetic structure attributable to subspecific designations. Evidence of genetic structure among putative subspecies was found. The amount of microsatellite variation (1-2 %) occurring within subspecies was significantly greater than zero. Moreover, the amount of allelic variance accounted for by subspecies comprised more than 50% of the among group variance measured ($\Phi ct / (\Phi ct + \Phi sc) = 0.53$). Additionally, the assignment test was moderately successful and assigned 60% of individuals to subspecies. Finally, the UPGMA dendrogram constructed from Dce showed separation of *pusillula* and the remaining populations. Comparisons of the heterogeneity in allele frequency between populations indicated significant differences between *pusillula* and *gouldii*, and between *pusillula* and a combined *samuelis-maxillaris-heermanni* group. This suggests that *pusillula* has diverged significantly from sister taxa in the region. Although the other song sparrow populations are not well resolved, further clustering into three groups, based on significant differences in allele frequencies, *pusillula*, *gouldii* and *samuelis-maxillaris-heermanni* may be warranted.

The song sparrows in the San Francisco Bay region have been studied extensively by previous researchers interested in plumage and morphological variation, erythrocyte antigen frequencies, and variation in song type (Aldrich 1984; Ferrell 1966; Marshall 1948b; Mulligan 1963). Overall, these studies demonstrate a lack of concordance between phenotypic traits, which brings into question the significance of current subspecific designations. However, the subspecies designation was historically meant to be a taxonomic device convenient for classifying geographic variability within species. Despite its misuse for a variety of other purposes, it was not an evolutionary unit (Mayr 1969). The traditional designation was used when 75% of individuals in one population could be distinguished from individuals of another population based on plumage or morphology (Mayr 1969). This sort of application resulted in the designation of subspecies of song sparrows in the San Francisco Bay region (Grinnell 1909; Grinnell 1913).

Grinnell first applied subspecific designations to populations of song sparrows that were morphologically distinct in the San Francisco Bay area and that occupied ecologically different habitats. However, an extensive study of plumage variation and morphology led Marshall (1948b) to conclude: "Because of the lack of correspondence between the various gradients for colour and measurements, entirely different races could be designated on the basis of some other character gradient"(p. 254). Marshall observed clinal variation emanating out from peaks of differentiation in plumage colour and morphological measurements in the bay song sparrows. Additionally, the geographic location of those peaks and the clinal variation of characters in the bay changed based on the character being examined. Despite these difficulties, the subspecific

designations of the tidal marsh song sparrows have remained and for this study serve as a basis for testing predictions of genetic structure based upon approximate morphological distributions.

My analysis of genetic structure tested for subspecific groupings and did find a significant amount of variance accounted for by subspecies. A closer examination of the subspecific groupings revealed that the divergence of *pusillula* was relatively high compared to all other population comparisons in Dce, and was supported across loci by 100% of bootstrap replicates (Figure 1.2). Secondarily, tests of differences in allele frequencies between population comparisons indicated two further groupings are warranted, *samuelis-maxillaris-heermanni* and *gouldii.*

One of the goals of this study was to define conservation units in order to guide management decisions. Conservation below the species level is focused primarily on maintaining intraspecific variation to conserve biodiversity as well as the evolutionary processes responsible for generating it (Moritz 1994b). The U.S. Endangered Species Act of 1973 together with the Distinct Population Segment (DPS) amendment in 1978 protects distinct species, subspecies, and populations. Since designation provides large financial resources as well as immediate protection from hunting, habitat exploitation, and other anthropogenic impacts that threaten population viability, much debate surrounds the criteria used to designate units of management and conservation (O'Brien and Mayr 1991).

The criteria and justification for designation of conservation units can be divided into two separate ideas which Moritz et al. (1995) describes as: 1) gene conservation, for the maintenance

of genetic diversity, and 2) molecular ecology, where patterns of genetic diversity are used to complement ecological studies. Gene conservation seeks to maintain the genetic structure and preserve the evolutionary potential of species. This approach sought mainly to identify populations with independent evolutionary trajectories (Moritz 1994b). To this end, several definitions (O'Brien and Mayr 1991; Moritz 1994b) attempt to incorporate meaningful criteria to identify groups of populations with distinct evolutionary potential. Most of these are based on phylogenetic distinctness. Subspecific designation now attempts to reflect long-term historical gene-pool separations as indicated by concordance at multiple independent loci (Avise and Ball 1990; Ball and Avise 1992), this is because phylogenetic partitioning results from accumulation of differences due to the lack of gene flow (O'Brien and Mayr 1991). Currently, the explicit criteria for designation as a subspecies are: "members of a subspecies share a unique geographic range or habitat, a group of phylogenetically concordant phenotypic characters, and a unique natural history relative to other subdivisions of the species" (O'Brien and Mayr 1991; pp. 1188).

Another widely used criterion for designation of conservation units is the Evolutionarily Significant Unit (ESU) (e.g. Firestone et al. 1999; Moritz and Faith 1998; Pierson et al. 2000; Wenburg et al. 1998; Zhu et al. 1998). Ryder (1986) originally described an ESU as: 1) a set of populations that is morphologically and genetically distinct from other similar populations; and 2) a set of populations with a distinct evolutionary history. However, Moritz et al. (1995) advocated the use of phylogeographic patterns to define ESU's to identify populations that were isolated historically and have potential for independent evolution. Moritz's (1994b) criteria for designation was based solely on genetic criteria: "ESUs should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci" (p. 373).

The song sparrows of the San Francisco Bay region display a lack of concordance between different phenotypic characters (song variation, blood group frequencies, morphology, plumage), as well as microsatellite variation. All differences between populations examined so far comprise quantitative rather than qualitative distinctions. No phylogenetic differences between the subspecies in San Francisco Bay have yet been established. This suggests that song sparrow populations in San Francisco Bay lack long-term historical isolation. However, the conservation of song sparrow populations in San Francisco Bay is still justified under molecular ecology.

The second conservation unit, based on molecular ecology, was deemed by Moritz et al. (1995) as the Management Unit (MU). MU's are populations or groups of populations that are functionally separate and therefore the criteria for MU's aim at identifying the geographic scale for monitoring and managing populations (Moritz et al. 1995). The only criterion for a MU is statistically significant divergence of allele frequencies. The qualitative divergence of allele frequency is irrespective of the phylogeny of alleles. The results from my analysis on song sparrow populations in San Francisco Bay indicate three groupings, which are divergent in microsatellite allele frequencies, *pusillula*, *gouldii*, and *samuelis-maxillaris-heermanni*.

One difficulty when identifying MU's based on statistically significant differences in allele frequencies is distinguishing between statistically significant and biologically significant differences (Hedrick 1999; Waples 1998). For instance, with larger sample sizes and the higher sensitivity of hypervariable loci, very small differences in allele frequency are expected to be judged as statistically significant (Moritz et al. 1995). To avoid attributing biological

significance to statistical significance in weakly diverged groups, Moritz (1995) recommended integrating genetic and ecological evidence.

Ecological evidence strengthens the argument for establishing conservation units based on the groupings listed above. Despite some clinal variation, extremes of subspecific designations still differ markedly in plumage and size (Aldrich 1984; Ferrell 1966; Grinnell 1909; Grinnell 1913; Marshall 1948b). *Samuelis* is small in size and blackish olive in dorsal coloration. It is the blackest subspecies of song sparrow in North America (Marshall 1948b). *Pusillula*, is slightly smaller than *samuelis*, has a yellowish-grey dorsal color and is the only song sparrow subspecies with a yellowish wash to the belly (Marshall 1948b; Ridgeway 1899). In addition, *pusillula* has been shown to maintain their bodyweight while drinking saline solutions, whereas *gouldii* were unable to do so (Basham and Mewaldt 1987). *Maxillaris* is the largest of the tidal marsh subspecies with a laterally flared bill at the nostrils. It is similar in coloration to *gouldi* but more blackish-brown on the dorsal surface (Marshall 1948b). *Gouldi* is intermediate in size between maxillaris and the other two marsh subspecies. It has a reddish brown dorsal coloration (Marshall 1948b).

The marked difference in phenotypic traits, despite low estimates of genetic divergence at microsatellite loci, beg the question: How can morphological differences be maintained in the face of high gene flow? Several explanations may account for the weakly resolved pattern of variation at microsatellite loci and large amount of variation in plumage and morphology: 1) recency of divergence and insufficient lineage sorting, due possibly to large effective population sizes and short geological time scale; 2) high current gene flow at neutral loci, but strong

selection at loci controlling morphological or plumage characteristics; 3) high current gene flow but low historical gene flow with low present-day differentiation being a result of introgression between subspecies or; 4) high gene flow with morphological and plumage variation resulting from phenotypic plasticity. If the last explanation were responsible for the observed pattern of genetic and phenotypic divergence in Bay song sparrows, it becomes difficult to attribute biological significance to the statistically significant differences in allele frequencies that I found. However, if there is a heritable component to morphology, and observed variation in morphological traits is the result of selection, then the divergence of populations in morphological and plumage characteristics strengthens the biological significance of my genetic results because adaptive traits may not be measured at neutral loci (Karhu et al. 1996).

The conservation status of song sparrow populations in San Francisco Bay as MU's rest on significant differences in microsatellite allele frequencies and possible adaptive differences in morphology. Moritz et al. (1995) proposed to include both ESU's and MU's as Distinct Population Segments protected under US Endangered Species Act. Among the putative subspecies studied here, *pusillula* is the most threatened by habitat loss and fragmentation (Marshall and Dedrick 1994). In addition, the diversion of freshwater into South San Francisco Bay has resulted in a reduction in salinity that has been accompanied by changes in plant composition of the salt marshes, which may result in changes in the selective pressures (Basham and Mewaldt 1987). These factors combined with the divergence in microsatellite allele frequencies found in this study suggest a prioritization of *pusillula* for conservation efforts.

The next task for conservationists is to figure out what to do with the grouping of *samuelis-maxillaris-heermanni*. Marshall described strong clinal variation in phenotypic traits that could be seen as evidence of an equilibrium between gene flow and natural selection in tidal marshes along the northern part of San Francisco Bay. Along the northern part of San Francisco Bay is a salinity gradient created by the drainage of the Sacramento-San Juaquin Delta through Suisun Bay, the Carquinez Straight, San Pablo Bay, exiting out the Golden Gate. These changes in salinity are accompanied by changes in tidal marsh vegetation along this gradient (Atwater et al. 1979). If the differences in morphology and plumage Marshall described are heritable and the result of local adaptation and selection, then it seems logical to make an arbitrary break in the MU according to habitat. However, until further work is conducted on the heritability of morphology and plumage colouration, and the potential for directional selection to act on these traits differently in relation to habitat, the designation of a separate MU for *samuelis* and *maxillaris* remains weak.

My analysis of variation at microsatellite loci in song sparrows in the San Francisco Bay region revealed low levels of differentiation between populations and subspecies. However, significant differences in allele frequencies clustered song sparrow populations into three MU's, *pusillula, gouldii,* and *samuelis-maxillaris-heermanni*. I propose *pusillula* be prioritized for conservation efforts based on the larger amount of genetic divergence shown by Dce and greater extent of habitat loss and alteration. Additionally, I propose *samuelis-maxillaris* be designated an MU despite low differentiation between *heermanni* and *samuelis-maxillaris* due to possible adaptive differences which may not have been detectable given my use of neutral loci.

Table 1.1. Sampling locality for microsatellite analysis.

Subspecies	Population locality	Acronym	County, State
Melospiza melodia gouldii	Mark's Marsh, Tomales Bay, Audubon Canyon Ranch	MM	Marin County, CA
	Los Gatos Creek County Park	LG	Santa Clara County, CA
Melospiza melodia pusillula	Palo Alto Baylands Nature Preserve	PB	San Mateo County, CA
	Dumbarton Marsh, Don Edwards San Francisco Bay National Wildlife Refuge	DM	Alameda County, CA
Melospiza melodia samuelis	Petaluma River Mouth, California Department Fish and Game	PM	Sonoma County, CA
	Sonoma Creek, San Pablo Bay National Wildlife Refuge	SC	Solano County, CA
Melospiza melodia maxillaris	Goodyear Slough Unit, Grizzly Island Wildlife Area	GS	Solano County, CA
	Rush Ranch Open Space, Solano County Farmlands and Open Space Foundation	RR	Solano County, CA
Melospiza melodia mailliardi	The Nature Conservancy Cosumnes River Preserve	CO	Sacramento County, CA

Table 1.2. Song sparrow microsatellite loci and PCR conditions.

Locus	Primer sequence	Repeat motif	Anneal temp	Mg (Mm)	Product size	No. alleles	Source
MME 1	F1: AGGAAAAGGGAGGGAGAGGGTG R1: GGGAGTGCAGAATGTGCAAATG	(TG)7TC(TG)15	50	2.0	140-164	13	Jeffery *et al* 2000
MME 3	F1: CCTCAGATTGGCATTGAAAGTTG R1: GGTCAGTTTGCTTGGGTGTTTTC	(TG)14	53	1.5	162-182	10	Jeffery *et al* 2000
MME 7	F1: TGCGAGCCTTTCCAAGTTTG R1: AACCCCACATGAAACAGGTCAC	(CA)2TA(CA)18	54	1.5	110-140	16	Jeffery *et al* 2000
MME 8	F1: TCATGGAGATGGGTGAATGCC R1: TGAATCAGCAGCACACACAACC	(TG)3TC(TG)13	56	1.5	208-234	20	Jeffery *et al* 2000
MME 12	F1: AGGGACTGTCACTGTGGGACTGAAG R1: TGGCTTTATGGAACAAGGCATC	(CCCACA)13	60	1.5	178-250	12	Jeffery *et al* 2000
ESCU1	F1: TTCTCTTGGTCTATGGAAGGTG R1: GCTTGAAAGACAGTCACCAGG	(CA)18	50	2.0	132-170	17	Hanotte *et al* 1994
GF2.35	F1: AAACACTGGGAGTGAAGTCT R1: AACTATTCTGTGATCCTGTTACAC	(CA)15	50	2.0	184-240	27	Petren 1998
PSAP335	F1: not published R1: not published	(TG)13	55	1.5	97-119	12	Temple and Leonard, pers. comm.
MME 2	F1: ATCAGAGATTCCTGCTACACACCC R1: GAAATTGTATCCGCCACCTCATTC	(TG)30	53	1.0	118-168	23	Jeffery *et al* 2000

Table 1.3. Mean sample size per locus, mean number of alleles per locus, and mean heterozygosity for song sparrow populations (SE). Heterozygosity measured as direct counts or unbiased estimate (Nei 1978).

Subspecies	Site	Mean sample size / locus	Mean number alleles / locus	Mean Heterozygosity	
				Direct count	Unbiased estimate
M. m. gouldii	MM	19.8 (0.6)	9 (1.0)	0.77 (0.049)	0.814 (0.025)
	LG	17.8 (0.2)	10 (1.1)	0.797 (0.036)	0.81 (0.031)
M. m. pusillula	PB	24.6 (1.3)	9.8 (1.6)	0.803 (0.055)	0.8 (0.048)
	DM	25.5 (1.1)	8.6 (0.9)	0.755 (0.041)	0.762 (0.046)
M. m. samuelis	PM	17.1 (1.4)	9.1 (1.2)	0.792 (0.055)	0.831 (0.032)
	SC	25.4 (1.4)	10.6 (1.5)	0.807 (0.028)	0.817 (0.039)
M. m. maxillaris	GS	19 (1.3)	10 (1.2)	0.796 (0.049)	0.815 (0.041)
	RR	28 (0.9)	10.3 (1.1)	0.776 (0.044)	0.813 (0.035)
M. m. mailliardi	CO	19.3 (1.0)	10.9 (1.4)	0.796 (0.031)	0.846 (0.025)
M. m. morphna	BB	23.1 (1.1)	9.6 (1.2)	0.651 (0.070)	0.777 (0.037)

Table 1.4. Results of AMOVA for song sparrow microsatellite loci.

		Among subsp.		Among pops within subsp.		Within pops.	
Locus		ΦCT	% variance	ΦSC	% variance	ΦST	% variance
Mme 1	Fst	0.0109	1.09	0.0022	0.22	0.0131	98.69
Mme 3		0.0457*	4.57	0.0146**	1.39	0.0596**	94.04
Mme 7		0.0196	1.96	0.0084**	0.83	0.0278**	97.22
Mme 8		0.017*	1.70	0.0068**	0.67	0.0237**	97.63
Mme 12		0.0078	0.78	-0.0019	-0.18	0.0060	99.41
Escu 1		0.0094	0.94	0.0257**	2.55	0.0349**	96.51
Gf 235		0.0072	0.71	0.0130**	1.29	0.0200**	98.00
Psap 335		-0.0077	-0.77	0.0326**	3.29	0.0252**	97.49
Mme 2		0.0200	1.99	0.0098**	0.96	0.0295**	97.05
Total		0.0137*	1.38	0.0122**	1.18	0.0257**	97.44
Mme 1	Rst	0.0111	1.11	-0.0111	-1.10	0.0001	99.99
Mme 3		0.1838**	18.38	-0.0238**	-1.94	0.16433**	83.57
Mme 7		0.0900	9.00	0.0494**	4.49	0.1350**	86.50
Mme 8		0.0288*	2.88	-0.0053	-0.51	0.0237	97.63
Mme 12		0.0020	0.20	-0.0078	-0.78	-0.0058	100.58
Escu 1		-0.0065	-0.65	0.0159	1.60	0.0095	99.05
Gf 235		-0.0163	-1.63	0.0116	1.17	-0.0046	100.46
Psap 335		0.0069	0.69	-0.0121	-1.21	-0.0051	100.51
Mme 2		0.1128*	11.28	0.0293**	2.60	0.1388	86.12
Total		0.0174	1.88	0.00433	0.28	0.0217**	97.84

*$p<0.05$, **$p<0.001$.

Table 1.5. Genetic assignment of individuals to subspecies, percent of total in ( ).

	Predicted subspecies					
Subspecies	*M. m. samuelis*	*M. m. maxillaris*	*M. m. pusillula*	*M. m. gouldii*	*M. m. heermanni*	Total
M. m. samuelis	29 (61.7)	5 (10.6)	4 (8.5)	5 (10.6)	4 (8.5)	47
M. m. maxillaris	10 (19.2)	27 (51.9)	4 (7.7)	7 (13.5)	4 (7.7)	52
M. m. pusillula	4 (7.1)	3 (5.3)	47 (83.9)	0 (0)	2 (3.6)	56
M. m. gouldii	4 (10.3)	11 (28.2)	0 (0)	20 (51.28)	4 (10.3)	39
M. m. heermanni	7 (33.3)	3 (14.3)	0 (0)	4 (19.0)	7 (33.3)	21

Table 1.6. Cavalli-Sforza and Edwards chord distance between song sparrow populations.

Subspecies		*M. m. gouldii*		*M. m. pusillula*			*M. m. samuelis*		*M. m. maxillaris*
	Population	MM	LG	PB	DM	PM	SC	GS	RR
M. m. gouldii	MM								
	LG	0.0597							
M. m. pusillula	PB	0.0654	0.0500						
	DM	0.0644	0.0556	0.0232					
M. m. samuelis	PM	0.0503	0.0600	0.0529	0.0517				
	SC	0.0400	0.0474	0.0407	0.0451	0.0250			
M. m. maxillaris	GS	0.0497	0.0396	0.0443	0.0494	0.0384	0.0296		
	RR	0.0358	0.0373	0.0446	0.0464	0.0326	0.0232	0.0251	
M. m. heermanni	CO	0.0537	0.0431	0.0510	0.0588	0.0358	0.0312	0.0392	0.0318

Figure 1.1. Subspecies locations and sampling sites. The purported range of *samuelis* (sites PM and SC) is San Pablo Bay, *maxillaris* is Suisun Bay (sites GS and RR), and *pusillula* is San Francisco Bay (sites PB and DM). *Gouldii* is found in the upland habitats surrounding the bay (sites MM and LG) and *heermanni* is located to the east of Suisun Bay (site CO). Adapted from Marshall (1948b).

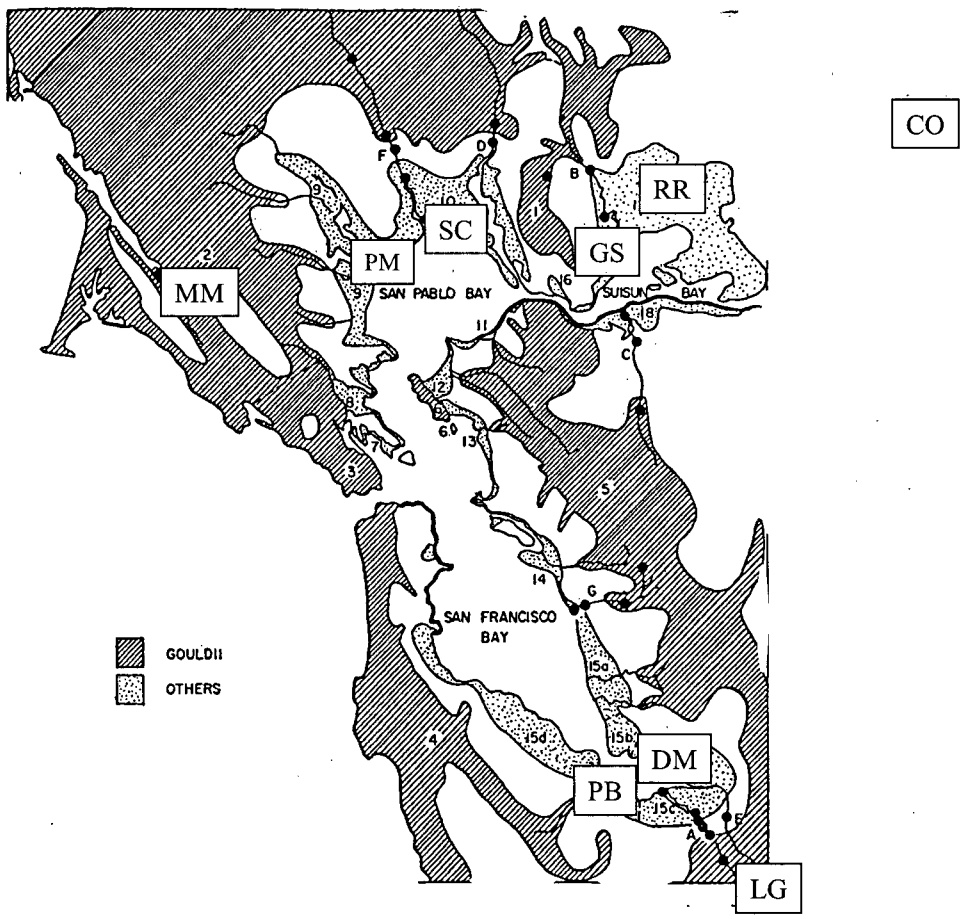
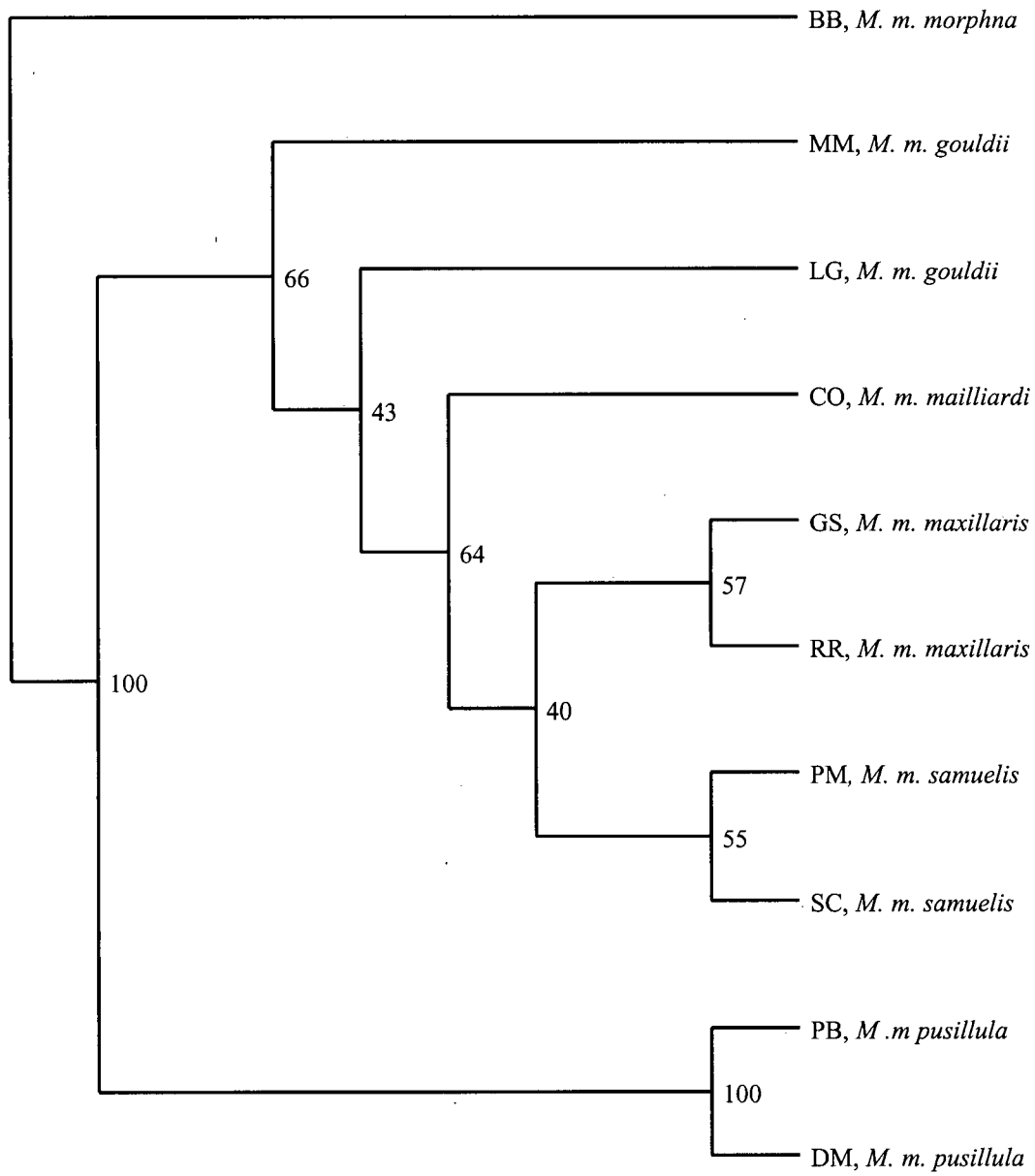


Figure 1.2. Cavalli-Sforza and Edwards (Cavalli-Sforza and Edwards 1967) chord distance UPGMA (Sneath and Sokal 1973) phenogram calculated from 8 microsatellite loci. Numbers to the right of branch indicate bootstrap support over 100 replicates.



CHAPTER 2

Comparison of morphological and microsatellite variation in Song Sparrows (*Melospiza melodia*) in San Francisco Bay

2.1 INTRODUCTION

Populations change over time as a result of two evolutionary processes, random genetic drift and natural selection. Both forces are potentially constrained by gene flow, which can reduce differentiation by moving genes between populations (Slatkin 1987). Although theoretical predictions about the importance of gene flow in evolution are clear, the effects of gene flow on genetic structure in natural populations is less well understood (Slatkin 1987). Historically, the study of geographic variation in phenotype and morphology has been central to understanding evolutionary processes, under the assumption that morphology reflected an underlying genetic structure (Mayr 1942; Mayr 1963). However, several recent studies have reported discordant patterns of genetic and morphological variation in plants (Podolsky and Holtsford 1995), fish (Turner 1974), and butterflies (Nice and Shapiro 1999) and in many species of birds (*Agelaius phoenicues*, Ball et al. 1988; *Molothrus ater*, Ball and Avise 1992; *Quiscalus quiscula*, Zink et al. 1991; *Carduelis* spp., Seutin et al. 1995; *Picoides pubescens*, Ball and Avise 1992; *Melospiza georgiana*, Greenberg et al. 1998; and *Zenaida macroura*, Ball and Avise 1992). These studies raise questions about the causes of geographic variation in morphology and our ability to link process with patterns of geographic variation in phenotype.

In this study I compared geographic variation in morphology and genetic differentiation at microsatellite loci to indirectly assess the extent of gene flow and examine the evolutionary

forces acting on song sparrows (*Melospiza melodia*) in the San Francisco Bay region. This region has four phenotypically distinct year-round resident subspecies of song sparrow described which provide a basis for studies of geographic variation. Three endemic putative subspecies are found in tidal salt marshes, each restricted to one of three sub-bays of the greater San Francisco Bay. The Samuel's song sparrow (*M. m. samuelis)* resides in San Pablo Bay, the Suisun song sparrow (*M. m. maxillaris)* in Suisun Bay, and the Alameda song sparrow (*M. m. pusillula*) in South San Francisco Bay. The Marin song sparrow (*M. m. gouldii*) occupies the uplands surrounding these bays (Grinnell and Miller 1944; Figure 1). These described song sparrow subspecies differ markedly in plumage and size at their greatest extent of phenotypic divergence. The Samuel's song sparrow is the blackest of all song sparrow subspecies in North America, the Suisun song sparrow has the thickest bill, and the Alameda song sparrow is one of the smallest subspecies and has a light dorsal colour and yellowish wash to the belly (Aldrich 1984; Marshall 1948b). In addition to these four named subspecies, I also studied the Modesto Song Sparrow (*M. m. heermanni*; *heermanni*) whose range borders *maxillaris* to the east. *Heermanni* inhabits freshwater riparian areas of California's Central Valley (Grinnell and Miller 1944; Marshall 1948b).

The source and maintenance of morphological differentiation among song sparrows in the San Francisco Bay region has been noted by many evolutionists (Grinnell 1913; Huxley 1942; Mayr 1963; Miller 1947; Miller 1956) and this has focused attention on the ecology, behaviour, and phenotypic variability of this group (Johnston 1956a; Johnston 1956b; Marshall 1948a; Marshall 1948b). However, no studies of the population genetic structure of Bay song sparrows or the potential concordance of genetic and morphological variation have been conducted. In this

study, I compared morphological and microsatellite variation in song sparrows in the San Francisco Bay region. Furthermore, I related morphological and genetic divergence to geographic distance and environmental differences in salinity in San Francisco Bay in order to investigate potential microevolutionary processes responsible for differentiation in this region. I now review briefly some of the evidence from earlier work that identifies potential mechanisms of differentiation in song sparrows in the San Francisco Bay region.

Mechanisms for differentiation in song sparrows

Two attributes of song sparrow species and the San Francisco Bay region geography are likely to have contributed to the microgeographic differentiation in this area. First, song sparrows possess a niche of moderate width. They characteristically inhabit vegetation found near permanent water such as streams, tidal sloughs, or coastlines (Marshall 1948a). This enables them to disperse into and take advantage of several different habitats, possibly with different associated selective pressures (Miller 1956). Second, the environments in the San Francisco Bay region form a mosaic of different isolated habitats in a small geographical area, some of which are habitable by song sparrows (riparian, coastal sage scrub, tidal salt marsh) and some that are not (dry grassland, open water) (Figure 2.1). It is possible, therefore, that the San Francisco Bay song sparrows exist in an arena of allopatric populations, subject to divergent selective forces (Marshall 1948a; Marshall 1948b).

Another attribute of the San Francisco Bay region that may constrain evolutionary forces acting on song sparrows is the young geological age of the tidal marshes surrounding San Francisco

Bay. Approximately 10,000 years ago the ocean entered the Golden Gate as the glaciers retreated and sea levels rose, and the current water level was reached about 5,000 years ago (Atwater 1979). The tidal marshes surrounding San Francisco Bay are probably 4,000-6,000 years old with South San Francisco Bay marshes being a bit younger, approximately 2,000 years old (Atwater et al. 1979). The apparent restriction of song sparrows to specific habitats, the spatial configuration of those habitats, and the young geological history of the region provide a framework within which drift, selection, and gene flow may facilitate local differentiation.

Based on prior studies of phenotypic differentiation, several mechanisms have been proposed which may have resulted in geographic variation in song sparrows in San Francisco Bay. These include: a) drift due to small effective population size (Miller 1947); b) geographic isolation preventing gene flow between populations (Marshall 1948a; Mayr 1942); c) strong selective forces favouring differentiation in morphological traits despite high levels of gene flow (Aldrich 1984; Zink and Dittman 1993); and d) phenotypic plasticity of morphological traits (Smith 1998; Zink and Dittman 1993). The extent of gene flow has a large impact on which evolutionary force will dominate in producing differentiation. If gene flow is extremely restricted then isolated neighbourhoods can be produced even in continuous habitat, and differentiation between neighbourhoods might be expected due to random genetic drift. If gene flow is less restricted, then barriers such as expanses of open water and unsuitable habitat may be required to facilitate differentiation. In this situation, moderate selection could also encourage differentiation between geographically isolated populations wherein environmental forces select for particular morphological traits. If gene flow throughout the region were high, then very strong selection would have to exist to produce phenotypic differentiation. In all these cases, morphological

differentiation unrelated to heritable variation in traits may also exist as a developmentally plastic response to environmental variation. None of these mechanisms is mutually exclusive and several of them may be operating to varying degrees. The purpose of this study is to provide insight into evolutionary processes causing phenotypic differentiation in song sparrows in the San Francisco Bay region. Conclusive evidence on evolutionary processes causing differentiation will have to come from detailed experiments examining actual selective forces within habitats in the San Francisco Bay region and an establishment of the environmental and heritable component of phenotypic variation in song sparrows in this region.

Random Genetic Drift

Population gene frequencies can change due to random processes alone (Slatkin 1987). Genetic drift can be a powerful evolutionary force, particularly in small populations when restricted gene flow limits the introduction of new alleles and random sampling of individuals can predominate. For example, microgeographic variation in genetic structure of house mice (*Mus musculus*) inhabiting barns on a single farm was attributed in part to genetic drift in small populations (Selander 1970). Divergence due to random genetic drift and small effective population size on the microgeographic scale has also been noted in other species (brown trout, *Salmo trutta*, Estoup et al. 1998; brook charr, *Salvelinus fontinalis,* Angers et al. 1995; woodmouse, *Apodemus sylvaticus,* Markov and Chassovnikarova 1999) including other bird species (Blondel et al. 1999; Freeman-Gallant 1996; Piertney et al. 1998). In his study of variation in erythrocyte antigen frequencies in Bay song sparrows, Ferrell (1966) found random fluctuations in blood group frequencies that may have resulted from drift. Supporting the idea that Bay song sparrows are relatively isolated genetically, Johnston (1956) reported a median juvenile dispersal distance in

samuelis of 185 meters, with 81% of juveniles settling within 360 meters of their natal territory. These are among the shortest dispersal distances recorded for song sparrows (Arcese 1989; Johnston 1956b). A negative correlation between population differentiation and dispersal distance has been found in a variety of organisms (see Bohonak 1999 for a review, e.g. marine organisms, Palumbi 1995; phytophagus insects, Peterson and Denno 1998; plants, Govindaraju 1988). Although these findings are generally consistent with the idea that small effective population size has influenced differentiation in song sparrows in the San Francisco Bay region some authors have suggested that geographic barriers to dispersal may be necessary to limit gene flow in the region.

Geographic Isolation

Marshall (1948b) proposed that differentiation in Bay song sparrows resulted mainly from geographic isolation due to physical barriers preventing effective dispersal and gene flow. He studied geographic variation in plumage across song sparrow populations within each putative subspecies and along the riparian corridors that connect the upland and tidal marsh habitat. In particular, he found areas within the range of each tidal marsh subspecies wherein populations appeared to be most distinct in their subspecific plumage characteristics. Surveying outward from those centres, Marshall found that plumage characteristics became less distinct and eventually intergraded predictably with adjacent subspecies based on their geographic connectedness. He concluded that interbreeding occurred among subspecies where they came into contact, and especially where upland and tidal marsh habitat were juxtaposed. In arriving at this conclusion, Marshall also made the unstated assumption that plumage variation among types reflected heritable variation in colouration.

Physical barriers to dispersal have resulted in genetic differentiation in bluegill sunfish (*Lepomis macrochirus*) between reservoirs and in a flightless waterstrider (*Aquarius remigis*) between streams (see Avise 1994 for review). In birds, genetic differentiation due to physical barriers to dispersal has been found at small geographic scales in the red grouse (*Lagopus lagopus scoticus;* Piertney et al. 1998) and savannah sparrow (*Passerculus sandwichensis;* Freeman-Gallant 1996). However, if song sparrows possess extremely high vagility then physical barriers to dispersal may not prevent gene flow, in that case, strong selection may be necessary to cause marked differentiation in phenotypic traits.

Selection

Strong selection for locally adapted genotypes may lead to phenotypic differentiation even in the presence of large amounts of gene flow (Endler 1977). For instance, despite the potential for large amounts of gene flow, selective differences between populations have resulted in morphological differentiation in the little greenbul (*Andropadus virens*; Smith et al. 1997) and to population specific variation in the timing of breeding in the blue tit (*Parus caeruleus*; Blondel et al. 1999). Important ecological differences clearly exist between habitats of song sparrows in the San Francisco Bay region, and some authors have suggested that these differences have been sufficient to promote local adaptation by divergent selection. Basham and Mewaldt (1987) compared salt tolerance of upland *gouldii*, which inhabits freshwater riparian areas, and tidal salt marsh-living *pusillula*. They found that *pusillula* better maintained its bodyweight when drinking saline solutions than did *gouldii*, and concluded that *pusillula* possessed adaptations that enabled it "to utilize hyperosmotic solutions, as least as saline as those found in San Francisco

Bay," whereas *gouldii* did not (Basham and Mewaldt 1987; p. 708). The annual tide cycle might also select for earlier breeding in marshes as compared with upland habitats. A primary cause for nestling mortality is tidal flooding (Johnston 1956a). Tidal marshes in San Francisco Bay experience two high and two low tides daily, with tide height varying throughout the year. The fact that salt marsh song sparrows breed on average 15 days earlier than upland song sparrows has been offered as evidence of local adaptation to avoid flooding. By breeding earlier the major nesting effort for tidal marsh song sparrows coincides with the lowest tides of the spring season and therefore avoids a primary cause of mortality for nestlings (Johnston 1954; Johnston 1956a).

Strong natural selection was proposed by (Zink and Dittman 1993) to account for the marked differentiation in morphology observed in song sparrows across North America despite a lack of genetic structure found at mitochondrial DNA (mtDNA). Ferrell (1966) also suggested that clinal variation in morphology within the San Francisco Bay song sparrows might be the result of variation in local selection pressures. However, for selection to cause adaptive divergence in phenotypic traits, those traits must be heritable. Thus, an alternate hypothesis to explain phenotypic differentiation in morphology is that morphological traits are phenotypically plastic and subject to environmental factors throughout the bay region.

Phenotypic plasticity

Apparent subspecific differences in morphology could result if song sparrows show phenotypic plasticity in response to variation in habitat (Smith 1998; Smith and Zach 1979). Examples of differentiation resulting from phenotypic plasticity have been found in tadpoles (*Rana sylvatica;* Van Buskirk and Relyea 1998), freshwater snails (*Physa heterostropha*; DeWitt 1998),

stickleback fish (*Gasterosteus* spp.; Day et al. 1994), skinks (*Bassiana duperreyi;* Elphick and Shine 1998), and pocket gophers (*Thomomys bottae*; Patton and Brylski 1987).

James (1982) used reciprocal transplant experiments on red-winged blackbirds (*Agelaius phoenicues*) to show that regional differences in morphology were non-genetic. In song sparrows, Smith (1993) also found that morphological variation between two subspecies of song sparrows was due to a developmental response to environmental variation. Smith transplanted nestlings of the eastern sierra subspecies to nests of *gouldii* in San Francisco Bay and found that nestlings diverged towards the foster population in morphology and exhibited high levels of environmental flexibility particularly in bill depth and width. Smith's transplant experiment combined with an allometric analysis of song sparrow morphology led her to conclude that body size was environmentally plastic in song sparrows. She hypothesized that local environment influences body size to produce dramatic changes in the suite of allometrically related traits.

In contrast to these experiments, however, Smith and Dhondt (1980) showed by reciprocal transplant within a single song sparrow population, that wing, tarsus, bill length, and bill depth were all significantly heritable. Overall, therefore while heritable variation in morphology exists in at least some populations of song sparrows, phenotypic plasticity is also present and may respond to the environment.

Clearly, random genetic drift, geographic isolation, selection, and phenotypic plasticity may all contribute to differentiation in song sparrows in the San Francisco Bay region. However, comparing genetic and morphological variation in this region may aid in assessing gene flow

between the song sparrow subspecies and the impact of gene flow on phenotypic differentiation. Furthermore, I related the morphological and genetic divergence to geographic distance and environmental differences in salinity in San Francisco Bay in order to investigate more directly the relationship between geography, environment and differentiation.

I address the following predictions with my analysis: First, under the hypothesis that apparent morphological and genetic differentiation is due to random genetic drift, I predicted that random fluctuations in morphology and allele frequencies between populations would be observed and would bear no relation to geographic separation. Second, if differentiation in song sparrows is due primarily to geographic isolation, I predicted morphological and genetic divergence among populations should be concordant. That is, dendrograms based on microsatellite measures of differentiation should match generally those based on morphological measures. Concordance between genetic and morphological distances would also be supported by a Mantel's test rejecting the null hypothesis of no correlation between matrices. Third, under the hypothesis that divergent selective forces between tidal salt marsh and freshwater riparian have resulted in phenotypic differentiation, there is no prediction of concordance between morphological and genetic divergence. In addition, correlation of morphological divergence with an environmental gradient, such as salinity, would be consistent with the selection hypothesis. However, the fourth hypothesis, that apparent differentiation is developmental in origin and results from phenotypic plasticity also predicts a lack of concordance between morphological and genetic divergence and a similar concordance with an environmental gradient. If phenotypic plasticity in body size were primarily responsible for apparent differentiation among song sparrow subspecies, then

differences in shape between apparent subspecies might be small or absent. Divergence in shape that is not related to overall size may result from selection in different environments.

2.2 MATERIALS AND METHODS

Birds of four putative subspecies from 14 different populations were sampled March-May, 1999, during the breeding season from tidal salt marshes and surrounding freshwater riparian areas in the San Francisco Bay region. Adults were captured in mist nets, measured, sampled for blood, and released. Twelve blood samples were also taken from nestlings (one per nest) at Petaluma Marsh (PM). The subspecies and locations from which birds were sampled are listed in Table 2.1 and shown on Figure 2.1.

For morphometric analysis, a total of 257 males from 14 populations were measured. Males were sexed by the presence of a cloacal protuberance or a heterozygous genotype according to z-linked loci. Seven morphological traits on each bird were recorded with digital callipers and electronic balance: weight to nearest 0.1 g (WGHT), unflattened wing to nearest 1 mm (WING), tarsometatarsus length to nearest 0.01 mm (TAR), tail to nearest 1 mm (TAIL), bill length to nearest 0.01 mm (from nares to tip of bill; BLGTH), bill width to nearest 0.01 mm (at nares; BWDTH), and bill depth to nearest 0.01 mm (nares to mandibular ramus; BDPTH). For complete descriptions of measurements taken see Pyle (1997). All measurements were made by the author to minimize observer bias.

For the genetic analysis, a total of 215 birds from four putative subspecies and nine populations were included: MM, LG (*gouldii*); PB, DM (*pusillula*); PM, SC (*samuelis*); GS, RR (*maxillaris*); CO (*heermanni*). Blood was collected and nine microsatellite loci, MME 1, MME 2, MME 3, MME 7, MME 8, MME 12, ESCU 1, and GF 235, amplified as described in Chapter 1.

Multivariate analysis of morphology and discrimination between subspecies

A principal components analysis (PCA) on the log-transformed covariance matrix was first performed to determine if subspecies grouped morphologically. Once groupings were established, multivariate analysis of variance (MANOVA) was used to determine the statistical significance of differences between population means. Geographic differentiation between sites was examined using canonical variates analysis (CVA), a multiple group discriminant analysis. CVA was performed on the log-transformed variables using the DISCRIM and CANDISC procedures in SAS version 6.12 (SAS institute 1996). Interpretation of significance tests in a multivariate analysis is dependent on two assumptions, multivariate normality and homoscedasticity of population variance-covariance matrices (Dillon and Goldstein 1984). Multivariate normality was approximated using tests of univariate normality (UNIVAR procedure) in SAS and equality of variance-covariance matrices was checked using Bartlett's modification of the likelihood ratio test (DISCRIM procedure, METHOD=NORMAL, POOL=TEST, SLPOOL=0.05; Morrison 1976). The reliability of the discriminant function was evaluated by splitting the data set in two, using half to create the function and the other half to validate it.

In her study of phenotypic variation in song sparrows, Smith (1998) proposed that multivariate body size might be phenotypically plastic. In order to discriminant between subspecies based on shape, excluding the contribution of size to the between group differences, a multiple group principal components analysis (MGPCA) may be performed followed by a CVA on only the shape components (MGPC scores 2-7; Thorpe 1983; Thorpe 1988). To perform this analysis, all measurements were log-transformed prior to analysis to linearize variables, stabilize variances, and preserve allometries. MGPCA was performed using the PRINCOMP procedure in SAS, using the within-group (in this case, population) pooled variance-covariance matrix. After determination that all variables were positively correlated with MGPC1, indicating that MGPC1 corresponded to overall body size, MGPC1 was removed from the analysis and a CVA on MGPC2-7 was performed using the CANDISC procedure (SAS institute 1996).

The results from the genetic assignment test (GENECLASS; Piry and Cornuet 1999), employed in Chapter 1 to assign individuals to the subspecies where their genotype is most likely to occur, were used for a genetic comparison to the morphological discriminant analysis.

Subspecific and population structure

A nested analysis of variance was used to examine the contribution of between subspecies, between population, and within population variances to the total variance in morphological traits (ANOVA; SAS institute 1996). The contribution of the components to the between group variation is assessed by their F-score (between/within group variance; Thorpe 1983).

The analysis of microsatellite variation was taken from Chapter 1 to compare with the morphological analysis of variance. In brief, Arlequin version 2.000 (Schneider et al. 2000) was used to calculate Φ -statistics analogous to F-statistics (that employed differences in allele frequencies only) and R-statistics (Slatkin 1995; that used an analysis of molecular variance (AMOVA) and accounts for variance in size between pairs of alleles; Excoffier et al. 1992). Genetic variance among populations in the total sample were calculated (Fst, Rst) as well as a hierarchical calculation which was used to partition variation into three components, "within populations" (Φ_{ST}), "among populations/within groups" (Φ_{SC}) and "among groups" (Φ_{CT}) (Excoffier et al. 1992).

Morphological and genetic divergence

Morphological divergence between populations was estimated using Mahalanobis D^2 calculated between populations in canonical variable space. The squared distance between two groups in variable space can be calculated by their Euclidean or Pythagorean distance, however, this measure does not take into consideration correlations between variables. Mahalanobis distances are normalized by the pooled within-group variance and were chosen because they incorporate the effects of variable correlations (Campbell and Atchley 1981). Distances were jackknifed over individuals by randomly selecting 80% of the data set and recalculating the distances ten times. Unweighted pair group cluster analysis (UPGMA) was used to examine relationships between populations in morphology. A consensus tree of ten distance matrices were combined using NEIGHBOR and CONSENSE in PHYLIP version 3.57c (Felsenstein 1995).

Dce, Cavalli-Sforza and Edward's chord distance (1967), was taken from Chapter 1 to compare with morphological divergence (calculated using the GENEDIST program in PHYLIP version

3.57c; Felsenstein 1995). Genetic relationships between populations were constructed using UPGMA (program NEIGHBOR) and compared to morphological relationships.

Matrix correlations

Associations between morphological, genetic, and geographic distance matrices were examined using Mantel (1967) tests and partial Mantel tests (Manly 1991). In addition, a dissimilarity matrix of differences in salinity between sites was used to investigate the possible role of environment in differentiation (Smith 1999). Although differences in salinity between sites may result in different selective pressures on song sparrow physiology due to differences in salt tolerance (Basham and Mewaldt 1987), salinity is also correlated with vegetation differences between sites (Atwater et al. 1979). Mantel procedures use random permutations of matrix rows and columns to estimate whether an association between two distance matrices is stronger than that due to chance (Sokal and Rohlf 1995). First, genetic and morphological distance matrices were compared to each other and then to the geographic and salinity matrices individually. Second, Partial Mantel tests, which allow multiple matrices to be compared simultaneously, were used to examine correlations between genetic and morphological matrices while controlling for the effects of geography and salinity respectively. Calculations were carried out using the FORTRAN subroutine found in Manly (1991). Distances were first standardized to a mean of zero and variance of one and the significance of the estimated coefficients and R^2 was determined by approximating the randomization distribution by the observed values and 1000 other values obtained by permuting the rows and columns of the dependent matrix.

2.3 RESULTS

Song sparrows in the San Francisco Bay region display a large amount of morphological variation in the measurements reported here (Table 2.2) and in principal component scores derived from these measurements (Figure 2.2, Table 2.3). Moreover, these data suggest that populations that I sampled separated roughly along putative subspecific lines. Results from the MANOVA showed all subspecies centroids to be significantly different at $p = 0.0001$ (Wilks' Lamda = 0.06998, Pillai's Trace = 1.5678, Hotelling-Lawley Trace = 5.6636, 28 df; Bonferroni adjusted p-level = 0.005). Subspecific mean values of single traits also differed markedly (all $p<0.0001$, univariate ANOVA). Variance-covariance matrices were similar across groups (Chi-square = 110.826, 112 df, $p>0.05$). Four significant tests for univariate normality rejected the null hypothesis of normality at $p = 0.05$, but no test was significant at the Bonferroni adjusted level ($p = 0.0014$). Nevertheless, as three of the four offending tests were for wing measurements, I performed the CVA with and without wing included. The classification of birds identified to correct subspecies was 77.98 % with wing included and 74.3% without (Table 2.4). A plot of canonical axes 1 and 2 show grouping of populations to subspecies, although *maxillaris* populations were less grouped (Figure 2.3). Despite a slight decrease in the reliability of the discriminant function when the size axis was excluded, discriminant analysis of only the shape components (MGPC 2-7) still showed that 71.65% of birds were identified correctly to subspecies. The genetic assignment test was less reliable, but still correctly assigned individuals to their presumptive subspecies 60.19% of the time (comparing 5 groups, Table 2.4).

Subspecific and population structure

I found substantial subspecific differentiation in morphological traits by nested ANOVA (Figure 2.4). Subspecies accounted for 74.2% of the variance in bill depth, 62.9% of the variance in bill width, and approximately 50% of the variance in weight, wing, tail length, bill length, and tarsus. In contrast, population within subspecies accounted for 0-5% of the variance for wing, weight, tail length, and tarsus, but ~10% of the variance in bill width and depth.

The analysis of microsatellite variation by AMOVA indicated that most of the variation occurred within populations (97-98%), whereas only ~1% variance occurred among subspecies (Figure 2.4). Differentiation between subspecies, $\Phi_{CT,}$ was 0.0137 for the Fst analog (which was significant at $p<0.05$), and 0.0174 for the Rst analog, slightly larger than the values between populations, $\Phi_{SC,}$ which were 0.0122 for the Fst analog and 0.00433 for the Rst analog.

Morphological divergence

Mahalanobis distances indicated the divergence of populations and subspecies in canonical variate space (Table 2.5). A UPGMA cluster dendrogram based on Mahalanobis distances showed groupings of sampled populations into recognized subspecies except for RR (*M. m. maxillaris*), which groups with CO (*heermanni*), and SB (*maxillaris*), which groups with *gouldii* (Figure 2.5). I calculated the dendrogram and jackknife estimates to illustrate the relationships between populations, however, it is necessary to point out that dendrograms artificially force data to conform to an hierarchical arrangement when it is doubtful that such an hierarchy exists at the intraspecific level. In addition, sample sizes for these analyses were low (11-25 individuals). As a result, jackknife estimates across individuals may be unreliable.

Topology of the UPGMA dendrogram calculated from the pairwise Dce matrix was not concordant with the morphological dendrogram derived from Mahalanobis distances. The morphological dendrogram displayed short branch lengths between populations and long branch lengths separating each of the subspecies. Although the genetic dendrogram displayed short branch lengths between populations for the three tidal marsh subspecies, *gouldii* showed comparatively shorter branch lengths separating populations. Topology of the genetic dendrogram displayed populations grouped into *pusillula* group, and one that contained all the others (Figure 2.5).

Comparison of morphology and genetics

No correlation was found between morphological and genetic distance matrices ($r = 0.115$) and the coefficient of correlation was weakly negative when controlling for geography ($r = -0.156$) or salinity ($r = -0.049$). However, I did find significant positive correlations between genetic distances and morphological distances and geographic and salinity distances (Table 2.6). The associations between the genetic and morphological distances and the geographic and salinity distances were strong, despite the lack of correlation between the geographic and salinity distances themselves, suggesting salinity and geographic variation were independent estimates of different factors.

2.4 DISCUSSION

Comparisons of morphological variation and microsatellite variation in song sparrows in the San Francisco Bay region consistently showed greater differentiation between subspecies in morphology than at neutral loci. The discriminant analysis based on morphological traits identified correctly ~75% of individuals to putative subspecies, while the genetic assignment test identified 60% to correct subspecies (Table 2.3). The proportion of variance accounted for by subspecies in morphological traits ranged from 45-74%, however, ~1% of the microsatellite variation was among subspecies (Figure 2.4). Finally, the UPGMA dendrogram based on Mahalanobis distances displayed relatively long branch lengths between subspecies compared with between populations within subspecies. However, the UPGMA dendrogram based on Dce showed one long branch length between *pusillula* and the remaining populations, with branch lengths separating populations of the other subspecies being of approximately equal length (Figure 2.5). Matrix correlation tests indicated a lack of concordance between morphological and genetic distances.

Many biologists have remarked on the amount of morphological differentiation on a microgeographic scale demonstrated by the song sparrows of the San Francisco Bay region (Marshall 1948a). Factors cited as potentially responsible for this differentiation include non-adaptive variation due to small effective population size (Miller 1947), spatial isolation as a prelude to their present ecological differentiation (Marshall 1948a; Mayr 1942), and continued isolation due to habitat selection and ecological preferences (Grinnell 1913; Huxley 1942). Studies performed by Marshall (1948a, 1948b), Johnston (1956a, 1956b), Ferrell (1966), and

Mulligan (1963) were all aimed at investigating the ecological and evolutionary processes responsible for differentiation. This study compares genetic and morphological structure and adds insight to previous hypotheses based on phenotypic differentiation; these will be addressed in the discussion that follows.

Random genetic drift

If random genetic drift due to small population size and highly restricted gene flow were the primary cause for differentiation in song sparrows, I predicted random fluctuations in allele frequencies and morphological measurements. The results of this study agree with Marshall (1948b) in that morphological traits do not show random fluctuations. Also low differentiation between populations and a high Nm of seven migrants per generation indicate that gene flow is high between populations. Therefore it is unlikely that random genetic drift has played a dominant role in the subspecific differentiation in song sparrows in San Francisco Bay. However, Marshall had previously noted drift might have played a substantial role in morphological differentiation of a particular population, SB (*maxillaris*). I did not sample SB at microsatellite loci and therefore cannot add genetic insight into that hypothesis. However, SB is unusual in that it groups closely with *gouldii* morphologically (Figure 2.5). The tidal marsh of the SB population has recently undergone changes in vegetation due to freshwater runoff from an adjacent suburb (Marshall and Dedrick 1994), this may have altered the evolutionary forces responsible for the distinctness of the population previously. The changes in this population warrant closer examination.

Geographic Isolation

Based on the well-structured variation in plumage, Marshall (1948b) focused on isolation and prevention of gene flow by geographic barriers as the major factor responsible for subspecific differentiation. If geographic isolation were the primary cause for subspecific differentiation in song sparrows in the San Francisco Bay region then I predicted a similar pattern of divergence between morphological and microsatellite analysis. However, I found a lack of concordance between morphological and genetic structure, which makes it unlikely that spatial isolation alone was the major force responsible for subspecific differentiation. Ferrell (1966) also set out to study the influence of avenues and barriers to gene flow and he found no correlation between apparent degree of isolation and statistically significant divergence of blood group frequencies.

Lack of concordance between genetic structure at a highly polymorphic neutral marker and morphological divergence makes selection or phenotypic plasticity necessary to account for the differences. If drift were the major factor in differentiation, all loci should be similarly affected. However, if selection influenced differentiation, then the loci under selection would be affected while non-selected loci would diverge due to drift. Therefore, differences in patterns of divergence measured by genetic or morphological traits should only result from selection or phenotypic plasticity (Slatkin 1987).

Selection

If the rate of morphological divergence were greater than the rate of divergence at neutral loci, then selective pressures between sites may differ and must be large enough to result in divergence morphologically despite lack of divergence at neutral microsatellite loci. If selection

has a major influence on morphology there should be evidence for heritability of morphological traits and differential environmental conditions causing habitat specific selective forces.

Other studies have found heritability of morphological traits in birds (Larsson et al. 1997; Merila et al. 1998; Merila J 1996). Previous studies on an insular population of song sparrows in British Columbia (*M. m. morphna*) also describe heritable variation in morphological traits (Smith and Dhondt 1980; Smith and Zach 1979). Heritability for tarsus length was moderately high (h^2 = 0.317, Smith and Zach 1979; $h^2 = 0.71$, Smith and Dhondt 1980; $h^2 = 0.90$, Smith and Dhondt 1980), while heritability for bill measurements varied widely (bill length $h^2 = -0.04 - 0.71$; bill depth $h^2 = 0.39 - 1.23$; bill width $h^2 = 0.30 - 0.59$, Smith and Dhondt 1980; Smith and Zach 1979). These studies have demonstrated that heritable variation in morphology is available for selection to operate in this song sparrow population and selection on morphological traits in nature has been documented in one song sparrow population (Schluter and Smith 1986).

Other evidence for selective differences between subspecies can be found in the morphological allometric trajectories. In her study of allometry in the song sparrow, Smith (1998) concluded that "the salt marsh [*maxillaris*] bill length represents an alteration of developmental integration with respect to other song sparrows and thus highlights the action of evolutionary forces." Although there is evidence that body size may be plastic in vertebrates (Patton and Brylski 1987; Smith and Patton 1988), there is also evidence that it is significantly heritable (Thorpe and Leamy 1983). Song sparrows in San Francisco Bay differed not just in size, which may have an environmental influence but also in shape. The results of my discriminant analysis revealed that

the song sparrows found in San Francisco bay can be assigned to correct subspecies 72% of the time based on shape variables alone.

If morphological differences between subspecies are a result of selective forces, then environmental conditions should be different in each of the respective subspecies habitats. The subspecies with the greatest evidence of differentiation, *pusillula*, is particularly tolerant of drinking water much more saline than can be tolerated by upland subspecies (Basham and Mewaldt 1987). Interestingly, I found that both morphological and genetic divergence were correlated with salinity of seawater within San Francisco Bay. Vegetation within marshes may also differ depending on the salinity. Brackish marshes found in Suisun Bay are often dominated by common tule (*Scirpus acutus*), Olney's bulrush (*Scirpus olneyi*), cat-tails (*Typha* spp.), common reed (*Phragmites communis*) and arroyo willow (*Salix lasiolepis*) as opposed to the common pickleweed (*Salicornia pacifica*) and California cordgrass (*Spartina foliosa*) dominated marshes of San Pablo and South San Francisco Bay (Atwater et al. 1979). The difference in vegetation could also affect the dominant types of food available to song sparrows in each major sub-bay, and, thus, contribute to selection on bill morphology. Furthermore, tidal marsh vertebrates compared with upland species tend towards greyish or blackish coloration (Greenburg and Droege 1990; Grinnell 1913) that may be a result of selection based on substrate colour due to the oxidized and gleyed soils of tidal salt marshes. In this study, both morphological and genetic divergences were correlated with salinity, which supports selection as a major force in subspecific differentiation.

Further research could focus attention on the mechanism of selection in tidal marsh song sparrows. Additionally, an examination of genetic markers that may reflect adaptive differences such as quantitative trait loci (QTL) and major histocompatibility complex genes (Hedrick 1996), could estimate differentiation at selected loci.

Phenotypic plasticity

Finally, it is also possible that differences in morphology not attributable to differences at neutral loci may be ecophenotypic in origin (James 1982). If subspecific differences are primarily due to phenotypic plasticity then I predicted a lack of concordance between morphological and genetic divergences, the same prediction as for selection. However, I also predicted that most of the subspecific variation would be due to the size component (MGPC 1), which was not supported by this study. A common garden or reciprocal transplant experiment examining the genetic and environmental basis for differences in morphology would be valuable in determining more conclusively if selection or phenotypic plasticity were the main cause for subspecific differentiation.

Other population genetic studies on other birds have found weak differentiation at microsatellite loci despite strong phenotypic differentiation (e.g Graputto et al. 1998). In song sparrows, lack of differentiation in mtDNA across North America and the Aleutian Islands was attributed to the recency of divergence among clades, and hypothetically a faster rate of evolution in phenotypic compared to molecular traits (Hare and Shields 1992; Zink and Dittman 1993). The weak differentiation observed between song sparrow subspecies in the San Francisco Bay region may result either from recent differentiation or from current high gene flow. The formation of tidal

marshes in the San Francisco Bay region is geologically recent (2,000-4,000 years old), therefore any differentiation detected would have been of recent origin. However, although the amount of variance in microsatellite allele frequency accounted for by subspecies was over half the among group variance, the low estimates may indicate that there is still gene flow present between populations. Regardless, the large amount of differentiation in morphological traits results from either different selective forces on morphology between the various song sparrow habitats of the San Francisco Bay region, or from developmental differences associated with the differences in habitat.

CONCLUSION

Based on significant differences in microsatellite allele frequencies and greater divergence at Dce I propose that the range of *pusillula* be designated a Management Unit. Additionally, I propose the ranges of *samuelis* and *maxillaris* be designated a MU despite low differentiation between *heermanni* and *samuelis-maxillaris* because it remains possible that adaptive differences between these types were not identified with neutral loci. Estimates of divergence based on morphological traits and microsatellite loci analysis were not concordant among subspecies of song sparrows in the San Francisco Bay region. Whereas morphological traits indicated highly structured variation in morphology, microsatellite loci analysis showed weak differentiation between subspecies. Low estimates of divergence indicate recency of differentiation between song sparrow subspecies in the San Francisco Bay region or high current gene flow. In any case, both differences in selection between habitats or phenotypic plasticity in morphological traits could each account for geographically structured morphology.

Table 2.1. Locality and sample size for morphological data.

Subspecies	Population locality	Acronym	n	County, State
Melospiza melodia gouldii	Mark's Marsh, Tomales Bay, Audubon Canyon Ranch	MM	20	Marin County, CA
	Tennessee Valley, Golden Gate National Recreation Area	TV	15	Marin County, CA
	San Pedro Valley County Park	SP	18	San Mateo County, CA
	Los Gatos Creek County Park	LG	17	Santa Clara County, CA
Melospiza melodia pusillula	Palo Alto Baylands Nature Preserve	PB	23	San Mateo County, CA
	Dumbarton Marsh, Don Edwards San Francisco Bay National Wildlife Refuge	DM	23	Alameda County, CA
Melospiza melodia samuelis	China Camp State Park	CC	11	Marin County, CA
	Petaluma River Mouth, California Department Fish and Game	PM	14	Sonoma County, CA
	Sonoma Creek, San Pablo Bay National Wildlife Refuge	SC	21	Solano County, CA
	Triangle Levy, San Pablo Bay National Wildlife Refuge	TL	19	Solano County, CA
Melospiza melodia maxillaris	Benicia State Recreation Area, Southhampton Bay	SB	15	Solano County, CA
	Goodyear Slough Unit, Grizzly Island Wildlife Area	GS	13	Solano County, CA
	Rush Ranch Open Space, Solano County Farmlands and Open Space Foundation	RR	23	Solano County, CA
Melospiza melodia mailliardi	The Nature Conservancy Cosumnes River Preserve	CO	25	Sacramento County, CA

Table 2.2. Morphological measurements of song sparrows by population and subspecies (mean ± SE (*n*)).

	M. m. samuelis				*M. m. maxillaris*		
	CC(11)	PM(14)	SC(21)	TL(19)	SB(15)	GS(13)	RR(23)
Wing	58.09 ± 1.30	59.64 ± 1.15	58.43 ± 1.16	58.26 ± 1.48	60.87 ± 1.46	61.69 ± 1.49	62.39 ± 1.73
Weight	17.94 ± 1.00	17.98 ± 0.70	17.95 ± 0.63	17.74 ± 0.63	19.37 ± 1.15	19.55 ± 1.08	20.03 ± 0.98
Tail	56.27 ± 2.37	57.14 ± 1.88	56.14 ± 1.74	56.53 ± 2.39	59.33 ± 2.64	61.15 ± 2.41	61.30 ± 2.80
Tarsus	20.89 ± 0.50	20.82 ± 0.70	20.68 ± 0.52	20.80 ± 0.46	21.70 ± 0.41	21.60 ± 0.56	21.93 ± 0.75
Bill length	9.27 ± 0.37	9.07 ± 0.42	9.10 ± 0.44	9.48 ± 0.35	9.34 ± 0.44	9.47 ± 0.32	9.72 ± 0.35
Bill width	5.16 ± 0.24	5.19 ± 0.27	5.20 ± 0.31	5.27 ± 0.22	5.36 ± 0.31	5.75 ± 0.30	6.02 ± 0.30
Bill depth	5.68 ± 0.19	5.68 ± 0.23	5.72 ± 0.22	5.84 ± 0.24	6.10 ± 0.18	6.33 ± 0.29	6.74 ± 0.25

	M. m. pusillula		*M. m. gouldii*				*M. m. heermanni*
	PB(23)	DM(23)	MM(20)	TV(15)	SP(18)	LG(17)	CO(25)
Wing	58.61 ± 1.73	57.96 ± 1.66	60.55 ± 1.50	60.47 ± 1.51	61.17 ± 1.76	61.76 ± 1.48	64.24 ± 2.01
Weight	18.56 ± 0.89	18.71 ± 0.92	18.85 ± 0.85	19.07 ± 1.08	18.86 ± 0.81	19.11 ± 0.91	20.18 ± 1.3
Tail	55.57 ± 2.86	54.78 ± 2.26	59.50 ± 2.24	58.07 ± 2.43	59.61 ± 3.84	61.94 ± 3.07	64.28 ± 2.72
Tarsus	20.89 ± 0.61	20.85 ± 0.59	21.41 ± 0.74	21.51 ± 0.54	21.38 ± 0.58	21.87 ± 0.56	22.34 ± 0.66
Bill length	8.69 ± 0.34	8.64 ± 0.36	8.71 ± 0.30	8.90 ± 0.24	8.92 ± 0.35	8.84 ± 0.38	9.65 ± 0.45
Bill width	4.76 ± 0.22	4.75 ± 0.21	5.08 ± 0.21	5.06 ± 0.26	4.99 ± 0.22	5.30 ± 0.19	5.91 ± 0.34
Bill depth	5.47 ± 0.18	5.36 ± 0.22	5.58 ± 0.18	5.86 ± 0.20	5.53 ± 0.21	5.76 ± 0.16	6.80 ± 0.28

Table 2.3. Principal component coefficients from principal components analysis of the log-transformed covariance matrix of seven morphological measurements and canonical loadings from discriminant analysis of subspecies. Proportion of variance and cumulative variance in ( ).

	PC1	PC2	PC3	CAN1	CAN2	CAN3
Wing	-0.21485	0.315928	0.140984	0.774385	-0.378003	-0.124882
Weight	-0.296534	0.433037	-0.757917	0.562431	-0.464544	0.475055
Tail	-0.314099	0.603393	0.592721	0.761917	-0.256553	-0.411439
Tarsus	-0.163982	0.190744	-0.193008	0.629006	-0.496726	-0.04921
Bill length	-0.291626	-0.409526	0.026307	0.669262	0.502256	0.034939
Bill width	-0.580046	-0.330681	0.113518	0.894977	0.172861	-0.037093
Bill depth	-0.564677	-0.187457	-0.059472	0.963982	0.056671	0.254134
eigenvalue	0.020455	0.003572	0.002383	4.3029	1.0641	0.2626
proportion	68.9 (68.9)%	12.0 (80.9)%	8.0 (88.9%)	76.0 (76.0)%	18.8 (94.76)%	5.0 (99.4)%

Table 2.4. A) Classification of song sparrows by discriminant function to subspecies. Percent of total in ( ). Overall, 77.98% of individuals classified to correct subspecies. Missclassification mostly within salt marsh or upland groups. B) Genetic assignment of individuals to subspecies, percent of total in ( ).

A. Morphological

	Predicted subspecies					
Subspecies	*M. m. samuelis*	*M. m. maxillaris*	*M. m. pusillula*	*M. m. gouldii*	*M. m. heermanni*	Total
M. m. samuelis	27 (69.2)	4 (10.3)	6 (15.4)	2 (5.1)	0 (0)	39
M. m. maxillaris	4 (15.4)	22 (84.6)	0 (0)	0 (0)	0 (0)	26
M. m. pusillula	1 (3.2)	3 (9.7)	27 (87.1)	0 (0)	0 (0)	31
M. m. gouldii	1 (3.7)	0 (0)	1 (3.7)	19 (70.4)	6 (22.2)	27
M. m. heermanni	0 (0)	0 (0)	0 (0)	3 (21.4)	11 (78.6)	14

B. Genetic

	Predicted subspecies					
Subspecies	*M. m. samuelis*	*M. m. maxillaris*	*M. m. pusillula*	*M. m. gouldii*	*M. m. heermanni*	Total
M. m. samuelis	29 (61.7)	5 (10.6)	4 (8.5)	5 (10.6)	4 (8.5)	47
M. m. maxillaris	10 (19.2)	27 (51.9)	4 (7.7)	7 (13.5)	4 (7.7)	52
M. m. pusillula	4 (7.1)	3 (5.3)	47 (83.9)	0 (0)	2 (3.6)	56
M. m. gouldii	4 (10.3)	11 (28.2)	0 (0)	20 (51.28)	4 (10.3)	39
M. m. heermanni	7 (33.3)	3 (14.3)	0 (0)	4 (19.0)	7 (33.3)	21

Table 2.5. Mahalanobis distances among populations of song sparrows.

Subspecies		*M. m. gouldii*				*M. m. pusillula*		*M. m. samuelis*				*M. m. maxillaris*		
	Pop.	MM	TV	SP	LG	PB	DM	CC	PM	SC	TL	SB	GS	RR
M. m. gouldii	MM													
	TV	3.086												
	SP	1.137	3.903											
	LG	2.211	5.005	4.365										
M. m. pusillula	PB	5.224	5.297	4.609	13.516									
	DM	7.245	9.283	6.635	17.108	0.807								
M. m. samuelis	CC	8.201	7.303	8.481	12.650	7.285	9.371							
	PM	4.455	4.451	4.778	8.081	5.901	8.679	1.814						
	SC	7.039	5.965	8.085	11.563	6.412	8.792	0.704	0.842					
	TL	12.895	9.700	13.234	16.024	12.547	15.993	1.075	3.397	1.786				
M. m. maxillaris	SB	7.135	2.541	8.252	5.874	12.754	17.862	7.263	5.840	6.729	7.127			
	GS	14.211	9.558	17.092	9.359	25.210	31.508	13.581	11.310	12.118	11.277	2.923		
	RR	28.973	19.874	32.904	20.823	42.336	50.853	26.551	24.199	24.563	21.547	9.503	2.970	
M. m. mailliardi	CO	32.972	23.424	36.389	22.050	50.052	60.393	36.610	32.097	34.558	31.618	13.241	6.736	2.648

Table 2.6. Simple regression coefficients of morphological, genetic, geographic, and salinity distance matrices. * indicates Mantel parameter value exceeded during 1000 iterations less than 50 times ($p<0.05$). Genetic and morphological distance matrices not correlated. However, each is correlated with geography and salinity.

	Morphology	Genetic	Geographic
Genetic	0.115		
Geographic	0.404*	0.533*	
Salinity	0.451*	0.342*	-0.062

Figure 2.1. Subspecies locations and sampling sites. The purported range of samuelis (sites CC, PM, and SC) is San Pablo Bay, maxillaris is Suisun Bay (sites SB, GS, and RR), and pusillula is San Francisco Bay (sites PB, DM). Gouldii is found in the upland habitats surrounding the bay (sites MM, TV, SP, and LG) and heermanni is located to the east of Suisun Bay (site CO). The range of gouldii and the salt marsh subspecies are separated by uninhabitable dry hillsides (in white), however riparian streams join the two habitats providing possible corridors for dispersal. Adapted from Marshall (1948b).

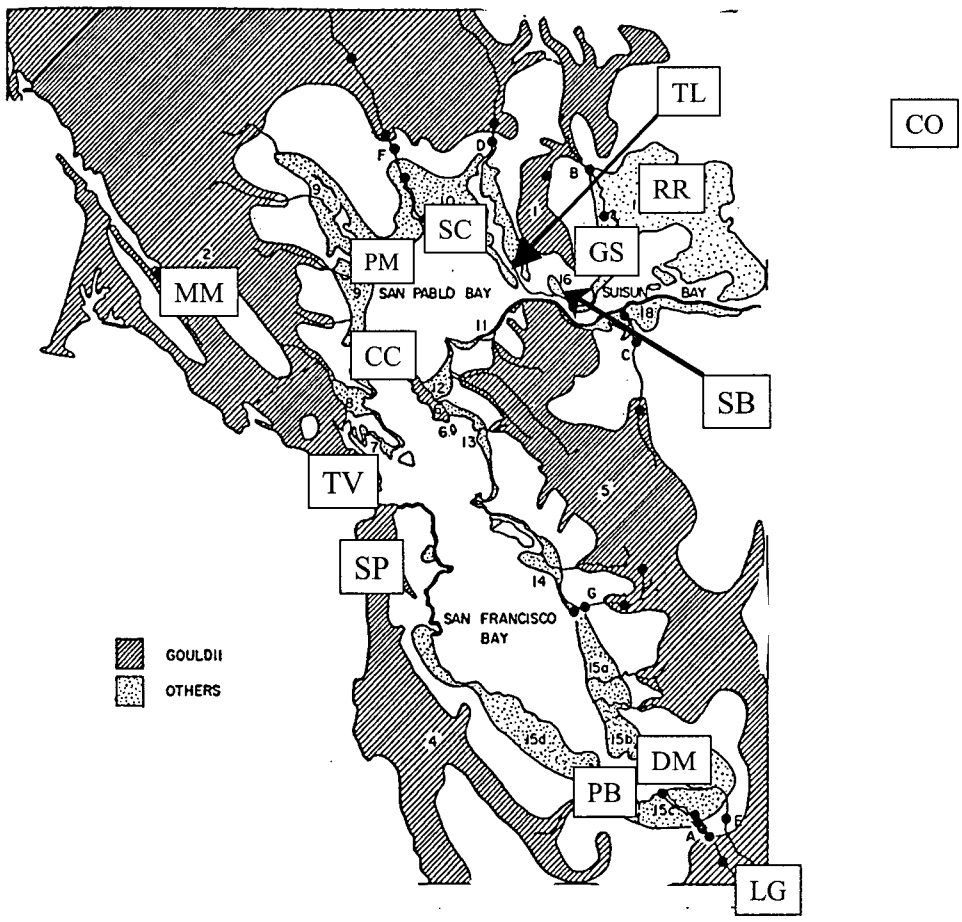


Figure 2.2. Plot of individual principal component scores 1 and 2. Subspecies group together although separation is not complete.

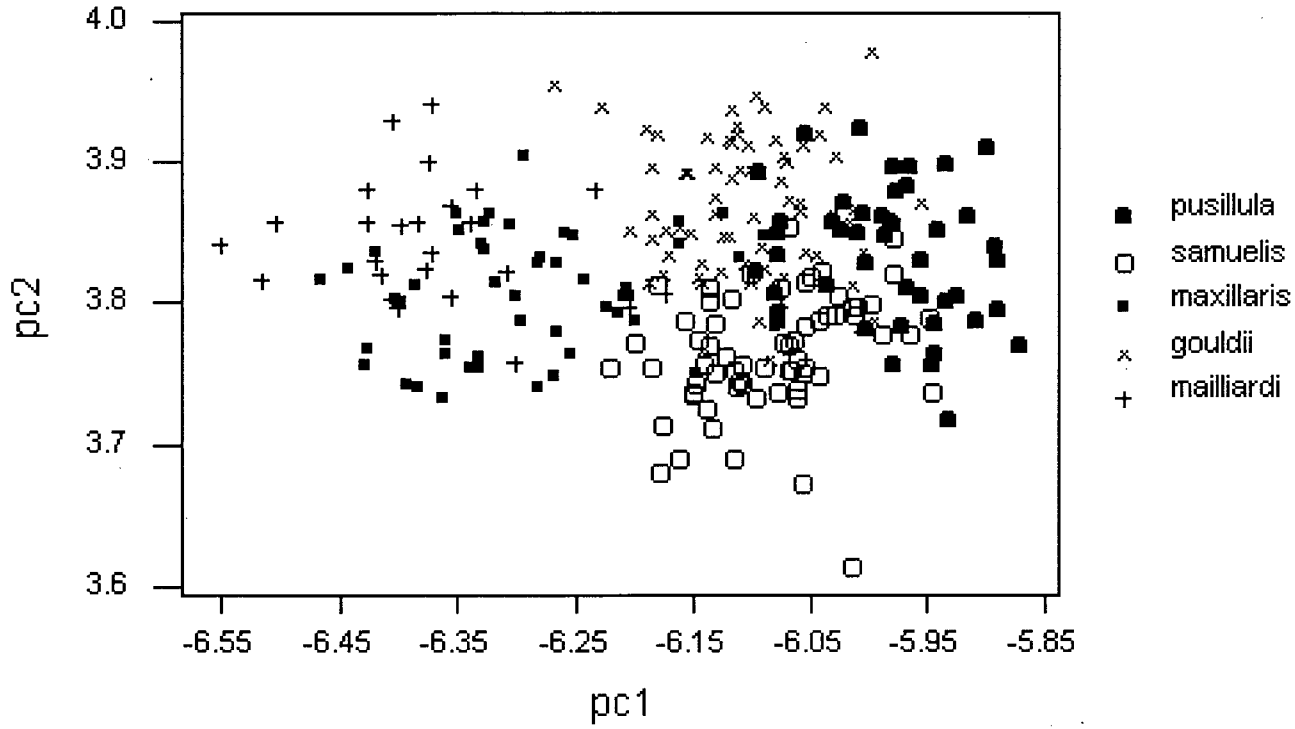


Figure 2.3. Group centroids for populations in canonical variate space. Populations group according to subspecies. (DM, PB = *pusillula*; PM, SC, CC, TL = *samuelis*; SB, GS, RR = *maxillaris*; SP, MM, TV, LG = *gouldii*, CO = *heermanni*). Distances between populations shorter than distances between subspecies. *Maxillaris* populations show clinal variation.

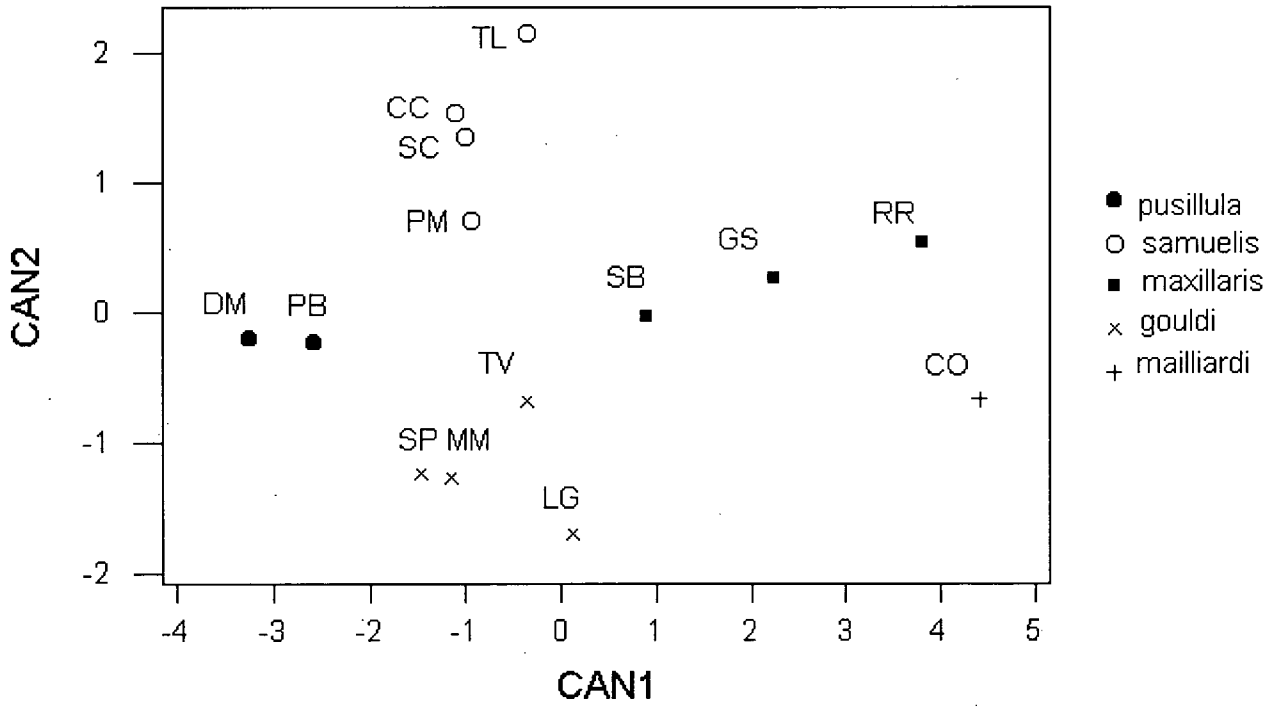


Figure 2.4. Hierarchical decomposition of variance components (%) for seven morphological traits from nested ANOVA adjacent to variance components derived from hierarchical AMOVA of 9 microsatellite loci. Variance between subspecies is high in morphological traits but low in microsatellites.

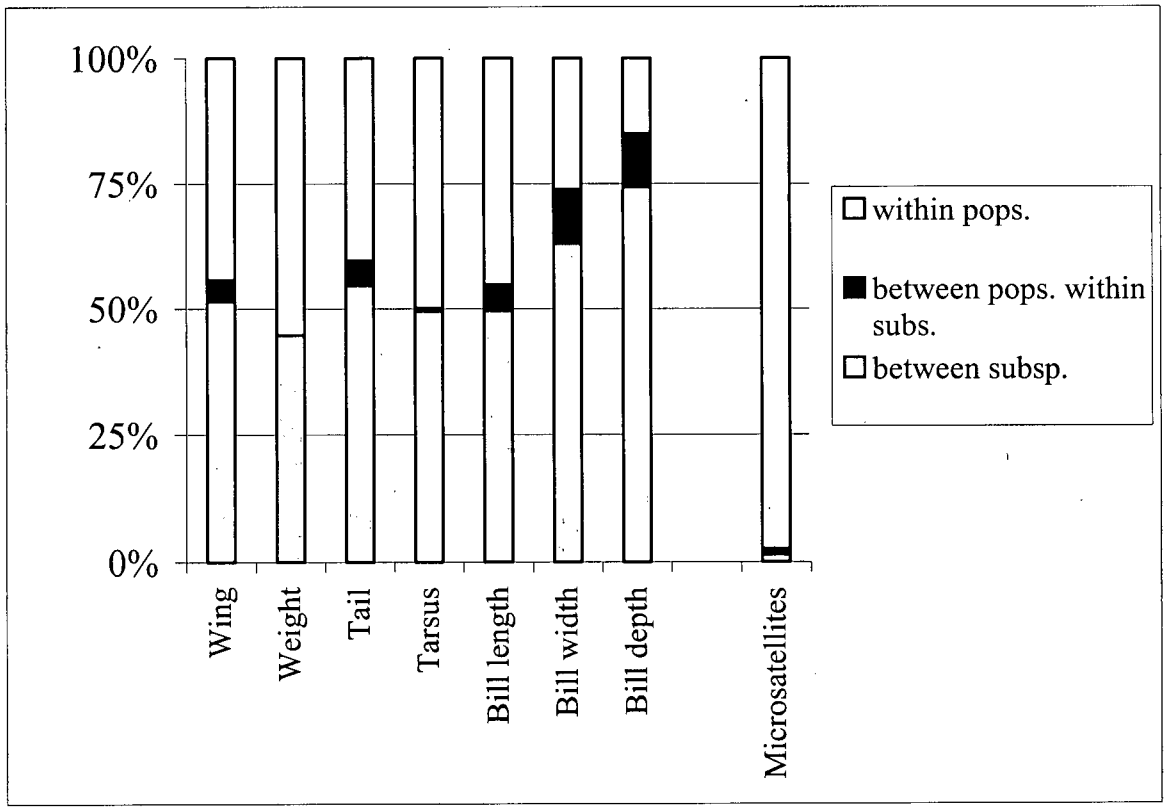
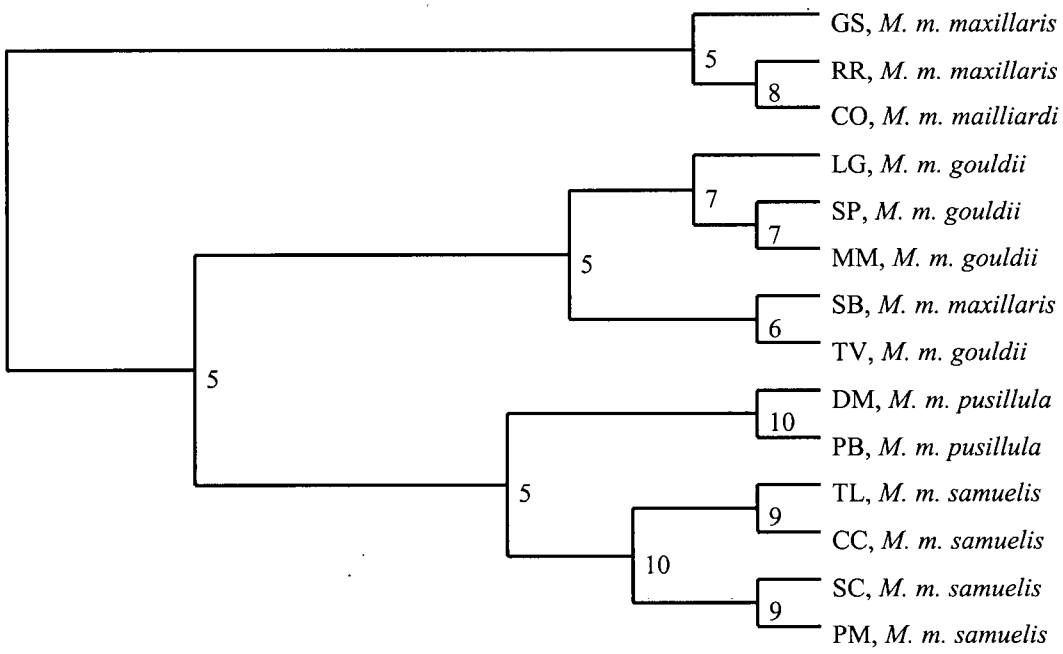
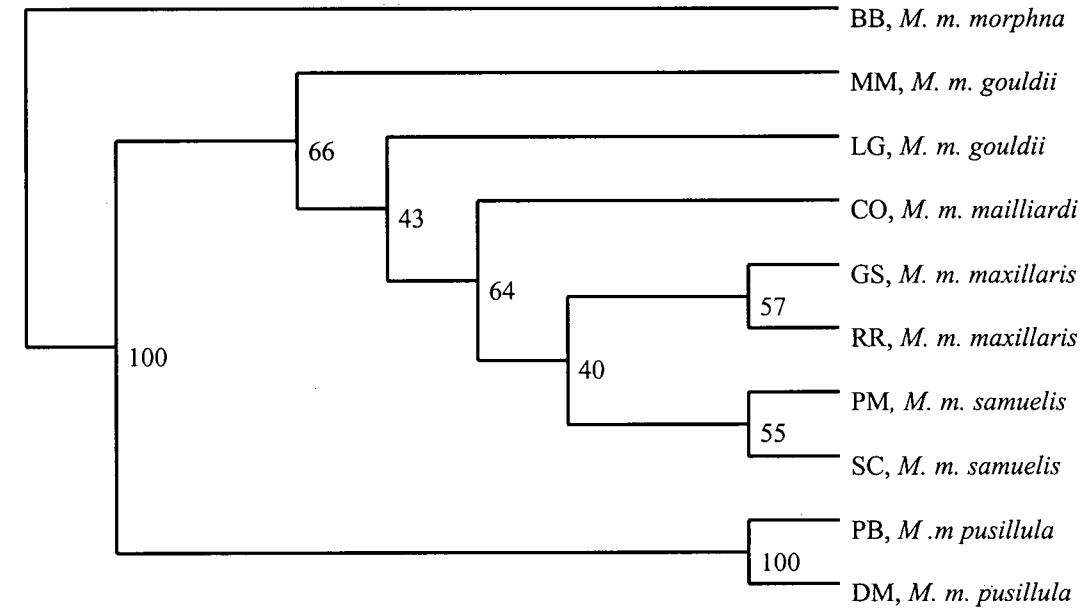


Figure 2.5. A) UPGMA dendrogram of 14 song sparrow populations based on Mahalanobis distances derived from discriminant analysis of seven morphological traits. Numbers to the right of the branch indicate jackknife support out of 10. B) Cavalli-Sforza and Edwards (Cavalli-Sforza and Edwards 1967) chord distance UPGMA (Sneath and Sokal 1973) phenogram calculated from 8 microsatellite loci. Numbers to the right of branch indicate bootstrap support over 100 replicates.

A.



B.



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