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Population Genetics of Island Endemics: Neutral and Major Histocompatibility Loci

By

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A dissertation submitted to the Graduate School of the University of Missouri–St. Louis in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology with an emphasis in Evolution

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Dissertation Abstract

Island archipelagoes are ideal for the study of microevolutionary forces due to their multiple, closely related but geographically disjunct populations. For my dissertation work, I used both neutral and major histocompatibility complex (MHC) loci to determine the population genetic structures of bird species endemic to the Galápagos Islands. MHC molecules play an integral role in the immune system by recognizing foreign pathogens. As a result, the high variability found at these loci is maintained primarily through selection for resistance to parasites. In addition to selection, MHC loci are also affected by neutral forces: mutation, gene flow, and genetic drift. I described variability at MHC class II genes in two bird species endemic to the Galápagos Islands and compared their MHC diversity with diversity at neutral loci, as well as MHC variability in their closest mainland relatives. Small island populations are predicted to have reduced genetic variability due to the effects of genetic drift; however, selection may be strong enough to prevent the loss of variability at MHC loci.

The Galápagos hawk (*Buteo galapagoensis*) has small breeding populations on eight islands. Analyses of both neutral nuclear VNTR (Chapter 1) and mitochondrial (Chapter 2) loci showed low within-population variability but high between-population differentiation. The mitochondrial analyses in Chapter 2 also indicated that Galápagos hawks split from their mainland sister species, the Swainson's hawk (*B. swainsoni*), relatively recently, likely less than 300,000 years ago. In Chapter 3, we found that smaller, more inbred populations had birds with higher louse loads and, in general, lower and less variable natural antibody titres than the larger, more genetically variable hawk populations.

Chapter 4 presents MHC work done on the Galápagos penguin (*Spheniscus mendiculus*), a seabird whose breeding colonies experience population bottlenecks associated with El Niño events. Previous work by others using neutral microsatellite loci showed that the penguins have very little genetic structuring among colonies, and they have low allelic richness. Their MHC diversity was correspondingly low and lower than that in their sister species, the Humboldt penguin (*S. humboldti*). Galápagos penguins had only three MHC alleles, which differed by only a few base pairs.

MHC work on the Galápagos hawks (Chapter 5) revealed similarly low variability. Galápagos hawks had fewer and less divergent alleles than the Swainson's hawk, their closest mainland relative. A subset of their alleles formed a low diversity cluster similar to ones documented in other species, though its function is unknown.

The MHC diversity in both the Galápagos penguin and hawk was lower than in the mainland species and similar to the low variability at neutral loci, indicating that genetic drift has had an overwhelming effect. Overall, these results, as well as analyses of the relationships among alleles from the pairs of closely related species, give us added insight into the relative strengths of the forces shaping MHC variability and more information about the evolution of MHC genes, which is still poorly understood in birds.

Lastly, in Chapter 6, I characterized the neutral population genetic structure of six Galápagos mockingbird (*Mimus* spp.) populations. Genetic variability increased with island area and we found a pattern of isolation by distance, both indicating the influence of genetic drift. Significant levels of genetic and morphological differentiation existed among all six populations, though morphological distances were smaller between islands of similar area suggesting the influence of natural selection.

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I will be forever grateful to my major advisor and mentor, Dr. Patricia Parker. Her infectious enthusiasm for science and her encouragement of undergrads to get involved started me along this path, and I have continued to benefit from her knowledge, experience, and generosity. Patty has contributed immeasureably to this dissertation through her astonishing intellect, insightfulness, and ability to ask questions, and I am grateful for all the support she has given me with her time, energy, and resources. I feel very fortunate to have been able to study in such a special place as Galápagos, work with an incredibly supportive group like the Parker Lab, and learn from a researcher as talented as Patty. As I grow as a scientist, I hope to cultivate in myself those qualities that have made Patty so successful in her career, and I hope we will continue to have a long and productive relationship.

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I want to thank the Charles Darwin Research Station and Galápagos National Park for allowing us to work in such a beautiful and truly special place and for logistical support, which greatly facilitated our fieldwork. Funding for this project was provided by the Saint Louis Zoo's Field Research for Conservation Program, the Harris World Ecology Center (formerly the International Center for Tropical Ecology), the American Ornithologists' Union, and the E. Desmond Lee and Family Fund's Collaborative Vision in Zoological Studies.

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Chapter 1

Population genetics of the Galápagos hawk (*Buteo galapagoensis*): genetic monomorphism within isolated populations

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ABSTRACT

Because of their smaller size and isolation, island populations tend to be more divergent and less genetically variable than mainland populations. We collected DNA samples from nine Galápagos hawk (*Buteo galapagoensis*) island populations, covering the species' entire range. Neutral minisatellite DNA markers were used to calculate within-island genetic diversity and between-island genetic differentiation (F_{ST}). Typically, these markers mutate too quickly to be informative in such studies. However, in very small, isolated populations, concerns about high mutational rate are obviated by the relative force of genetic drift. Individuals within islands had the highest levels of reported genetic uniformity of any natural bird population, with mean within-population band-sharing similarity values ranging from 0.693 to 0.956, increasing with decreasing island size. Galápagos hawks exhibit cooperative polyandry to varying degrees across islands; however, we did not find an association between degree of polyandry and genetic variability. Between-island F_{ST} values ranged from 0.017 to 0.896, with an overall archipelago value of 0.538; thus, most populations were genetically distinct. Also, we documented higher levels of genetic similarity between nearby populations. Our results

indicated negligible gene flow among most Galápagos hawk populations, and genetic drift has played a strong role in determining structure at these minisatellite loci.

KEY WORDS: *Buteo galapagoensis*, cooperative polyandry, Galápagos Hawk, Galápagos Islands, genetic drift, minisatellites

INTRODUCTION

Population genetic structure reflects a number of processes, such as mutation rate, genetic drift, gene flow, natural selection, and phylogeographic history (Bohonak 1999; Ouborg et al. 1999). Genetic variability is lost via genetic drift and selection against some genotypes. Generally, genetic drift has a stronger effect in smaller populations; thus, a positive relationship between population size and genetic variation is expected (Nevo et al. 1984; Frankham 1996). Populations may diverge due to random fixation of different alleles, differences in selective pressures, or the addition of novel mutations. Gene flow, however, can have a homogenizing effect among populations and mitigate the loss of intra-population variation by adding new alleles or replacing alleles lost due to drift (Slatkin 1985).

Populations on islands often have lower levels of genetic variation than those on the mainland (Frankham 1997). Populations of birds on island archipelagos tend to be more strongly differentiated than geographically separate mainland populations because water acts as an effective barrier to gene flow for many species (Williamson 1981; Boag 1986; Baker et al. 1990). These patterns of decreased genetic variation and increased differentiation may result from founder events that occurred at the time of colonization. In many cases, though, founding flock sizes may be large enough that founder effects are negligible (e.g. Clegg et al. 2002). Even when the number of founders is known to be

quite small, subsequent arrival of additional immigrants may prevent a measurable founder effect (Grant et al. 2001). Alternatively, lower variability and increased differentiation on islands may be due to sequential founder events (Clegg et al. 2002), long-term genetic drift working in small, isolated populations (Baker et al. 1990; Mundy et al. 1997), or a combination of the two.

The Galápagos hawk (Aves: Falconiformes: *Buteo galapagoensis*) is endemic to the Galápagos archipelago located almost 1000 km west of South America. The islands are volcanic in origin, having arisen from a mantle hotspot (Morgan 1971), and they have never been connected to the mainland. The oldest of the present islands is approximately four million years old (White et al. 1993). However, older, now submerged seamounts to the southeast of the archipelago indicate that islands have been present over the hotspot for at least seventeen million years and probably for much longer (Christie et al. 1992; Werner and Hoernle 2003).

Hawks are presently found on nine islands: Santa Fe, Española, Pinzón, Santiago, Santa Cruz, Isabela, Fernandina, Pinta, and Marchena (Fig. 1). Historically, humans have shot hawks, and the hawks are now extirpated from two human-inhabited islands, San Cristóbal and Floreana. The population on Santa Cruz (another human-inhabited island) may also have been extirpated; no adults have been seen on the island in recent years, but juveniles are seen periodically. Distances of less than 5 km up to around 240 km separate islands with Galápagos hawk populations (Fig. 1). The level of hawk migration between islands is unknown but presumed to be low (de Vries 1975), as most *Buteos* are reluctant to cross large bodies of water (Kerlinger 1985). Swainson's hawks (*Buteo swainsoni*) are the Galápagos hawk's closest mainland relatives (Riesing et al. 2003), and they migrate

long distances over land (from North America to Argentina) but avoid flying over water (Fuller et al. 1998).

Galápagos hawk populations vary morphologically and behaviorally, also suggesting genetic isolation. They differ in overall body size, and in allometry to a lesser degree, across islands (de Vries 1973; Bollmer et al. 2003). Galápagos hawks exhibit cooperative polyandry, where territorial groups consist of one female and up to eight (usually two or three) unrelated males (Faaborg and Patterson 1981; Faaborg et al. 1995). Paternity is shared within and among broods, though there are often more males in a group than the number of chicks produced per brood (1-2); all birds in the group defend the communal territory and care for the brood, including males that are not the genetic sires of the offspring (Faaborg et al. 1995; DeLay et al. 1996). One Galápagos hawk population appears to be monogamous (Española), while the rest exhibit cooperative polyandry to varying degrees, with mean group sizes ranging from 2.5 to 4.5 birds (de Vries 1975; Faaborg et al. 1980; Bollmer et al. 2003). The factors contributing to this variation in mating system (e.g. sex ratio, survivorship) are unstudied but are likely associated with differences in habitat structure and resource availability.

In this study, we described the genetic structure of all nine populations of Galápagos hawks (thus sampling the entire range of the species) using multilocus minisatellite DNA markers. Minisatellites are hypervariable regions of DNA consisting of tandem repeats of short units of nucleotides (Jeffreys et al. 1985), which have been used to characterize population structure (e.g. Freeman-Gallant 1996; Carneiro da Silva and Granadeiro 1999; Gullberg et al. 1999; Tarr and Fleischer 1999). We described the amount of genetic variation present in populations and measured the degree of

differentiation among populations using Wright's F_{ST}, the standardized variance in allele frequencies among populations (Wright 1951, 1978). We tested the prediction that genetic variation increases with population size by using total island area and total area of appropriate habitat as indices of population size. In addition to population size, variation in mating system is predicted to partly determine genetic variability by impacting effective population size, mostly through biased sex ratios and variance in reproductive success (Nunney 1993; Parker and Waite 1997). In the Galápagos hawk, there may be increased variance in reproductive success and more skewed sex ratios in the more polyandrous populations, which would lead to decreased effective population sizes relative to total population size and a more rapid loss in variation. We tested for an effect of mating system (degree of polyandry) on genetic variability after first controlling for island area. Finally, we asked whether populations closer in geographic proximity are more similar genetically due to increased gene flow or more recent separation (isolation by distance).

METHODS

Field methods. —We visited the Galápagos Islands for two to three months each year between May and August from 1998 to 2003. Hawks (n = 541) were captured on nine islands: 25 individuals from Santa Fe, 23 from three sites on Española (Gardner Bay, Punta Suarez, and Punta Cevallos), 287 from three sites on Santiago (James Bay, Sullivan Bay, and the highlands), 93 from Volcan Alcedo on Isabela, 41 from Pinta, 26 from Marchena, 10 from Pinzón, 32 from Fernandina, and 4 from Santa Cruz. The hawks were caught using two methods: a balchatri trap baited with a live prey animal

such as a rat (Berger and Mueller 1959) or a rope noose on a stick to capture perched birds (Faaborg et al. 1980). We banded each hawk with an aluminum and/or anodized color band and took two 50 µl blood samples via venipuncture of the brachial vein. Samples were immediately put into 500 µl of lysis buffer (100 mM Tris, pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al. 1988), shaken, and stored at ambient temperature.

Minisatellite DNA markers.—The use of hypervariable multi-locus minisatellite profiles (VNTRs) in studies of population genetic differentiation is typically problematic due to constraints imposed in part by a high mutational rate at these loci (Flint et al. 1999). Moreover, Flint et al. (1999) cautioned that calculating F_{ST} values between human populations using minisatellites yielded an underestimate of genetic differentiation when compared to the level found via other markers. Therefore, their use in characterizing population genetic differentiation, at least in light of this finding, is a statistically conservative methodology. However, in special cases, such as those involving isolated island vertebrate populations, "the fixation of restriction-fragment polymorphisms can outpace the generation of fragment-length variability through recombination" (Gilbert et al. 1990). This claim was buttressed by the finding that all bands were fixed within one population of the Channel Island fox, and that individual foxes within each island had diagnostic, island-specific bands. Clearly in this and analogous special cases, "differences among hypervariable restriction-fragment profiles can be used to estimate relative genetic variability and to reconstruct the evolutionary relationships of natural populations" (Gilbert et al. 1990) because concerns related to a high mutational rate are largely obviated by the relative force of genetic drift in small populations.

In this study, we extracted DNA and performed multilocus minisatellite DNA fingerprinting using the restriction endonuclease *Hae*III and Jeffreys' probe 33.15 (Jeffreys et al. 1985) following procedures described in Parker et al. (1995). After hybridization, we used a Storm 820 Phosphorimager to visualize fingerprints. For most populations, we used only a subset of the samples (n = 163) for genetic analyses: 15 from Santa Fe, 15 from Española, 37 from Santiago, 22 from Isabela, 20 from Pinta, 20 from Marchena, and 20 from Fernandina. From Pinzón and Santa Cruz, we used all birds sampled (10 and 4, respectively), and they were all juveniles. For the other populations, we randomly selected individuals from the pool of sampled territorial adults (the class most likely to consist of non-relatives). We did not run all samples; however, fewer individuals are necessary to get a representative sample when populations (such as these) are lacking in genetic variability. We ran a total of nine gels, with 17 to 26 lanes each. We ran samples in alternating blocks of three to seven individuals from each island, so that multiple islands were represented on each gel. We chose four individuals from different islands as ladders and ran them on each of the gels. From the banding patterns, we created a presence-absence matrix of bands (alleles) encompassing all individuals. Due to high within-population genetic uniformity, the presence of a number of bands fixed across populations, and the ladders on each of the gels, we were able to reliably score across gels.

We assumed that bands were assorting independently and calculated within- and between-island similarity indices as $S = 2S_{AB} / (2S_{AB} + N_A + N_B)$, where S is the proportion of bands shared, S_{AB} is the number of bands shared by individuals A and B, N_A is the number of bands unique to individual A, and N_B is the number of bands unique to

individual B (Wetton et al. 1987; Lynch 1988, 1990). We calculated these from our presence-absence matrix using the program GELSTATS v. 2.6 (Rogstad and Pelikan 1996).

In fingerprinting, individuals are often used in multiple pairwise comparisons, thus resulting in nonindependence of band-sharing values (Danforth and Freeman-Gallant 1996; Call et al. 1998; Leonard et al. 1999). We used the *p-dif* test (Bertorelle et al. 1999) in the program Watson (Bucchini et al. 1999), a test that permutes individuals, not band-sharing values, to ask if within-island band-sharing values significantly differed from between-island values. We calculated F_{ST} values for each pairwise comparison of islands, as well as an overall archipelago value, according to Lynch (1990, 1991). F_{ST} values attain a maximum value of one when two subpopulations are fixed for different alleles (complete differentiation) and fall to zero when alleles are distributed randomly among subpopulations (no differentiation).

We used a linear regression to test the prediction that population genetic uniformity (as measured by within-island similarity indices) decreases with increasing island area. We calculated total island area in the program ArcMap 9.0 using digitized vegetation coverage maps obtained from the Charles Darwin Research Station. The projections were in decimal degrees, so we converted the areas to square kilometers (1 degree ≈ 111 km) and used the log of island area in the regression. Large portions of some of these islands (up to 75% of total island area) are barren of vegetation, making them less suitable for hawk territories. Total island area may therefore overestimate population size in some cases, so we performed a second regression using the log of total vegetated area (excluding lava and beaches). We tested for an effect of mating system

with a general linear model, using band-sharing values as the dependent variable, mean group size as a fixed factor, and log of total island area as a covariate. Due to the non-independence of minisatellite band-sharing values, we first randomly selected a subset of independent values (using each individual once) from each population. For mating system, we classified each island as having a mean group size of less than two males or more than two males using published data from de Vries (1975) and Bollmer et al. (2003) and new data collected from Fernandina in 2003 (1.4 ± 0.5 males per group, *n* = 10 groups). So, we classified Española, Santa Fe, Pinzón, and Fernandina as less polyandrous (mean group sizes of 1-1.5 males) and Isabela, Santiago, Marchena, and Pinta as more polyandrous (mean group sizes of 2.3-3.5 males). We used a Mantel (1967) test to examine isolation by distance (Slatkin 1993), testing the prediction that genetic differentiation among populations (F_{ST}) should increase with increasing geographic distance between them. We log-transformed the distance between islands as measured between nearest points. We performed these analyses in SPSS v. 10.0.5 for Windows (SPSS Inc. 1999) and IBDWS v. 2.0 beta (Jensen et al. 2004). We excluded Santa Cruz from the above analyses due to its small sample size.

Because there does not appear to be a breeding population on Santa Cruz, we performed an assignment test to see whether the juveniles we captured on Santa Cruz closely matched any of the other populations, which would indicate they could be migrants. While there are no tests designed for codominant minisatellite data, the online program Doh (Brzustowski 2002) as first described in Paetkau et al. (1995) can accommodate data from dominant markers by treating each band as a separate locus. We performed a segregation analysis by tallying, within each population, the co-occurrences

of each band with every other band in order to note cases of linkage (bands always appearing together within individuals) and allelism (individuals always having one or the other band but never both, indicating they belong to the same locus). We found no cases of linkage, and we eliminated all cases of allelism (most due to rare bands) by removing the less frequent band from each allelic dyad. We entered the remaining 23 independent bands into the Doh program as presence/absence data for each individual. The program assigns each individual into the population in which its genotype has the highest probability of occurring.

RESULTS

Within-population similarity.—We scored an average (± SD) of 14.1 ± 1.42 bands for each individual. Within-island similarity indices were high, ranging from 0.693 for Isabela to 0.956 for Santa Fe (Table 1; Fig. 2). The mean similarity index for Santa Cruz was slightly lower (0.657), but this is based on only six pairwise comparisons. Birds from Santa Fe were particularly lacking in genetic variation, having only a few variable bands. Specifically, 13 of the 16 Santa Fe bands scored were fixed in the population. All 15 Santa Fe birds were identical to two or three other birds, resulting in only four different genotypes in that population. In addition, four of the 10 birds on Pinzón were identical, while there were two sets of identical birds (two and three birds each) out of 15 individuals sampled on Española and four sets of identical birds (two or three birds each for nine total) on Marchena. The other populations (Isabela, Fernandina, Santiago, and Pinta) were more variable and had no identical individuals.

Regression analyses supported our prediction that genetic similarity among individuals in a population decreases with increasing total island area (r = -0.844, df = 7, P = 0.008; Fig. 3) and vegetated area (r = -0.846, df = 7, P = 0.008), though there was no substantial difference between the two measures. A general linear model showed there was no effect of degree of polyandry on genetic variability after controlling for island area (F = 0.537, P = 0.466, n = 78), while there was still a strong island area effect after controlling for mating system (F = 32.1, P < 0.0001, n = 78).

Population differentiation.—Between-island F_{ST} values ranged from 0.017 to 0.896 (Table 2) with an overall archipelago value of 0.538. We performed pairwise permutation tests to test whether populations were significantly distinct from each other. There were 28 pairwise comparisons, so we used a Bonferroni correction to avoid Type I errors, which brought our alpha level down to 0.002. Twenty-three of the 28 comparisons still showed significant differences among populations (P < 0.001 for all). Four of the five nonsignificant values involved Pinzón compared to Isabela (P = 0.058), Fernandina (P = 0.021), Santiago (P = 0.820), and Pinta (P = 0.006). The remaining comparison, Isabela vs. Fernandina (P = 0.203), had the lowest F_{ST} value (0.017; Table 2). Three of the five nonsignificant values also represent the three smallest interisland distances.

We had predicted that populations would exhibit isolation by distance. A Mantel test confirmed this, showing a significant pattern of increasing genetic differentiation with increasing distance between islands (r = 0.626; $P \leq 0.003$; Fig. 4).

Between-island dispersal.—Over the past few decades, juveniles have occasionally been seen on islands where there was no resident hawk population, but no

individual banded on one island had ever been observed on another island. In 2003, however, we observed two banded individuals on Fernandina, an island where hawks had not previously been studied. One individual, a territorial adult female, had been banded by us as a second-year juvenile on Volcan Alcedo, Isabela in 1998. The other bird was a territorial male whose band could not be read. It is very likely he was also banded as a juvenile on Alcedo in 1998, since 70 birds were caught there in two days, 64 of which were juveniles. Also, it is unlikely he could have come from an island other than Isabela, because Isabela separates Fernandina from all the other islands (Fig. 1).

In Table 3 we present the results of the assignment test for each population. The program accurately assigned all the individuals from the more genetically monomorphic Española, Santa Fe, Pinzón, and Marchena populations to their home islands, while there were misassignments among the larger populations, likely due to their greater genetic variability. The assignment test placed the four Santa Cruz juveniles into the populations they most closely matched. One of the four individuals caught on Santa Cruz had a banding pattern identical to one of the Santa Fe genotypes, and the assignment test placed it within the Santa Fe population. Another of the Santa Cruz individuals had a banding pattern very similar to those on Pinzón (mean band-sharing between it and the Pinzón individuals was 0.911 ± 0.03), and the assignment test placed it within the Pinzón population. The last two Santa Cruz individuals matched Santiago best, though the chance for an assignment error is higher for the more variable populations.

DISCUSSION

Genetic variation within populations.—In this study, we were able to characterize population genetic structure of nine Galápagos hawk populations, covering their entire species range. The hawk populations exhibited very little genetic variation, having within-population similarity indices ranging from 0.6 to over 0.9 at hypervariable minisatellite loci. To our knowledge, the smaller Galápagos hawk populations have the highest reported levels of monomorphism at minisatellite loci of any natural bird population, though some populations of New Zealand birds (reviewed in Miller et al. 2003) and other endangered island bird species (e.g. Rave 1995; Caparroz et al. 2001) are nearly as inbred. Gilbert et al. (1990) found even higher mean band-sharing values for populations of Channel Island foxes (*Urocyon littoralis*), another top predator, ranging from 0.75 up to 1.00. In contrast, unrelated birds in outbred mainland populations typically have band-sharing values around 0.2 and 0.3 (Parker Rabenold et al. 1991; Papangelou et al. 1998). Although there are no published studies using minisatellites in other *Buteos*, mean band-sharing within a small sample of overwintering Swainson's hawks was 0.374 ± 0.10 ($n = 8$; unpubl. data). So, the Galápagos hawk's ancestral mainland polymorphism was likely much higher.

Extremely low genetic variability within this species is probably the result of a single founder event coupled with long-term genetic drift. The *Buteo* phylogeny by Riesing et al. (2003) shows a very recent divergence between Galápagos and Swainson's hawks, and mtDNA work underway on the Galápagos hawks indicates a single colonization event from the mainland (Bollmer, Kimball et al., unpubl. data). Although there is evidence that island colonizations may not always result in a significant decrease in genetic diversity (Clegg et al. 2002; Grant 2002), in this case, the founding population

of hawks may have been small enough that a severe bottleneck occurred. The high mean inter-island band-sharing (0.617) and the presence of bands that are fixed across all populations (even though most populations are currently genetically isolated) suggest that hawks became inbred early on in their colonization of the islands. The close relationship between island area and genetic variation across populations indicates that long-term genetic drift has also been an important factor influencing the level of variability in the Galápagos hawk. The smallest populations have become fixed or nearly fixed for many of their bands, with different bands being common in different populations.

Within-island genetic uniformity decreased significantly with increasing population size, as approximated by total island area and vegetated area. While total island area explained a large portion of the variance in genetic similarity ($r = -0.844$), we had supposed that population size (and thus genetic variability) would correlate even more strongly with vegetated area due to the presence of large tracts of barren lava on some islands. Using only vegetated area, however, did not substantially improve the correlation ($r = -0.846$), even though five of the islands are less than 70% vegetated, two greatly so. We excluded Santa Cruz from this analysis because it differs from the rest of the islands in that it has an artificially small population on a large island due to the human impact there. Even though the Santa Cruz population is almost certainly the smallest in the archipelago, the four juvenile hawks sampled there exhibited the lowest mean similarity of any of the populations, probably due to inter-island movements of birds, which will be discussed below.

We found that there was no effect of mating system on genetic variability of Galápagos hawk populations. We had predicted that increased polyandry might result in

lowered effective population sizes relative to total population size due to increased variance in male reproductive success or more strongly biased sex ratios. The lack of difference between low and high polyandry populations shows that mating system is not a strong determinant of genetic variability in the Galápagos hawk; shared paternity may mitigate the effects of increased polyandry. Also, population size accounts for such a large portion of the variance in within-island genetic similarity that there is little remaining variability upon which other forces could act.

Genetic divergence among populations.—Overall, the high F_{ST} values indicate that Galápagos hawks are reluctant to cross large stretches of water, which is consistent with the migratory behavior of their closest mainland relatives (Fuller et al. 1998). Most hawk populations appear to be significantly genetically different from each other, with the exception of the interaction between Isabela and Fernandina and four comparisons involving Pinzón. The comparisons involving Pinzón are more suspect given that we sampled only 10 individuals on Pinzón, all of which were floater juveniles instead of territorial adults. Also, the use of the Bonferroni correction increased the probability of Type II errors, especially for the two comparisons with *P*-values of 0.006 (Pinzón vs. Pinta) and 0.021 (Pinzón vs. Fernandina). These two comparisons are also the most geographically distant of the nonsignificant values.

The hawk populations were divergent to varying degrees, as indicated by the pattern of isolation by distance. Lower F_{ST} values between nearby populations may be the result of ongoing (albeit relatively rare in most cases) gene flow between them, more recent population separation, or a combination of the two. Española and Santa Fe were the most divergent from the rest of the archipelago, with F_{ST} values between them and the

other islands ranging from 0.5 to 0.9. Their relatively extreme divergence (especially from each other) is likely due to the random fixation of alleles in these populations that are not common on other islands.

Fernandina and Isabela were indistinguishable at these minisatellite loci. Of all island pairs, they are separated by the shortest distance (< 5 km), and we observed a bird banded on Isabela residing in a territory on Fernandina. The lack of differentiation between these two populations, therefore, could be due to ongoing gene flow. Alternatively, their similarity could be due to more recent separation or drift acting more slowly in larger populations. With the current data we are unable to distinguish among these scenarios.

The four juveniles we captured on Santa Cruz are likely migrants from neighboring islands. When fledglings leave their territories, they spend at least three or four years in a non-territorial floater population, roaming all over their native island and occupying areas not used by territorial birds (de Vries 1975). Because of this nomadic behavior, we suggest that juveniles are much more likely than adults to move between islands. Dispersal of juveniles to Santa Cruz could be more probable than movement to other islands, because Santa Cruz is mostly or entirely uninhabited by a territorial adult population, which means that suitable habitat is vacant, and juveniles are not likely to be harassed and driven away by adults. The assignment test placed two of the birds into the Santa Fe and Pinzón populations with high degrees of probability. The other two were most similar to Santiago, though there is more likely to be a misassignment when dealing with more variable populations. Santiago is a likely source population because it supports a large floater population and is an adjacent island. We cannot eliminate the

possibility that one or more of these birds was born on Santa Cruz since we could not compare them to a sample of resident Santa Cruz territorial birds, because of the lack of known breeding adults there.

Island archipelagoes are well known as arenas for radiations of species (e.g. Darwin's finches, Hawaiian honeycreepers). Although we have described morphological and behavioral differences among populations of Galápagos hawks (Bollmer et al. 2003), and now the genetic differentiation shown here, these differences are on a microevolutionary scale. Presumably, hawks are one of the more recent arrivals to the archipelago, and have not been there long enough to diverge into subspecies or new species. Drift has had a strong influence on divergence at these neutral minisatellite markers, but the importance of drift in speciation is debatable (Barton 1998). Given the genetic isolation of many of these hawk populations, the Galápagos hawk may one day match the patterns seen in other sedentary species groups in the archipelago (e.g. the Galápagos tortoises [*Geochelone elephantopus* subspp.], lava lizards [*Microlophus* spp.]), with multiple subspecies or species restricted to one or a few islands.

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Table 1 Mean within-island Galápagos hawk minisatellite band-sharing value (± SD), total island area, and percent of each island that is vegetated (not lava or beach); islands are listed in order of increasing area as calculated from the digitized maps.

Island	Within-Island *S*	Area (km^2)	% Vegetated
Pinzón	0.903 ± 0.067	18.1	95.2
Santa Fe	0.956 ± 0.032	24.8	100.0
Pinta	0.765 ± 0.083	59.4	62.0
Española	0.900 ± 0.052	61.1	98.2
Marchena	0.891 ± 0.047	128.8	25.4
Santiago	0.711 ± 0.086	577.5	68.6
Fernandina	0.719 ± 0.101	647.6	30.5
Santa Cruz	0.657 ± 0.157	984.1	100.0
Isabela	0.693 ± 0.086	4,710.7	66.5

Table 2 Pairwise comparisons of between-island differentiation in Galápagos hawks. Mean between-island band-sharing values (± SD) are above the diagonal, with total number and number of independent pairwise comparisons scored in parentheses. F_{ST} values are reported below the diagonal.

	Española	Santa Fe	Pinzón	Isabela	Fernandina	Santiago	Marchena	Pinta
Española	~	0.306 ± 0.03 (225, 15)	0.656 ± 0.04 (150, 10)	0.546 ± 0.08 (330, 15)	0.534 ± 0.10 (300, 15)	0.593 ± 0.08 (555, 15)	0.579 ± 0.05 (300, 15)	0.563 ± 0.70 (300, 15)
Santa Fe	0.896	~	0.489 ± 0.04 (150, 10)	0.485 ± 0.08 (330, 15)	0.443 ± 0.08 (300, 15)	0.509 ± 0.07 (555, 15)	0.404 ± 0.05 (300, 15)	0.470 ± 0.07 (300, 15)
Pinzón	0.714	0.862	~	0.702 ± 0.08 (220, 10)	0.716 ± 0.09 (200, 10)	0.737 ± 0.07 (370, 10)	0.753 ± 0.05 (200, 10)	0.748 ± 0.07 (200, 10)
Isabela	0.551	0.659	0.322	~	0.701 ± 0.09 (440, 20)	0.669 ± 0.09 (814, 22)	0.641 ± 0.08 (440, 20)	0.632 ± 0.09 (440, 20)
Fernandina	0.591	0.708	0.335	0.017	~	0.675 ± 0.09 (740, 20)	0.631 ± 0.08 (400, 20)	0.636 ± 0.10 (400, 20)
Santiago	0.522	0.661	0.266	0.100	0.123	~	0.672 ± 0.07 (740, 20)	0.667 ± 0.08 (740, 20)
Marchena	0.752	0.872	0.583	0.421	0.472	0.393	~	0.753 ± 0.08 (400, 20)
Pinta	0.617	0.737	0.341	0.264	0.291	0.213	0.304	~

Table 3 Results of Galápagos hawk assignment test using minisatellite data. Rows represent the populations in which we sampled the individuals, while columns represent the populations to which Doh assigned the individuals. Santa Cruz is listed only as an island of capture, because there is no resident hawk population there with which possible migrants could be compared.

	Española	Santa Fe	Pinzón	Isabela	Fernandina	Santiago	Marchena	Pinta
Española	15	0	0	0	0	0	0	0
Santa Fe	0	15	0	0	0	0	0	0
Pinzón	0	0	10	0	0	0	0	0
Isabela	0	0	2	10	8	2	0	0
Fernandina	0	0	0	5	13	2	0	0
Santiago	0	0	1	5	5	23	0	3
Marchena	0	0	0	0	0	0	20	0
Pinta	0	0	1	1	0	0	5	13
Santa Cruz	0	1	1	0	0	2	0	0

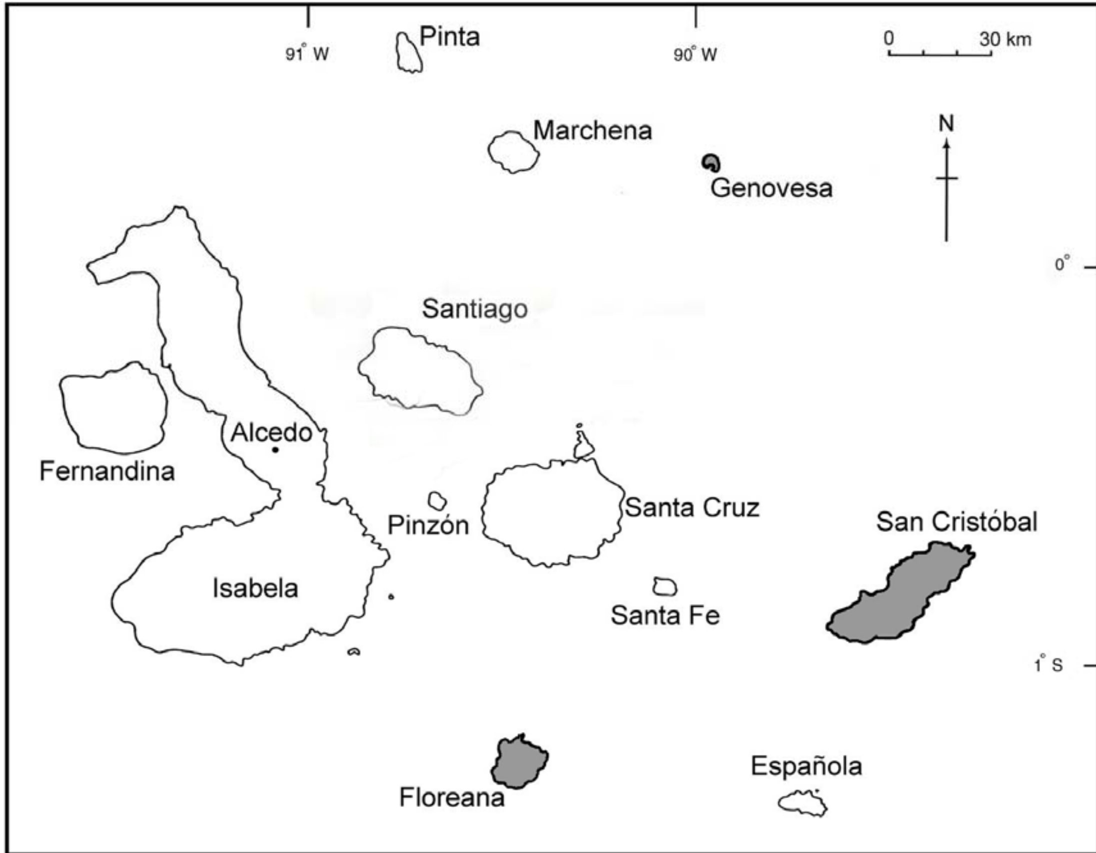
FIGURE LEGENDS

Fig. 1 Distribution of the Galápagos hawk on the Galápagos Islands. All labeled islands currently have hawk populations except for three islands that are shaded. Genovesa has never supported a hawk population, and the populations on San Cristóbal and Floreana have been extirpated by humans.

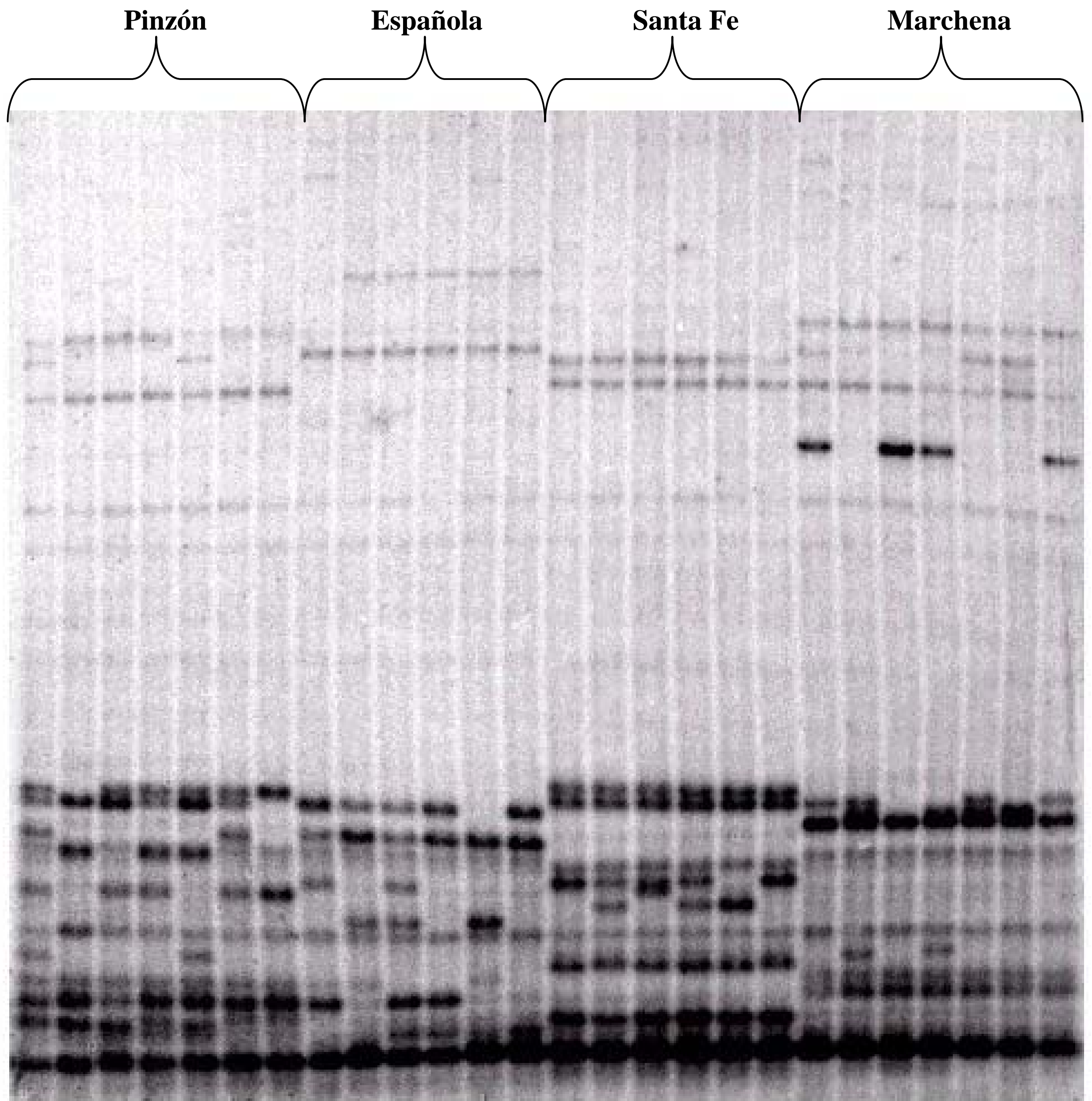
Fig. 2 An example of a multilocus minisatellite DNA fingerprinting gel of Galápagos hawks. Each lane represents the fingerprint of an individual randomly selected from those sampled on the four study islands named above the gel. Some of these populations exhibit the highest levels of monomorphism at minisatellite loci of any natural bird population studied. Note that several bands are unique to and/or fixed in their respective island populations, highlighting the powerful effect genetic drift has had in this system in limiting neutral genetic variance within-islands and increasing it among-islands.

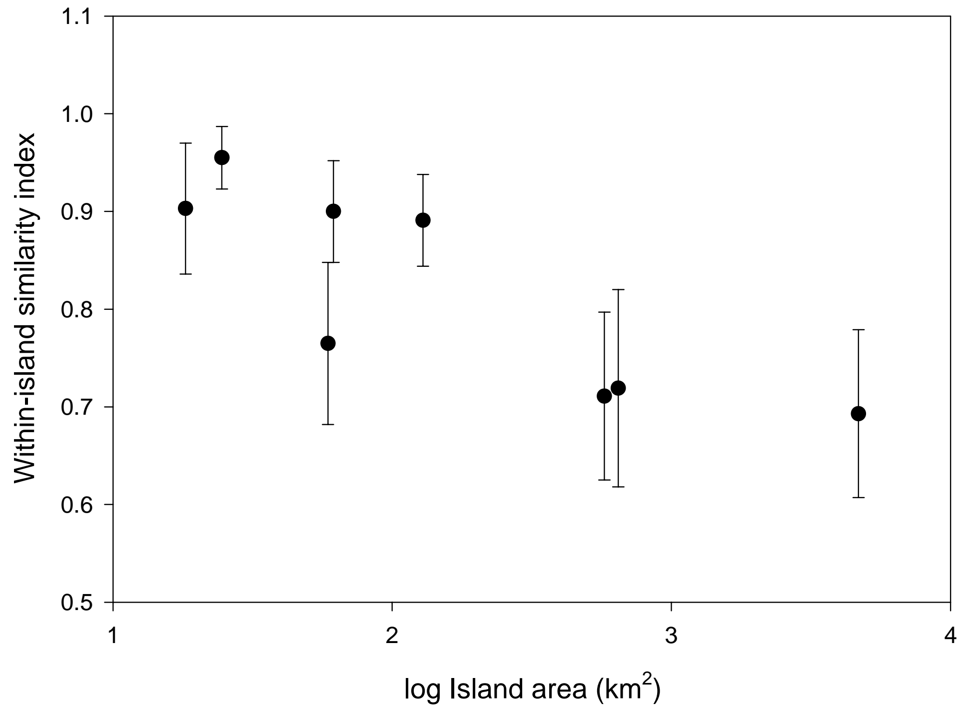
Fig. 3 Plot of mean genetic similarity (±SD) of Galápagos hawk individuals within islands against the log of island area (km^2). The data support our prediction that within-population genetic similarity should decrease with increasing island size.

Fig. 4 Plot of pairwise inter-island F_{ST} values against the log of geographic distances (km) between islands for Galápagos hawks. The degree of genetic differentiation between populations increases with increasing geographic distance.

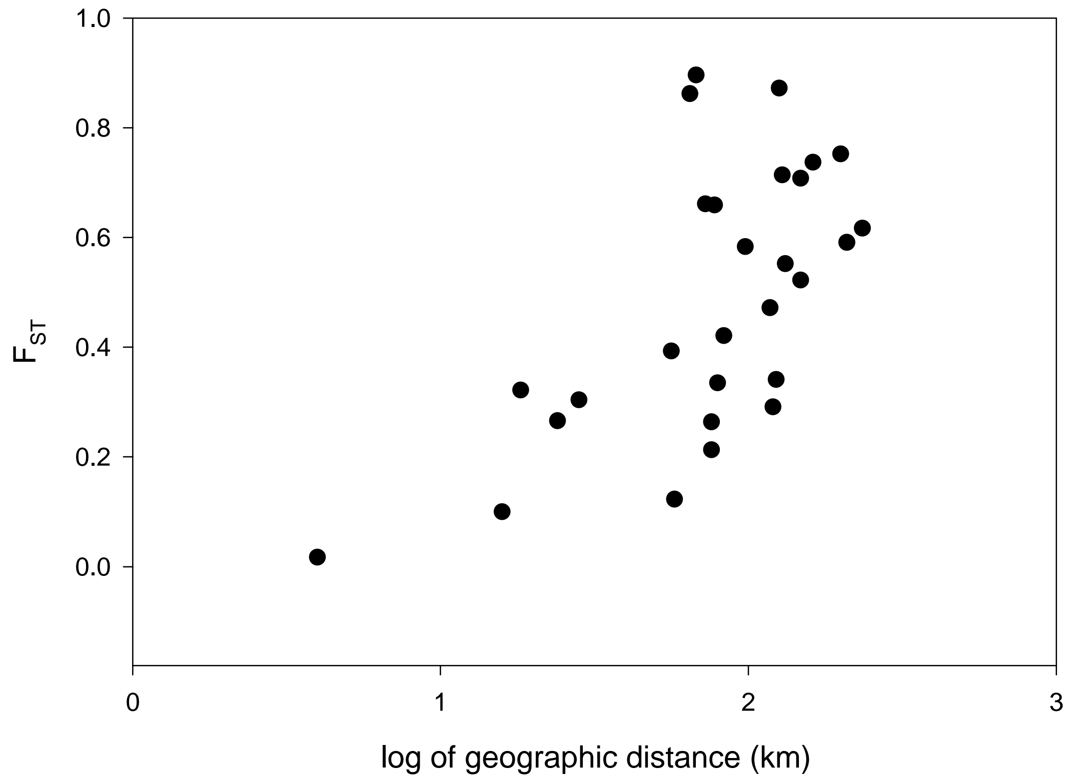


Pinta
91° W
90° W
0
30 km
N
Marchena
Genovesa
0°
Santiago
Fernandina
Alcedo
Pinzón
Santa Cruz
San Cristóbal
Isabela
Santa Fe
1° S
Española
Floreana


Pinzón
Española
Santa Fe
Marchena



Within-island similarity index
1.1
1.0
0.9
0.8
0.7
0.6
0.5
1
2
3
4
log Island area (km2)


1.0
0.8
0.6
0.4
0.2
0.0
F_ST
0
1
2
3
log of geographic distance (km)

Chapter 2

Phylogeography of the Galápagos hawk (*Buteo galapagoensis*): A recent arrival to the Galápagos Islands

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ABSTRACT

Galápagos hawks (*Buteo galapagoensis*) are one of the most inbred bird species in the world, living in small, isolated island populations. We used mitochondrial sequence and nuclear minisatellite data to describe relationships among Galápagos hawk populations and their colonization history. We sampled ten populations (encompassing the entire current species range of nine islands and one extirpated population), as well as the Galápagos hawk's closest mainland relative, the Swainson's hawk (*B. swainsoni*). There was little sequence divergence between Galápagos and Swainson's hawks (only 0.42% over almost 3 kb of data), indicating that the hawks colonized Galápagos very recently, likely less than 300,000 years ago, making them the most recent arrivals of the studied taxa. There were only seven, closely related Galápagos hawk haplotypes, with most populations being monomorphic. The mitochondrial and minisatellite data together indicated a general pattern of rapid population expansion followed by genetic isolation of hawk breeding populations. The recent arrival, genetic isolation, and phenotypic differentiation among populations suggest that the Galápagos hawk, a rather new species itself, is in the earliest stages of further divergence.

KEY WORDS: *Buteo galapagoensis*; Galápagos hawk; minisatellite DNA; mitochondrial DNA; phylogeography

1. Introduction

Island archipelagos have long been valuable for understanding evolutionary processes (Darwin, 1859; Grant, 1998; Whittaker, 1998). The relatively small size and isolation of populations on archipelagos often results in the occurrence of multiple, closely related yet distinct lineages on neighboring islands. There are numerous examples of radiations occurring in a variety of taxa on island systems around the world (e.g., Wagner and Funk, 1995). The refinement of phylogenetic techniques has opened up new avenues of investigation of these systems (Grant, 2001; Emerson, 2002), revealing mainland source populations and colonization patterns within archipelagos (e.g., Warren et al., 2003).

The Galápagos Islands, located on the equator 1000 km west of mainland Ecuador, are one of the most isolated archipelagos in the world and thus have a high degree of endemism. Almost a third of the plant species and half of the insect species are endemic (Tye et al., 2002). Fifty-nine percent of the vertebrates are endemic, including all of the native reptile and terrestrial mammal (rats) taxa (Tye et al., 2002). Endemism is high among the native terrestrial birds (84%) also, but it is much lower among the seabirds (26%) and shorebirds (23%; Tye et al., 2002). Though many taxa have speciated from their mainland ancestors, radiations within the Galápagos archipelago are

relatively rare compared to other, older archipelagos where taxa have had more time to speciate (Tye et al., 2002).

The islands in the Galápagos archipelago form over a mantle hotspot and drift in a southeasterly direction with the movement of the Nazca plate. The current islands range from less than half a million years old in the west up to 4 million years old in the east (White et al., 1993); however, older, now submerged islands indicate that islands have been present over the hotspot for at least 17 million years (Christie et al., 1992; Werner and Hoernle, 2003).

Radiations within Galápagos vertebrate lineages are skewed toward the reptiles and mammals, with few occurring among the birds (Table 1). There are about 40 recognized reptile taxa (including species and subspecies, depending on the latest taxonomic revisions). These 40 likely arose from only nine or ten original lineages from the mainland. The species and subspecies within taxa are generally isolated on different islands or volcanoes within an island. Within the mammals, the rice rats underwent a radiation, while neither of the two bat species have done so.

The pattern among the terrestrial birds is distinctly different from that of the reptiles. Only two of the founding bird lineages radiated into multiple species on the archipelago: the finches and the mockingbirds (Table 1). Two subspecies of Galápagos dove have been recognized (Swarth, 1931), but the rest of the taxa (even though they are all present on multiple islands) have not been subdivided. So, the 30 distinct lineages of terrestrial birds present now arose from only 14 colonizing lineages. This is a 2:1 ratio of current to colonizing lineages, whereas the reptiles are about 4:1. The 2:1 ratio is highly skewed by the finch radiation, the complexity of which is unique among Galápagos birds.

Excluding the finches, the relationship drops to 1.4:1. None of the 32 lineages of seabird or aquatic/shorebird have radiated within the Galápagos Islands. This striking difference between birds and reptiles has two possible explanations. First, birds are obviously more mobile, and so gene flow among populations might be preventing further divergence. Second, most of the bird species might have colonized the archipelago more recently and thus have not had time to diverge. Both explanations are supported by the lower degree of endemism seen among the birds, especially the waterbirds. It is possible that the lack of differentiation within bird lineages is due to their being not as well studied as the reptiles, but most Galápagos vertebrate lineages have been recognized for decades from extensive museum collections (long before genetic studies on particular taxa).

1.1. Galápagos hawk

Here, we characterize the population genetic structure and colonization history of one of these terrestrial bird species, the endemic Galápagos hawk (*Buteo galapagoensis*). The islands' only diurnal raptor, this hawk is widely distributed within the archipelago, currently inhabiting nine islands: Española, Santa Fe, Pinzón, Santiago, Isabela, Fernandina, Marchena, Pinta, and Santa Cruz. Once the "center of abundance" of the species distribution (Gifford, 1919), the Santa Cruz breeding population may now be extinct, though juveniles are occasionally seen there (Bollmer et al., 2005). To our knowledge, hawks have never existed on Genovesa, and their populations on Floreana (Steadman and DeLeon, 1999) and San Cristóbal were extirpated due to human activities. Morphological studies have been inconclusive as to the putative mainland sister species of the Galápagos hawk, focusing on several New World *Buteo* species (Brown and Amadon, 1968; Mayr and Short, 1970; Voous and de Vries, 1978). Molecular

phylogenetic studies suggest that Galápagos hawks are most closely related to the Swainson's hawk (*B. swainsoni*; Fleischer and McIntosh, 2001; Riesing et al., 2003), a Neotropical migrant which breeds in North America but migrates annually to southern South America (Fuller et al., 1998). Swainson's hawks are generally smaller and more slender than Galápagos hawks, and Swainson's adults have three color morphs as opposed to one dark morph in adult Galápagos hawks (Ferguson-Lees and Christie, 2001).

Island-populations of Galápagos hawks have extremely low levels of genetic variability as evidenced by mean similarity indices between 0.66 and 0.96 at hypervariable minisatellite loci, and genetic variation is positively correlated with island area, an index of population size (Bollmer et al., 2005). There is a significant amount of genetic differentiation among most populations; only two populations (Fernandina and Isabela) are statistically indistinguishable at minisatellite loci (Bollmer et al., 2005). Galápagos hawk populations vary behaviorally and morphologically (de Vries, 1973; Bollmer et al., 2003). The hawks breed in cooperatively polyandrous groups consisting of one female and up to eight males (Faaborg and Patterson, 1981; DeLay et al., 1996), and mean group size varies across islands (Bollmer et al., 2003). Galápagos hawks also vary in overall body size and shape across islands, with female mass in the smallest-bodied population averaging 22% less than in the largest-bodied population (26% in males; Bollmer et al., 2003).

In this study, we described the phylogeographic and population genetic structure of the Galápagos hawk, a species we know to be genetically monomorphic within populations but divergent between populations at nuclear loci. We collected

mitochondrial sequence data from all nine extant populations of Galápagos hawk. We were also able to obtain sequence data from a San Cristóbal hawk (a population now extirpated) collected during the 1905-1906 California Academy of Sciences expedition. In addition, we sampled migratory Swainson's hawks and investigated the degree of divergence between the two species to determine when the Galápagos lineage likely colonized the archipelago. Within Galápagos hawks, we examined relationships among different island populations at mitochondrial loci, using multilocus minisatellite data as a nuclear comparison, with the goal of elucidating the colonization history of the hawks in the archipelago.

2. Materials and methods

2.1. Field methods

We visited the Galápagos Islands for two to three months between May and August of each year from 1998 to 2003 and sampled 541 Galápagos hawk individuals from all nine extant populations (Table 2). We captured hawks using balchatri traps baited with rats (Berger and Mueller, 1959) and rope nooses on poles. We banded each hawk and took morphological measurements (see Bollmer et al., 2003) and two 50 μl blood samples via venipuncture. In addition, we captured and sampled thirty-four Swainson's hawks using balchatri traps placed in agricultural fields near the town of Las Varillas, in Córdoba province (Central Argentina) during January 2003.

The California Academy of Sciences in San Francisco, California has a single Galápagos hawk specimen collected in 1905 from the now extirpated San Cristóbal population. In order to obtain genetic data from this population, we visited the Academy in June 2004 and excised a toe pad from that specimen.

2.2. Laboratory methods

For most populations, we used a subset of the individuals in the genetic analyses (Table 2). When possible, we preferentially limited our pool of individuals to territorial, breeding adults, the class most likely to be genetically representative of the population and consist of nonrelatives (individuals within groups are unrelated [Faaborg et al., 1995]). On Pinzón and Santa Cruz, however, we captured only juveniles and used all of them in the analyses. Initially, we sequenced 26 hawks (Table 2) at four mitochondrial regions comprising 2860 bp. This included complete NADH dehydrogenase subunit 2 (ND2) sequences (1041 bp), 320 bases at the 3′ end of cytochrome *b* (CYB), 72 bp between CYB and the control region (CR), including tRNAthr, 415 bp of the 5′ end of CR (66 bp of the 5′ end of CR were problematic to sequence and are excluded from analyses), and 516 bp near the 5′ end and 496 bp near the 3′ end of cytochrome oxidase (COI). Among the Galápagos hawks sampled, most regions were invariant in this initial sample; therefore, we sampled 126 additional individuals (Table 2; 123 Galápagos and 29 Swainson's hawks) at only the variable 3′ end of COI and 415 bp of the CR.

The majority of sequences were single-stranded, though we obtained double-stranded sequences from those individuals where all gene regions were amplified, and for

sequences where there were uncertainties. Table 3 lists the primers used to amplify and sequence the CYB-CR, COI, and ND2 regions. Unless noted, primers are named to indicate light (L) or heavy (H) strand and the 3′ position of the primer numbered according to the complete mitochondrial genome of *Gallus gallus* (Desjardins and Morais, 1990). The CYB-CR region was amplified with L15662 and H15414 (name indicates the 3′ end of the primer numbered according to the complete mitochondrion of *Buteo buteo*). To double-strand sequences, we used the internal primers H16065 and L15004 (name indicates the 3′ end of the primer numbered according to the complete mitochondrion of *Buteo buteo*). COI was amplified in two reactions. The 5′ region was amplified with L6615 and H7539, and sequencing was done using L6615 or H7181. The 3′ region of COI was amplified with L7201 and H8214; sequencing was done using L7651 and H8214. ND2 sequences were obtained by amplifying and sequencing with primers L5216 and H6313. Sequences were double-stranded with internal primers L5716 and H5766.

PCR amplification followed standard protocols. We purified amplicons by precipitation using an equal volume of PEG:NaCl (20 %:2.5M) and washing with 70% ethanol. We sequenced purified amplicons using either ABI BigDye® Terminator v.1.0, BigDye® Terminator v.3.1, or Beckman DTCS Quickstart® chemistries. Manufacturers' recommendations were followed, except reaction volumes were cut to 1/2 - 1/6 of the recommended volume. Sequences were analyzed on an ABI Prism™ 310, ABI Prism™ 3100-Avant genetic analyzer (PE Applied Biosystems), or a CEQ™ 8000 (Beckman-Coulter™) genetic analysis system.

The 100-year-old San Cristóbal sample was processed in a lab dedicated to working with ancient DNA at the Florida Museum of Natural History located at the University of Florida. We extracted DNA from the toe pad and amplified the appropriate regions in the ancient DNA lab. Due to the poorer quality of the ancient DNA, we needed to sequence the regions in smaller segments using additional primers designed from Galápagos hawk sequences (primer sequences available from RTK upon request).

We performed multilocus minisatellite DNA fingerprinting using the restriction endonuclease *Hae*III and Jeffreys' probe 33.15 (Jeffreys et al., 1985) following procedures described in general in Parker et al. (1995) and specifically for Galápagos hawks in Bollmer et al. (2005). We visualized hybridized fingerprints using a Storm 820 Phosphorimager. We fingerprinted a total of 119 of the 122 Galápagos hawks sequenced at the variable mitochondrial loci (Table 2). From the resulting banding patterns, we created a presence-absence matrix of bands (alleles) encompassing all individuals.

2.3. Data analysis

We examined and compared sequences using Sequencher™ 4.1 (Gene Codes Corp.). We used DnaSP v. 4.0.5 (Rozas et al., 2003) to calculate within-population genetic diversity indices: haplotype diversity (Nei, 1987) and nucleotide diversity (π; Nei, 1987). We generated a 95% statistical parsimony-based haplotype network using TCS v. 1.18 (Clement et al., 2000). Mean genetic distances (number of variable sites and uncorrected *p*-distances) within and between species were calculated using *MEGA* v. 2.1 (Kumar et al., 2001). Standard errors were calculated via bootstrapping (500 replicates).

When the level of genetic differentiation between populations was ambiguous, we used pairwise differences to calculate F_{ST} values in Arlequin version 2.000 (Schneider et al., 2000).

To estimate divergence times, we assumed the mitochondrial protein-coding regions were diverging at 2% per million years (Shields and Wilson, 1987). There were six differences between Galápagos and Swainson's hawks (sites invariant within each species but variable between them) in the 2373 bp of protein-coding data used to determine divergence time: 3 in ND2, 1 in CYB, 1 in COI 5′, and 1 in COI 3′. There were other variable sites where some individuals from both species shared the same nucleotide, but these were not used to calculate the divergence between the two species. We estimated a 95% confidence interval for the divergence time assuming a Poisson model of evolution (e.g., Braun and Kimball, 2001). While this method does not correct for ancestral polymorphism, we were primarily interested in setting an upper limit on divergence time, making a correction unnecessary.

For the nuclear minisatellite data, pairwise similarity values were calculated from the presence-absence matrix (based on 46 characters) using the program GELSTATS v. 2.6 (Rogstad and Pelikan, 1996). Similarity values, the proportion of bands shared between any two individuals (Lynch, 1990), were converted to distances (1 – similarity value). We used the distances to construct a neighbor-joining tree in *PAUP** v. 4.0b10 (Swofford, 2002), using midpoint rooting and constraining it to non-negative branch lengths.

3. Results

3.1. Haplotype variation within and between Galápagos and Swainson's hawks

Sequence data is available in GenBank, accession nos. **AY870866** to **AY870892**. For the 26 individuals sequenced at the four mitochondrial regions, polymorphic sites were present in only two of those regions, the CR and the 3′ end of COI (911 bp total), while the other regions (1949 bp total) were invariant within each species, differing by 5 bp between species. Among the 151 individuals (excluding the San Cristóbal hawk) sequenced for the two variable regions, there were only 27 variable sites across all individuals: 6 found only within the 122 Galápagos hawks sampled, 16 only within the 29 Swainson's hawks, 3 in both species, and 2 monomorphic within species but variable between them (Table 4). There were a total of 19 haplotypes sequenced, 7 among the 122 Galápagos hawks and 12 among the 29 Swainson's hawks, indicating greater genetic variability in the Swainson's hawks (Tables 4, 5). The seven Galápagos hawk haplotypes differed from each other by an average of 3.14 ± 1.07 (SE) bases (mean uncorrected *p*-distance of 0.003 ± 0.001), while the 12 Swainson's hawk haplotypes differed by an average of 4.55 ± 1.10 bases (mean *p*-distance of 0.005 ± 0.001). The *p*-distances within Galápagos hawks ranged from 0 to 0.007, while they ranged from 0 to 0.011 in the Swainson's hawks. Including all the sampled individuals, the mean uncorrected *p*-distance was 0.002 ± 0.001 within Galápagos hawks and 0.003 ± 0.001 within Swainson's hawks. Galápagos and Swainson's hawk haplotypes differed from each other by an average of 10.43 ± 2.46 bases, with a mean *p*-distance of 0.011 ± 0.003, and *p*-distances ranged from 0.005 to 0.015. The smallest *p*-distance between Galápagos and

Swainson's hawks (0.005) is less than the largest distance within either one of them (0.007 in Galápagos and 0.011 in Swainson's hawks). Including all the sampled individuals, Galápagos and Swainson's hawks differed by an average of 10.20 ± 2.75 bases, with a mean p-distance of 0.011 ± 0.003.

Using DnaSP, we inferred the amino acid sequences from 492 of the 496 bp at the 3′ end of COI, which resulted in 164 codons in an open reading frame. Interestingly, within the 122 Galápagos hawks, of the five nucleotide substitutions, four were nonsynonymous and one was synonymous. Within the 29 Swainson's hawks, the only mutation in this region was synonymous.

Using a divergence rate of 2% per million years for the 2373 bp of coding DNA (Shields and Wilson, 1987), Galápagos and Swainson's hawks diverged approximately 126,000 years ago, with a 95% confidence interval between 51,000 and 254,000 years ago. While there is a large amount of error in molecular clock estimates (Arbogast et al., 2002; Lovette, 2004), our estimate still indicates that Galápagos hawks arrived in Galápagos very recently, likely less than 300,000 years ago.

3.2. Divergence among Galápagos hawk populations

There were only seven mitochondrial haplotypes present across the nine extant Galápagos hawk populations; multiple haplotypes were present in two populations (Isabela and Santa Cruz), while the other seven populations were fixed (Fig. 1). Three haplotypes were present on multiple islands. One (black circles in Fig. 1) was found in all individuals from the northern and central islands of Pinta, Marchena, Santiago, and

Santa Fe, and in two of the four Santa Cruz birds. The second haplotype (black triangles) was shared among all Pinzón individuals, as well as five individuals from Isabela and one from Santa Cruz. The third haplotype (black squares) was found in all Fernandina individuals, the majority of the sampled individuals from Isabela, and the San Cristóbal individual (see below). The remaining four haplotypes were unique to individual islands: one present in all Española individuals, one in a single Santa Cruz individual, and two in two Isabela individuals. Interestingly, one Isabela haplotype was more similar to the common haplotype present on the five central and northern islands than it was to other Isabela haplotypes. The genetic distances between populations were small, with the average number of base pair differences ranging from 0 to 4.25 (mean uncorrected *p*-distances ranging from 0 to 0.005).

Due to the degraded nature of the San Cristóbal sample, we sequenced a subset of the COI 3′ and CR regions. We were able to sequence 281 of the 496 bp of COI 3′ and 308 of the 415 bp of the CR, covering 65% of the 911 bp sequenced from the other individuals. These two fragments encompassed all but one of the sites that were variable in the other Galápagos hawks; the one missing site was a site that separated the Española haplotype from all the rest of the haplotypes, including the Swainson's haplotypes (site number 22 in Table 4). At the regions sequenced, the San Cristóbal haplotype was identical to the Fernandina/Isabela haplotype. While we cannot rule out possible variable sites in the 311 bp not sequenced for the San Cristóbal hawk, the rest of the Galápagos haplotypes were all monomorphic at those sites (except for site 22). It is likely that this individual is representative of the former population on San Cristóbal given that seven of the other nine populations were fixed for a single haplotype.

We calculated F_{ST} values between Isabela and Fernandina and Isabela and Pinzón, because Fernandina and Pinzón were each fixed for haplotypes present on Isabela, though Isabela had additional haplotypes. Both Fernandina ($F_{ST} = 0.216$, $P < 0.01$) and Pinzón ($F_{ST} = 0.451$, $P < 0.01$) were significantly differentiated from Isabela.

The minisatellite data indicated some differentiation among populations (Fig. 2). Española and Santa Fe individuals formed independent, distinct clusters. Most of the Pinzón individuals also clustered, though not as distinctly as those from Española and Santa Fe. Marchena and Pinta individuals generally clustered together, with some differentiation between them. Only individuals from Santiago, Isabela, and Fernandina, the three largest and most variable populations, were indistinguishable from each other.

The four Santa Cruz birds were widely distributed in the tree. One individual fell within the Santa Fe cluster, having a banding pattern identical to four Santa Fe individuals. Another fell within the Pinzón cluster. These two birds also shared haplotypes with Santa Fe and Pinzón, respectively, suggesting these birds were born on those islands and subsequently dispersed to Santa Cruz. The other two Santa Cruz birds were not closely associated with any particular population.

The program TCS will estimate the root of a haplotype network based on the position of a haplotype in the tree and its frequency, which correlate with haplotype age (Castelloe and Templeton, 1994). When Swainson's hawk haplotypes were not included, TCS estimated that the most likely root of the Galápagos hawk haplotypes was the common one shared by Pinta, Marchena, Santiago, Santa Fe, and Santa Cruz. When Swainson's hawks were included, TCS still estimated that the most common Galápagos haplotype was the root, because the program does not take into consideration information

about outgroups. The haplotype network (Fig. 1) created by TCS, though, identified the haplotype shared by the Fernandina, Isabela, and San Cristóbal populations as the one most closely related to Swainson's hawks, indicating it is the oldest of the Galápagos hawk haplotypes.

4. Discussion

4.1. Recent divergence between Galápagos and Swainson's hawks

The mitochondrial data indicated that Galápagos hawks form a monophyletic clade; thus, there was likely a single colonization event. They showed remarkably little divergence from their mainland sister species, the Swainson's hawk, differing by only 0.42% over almost 3 kb of data. The divergence between Swainson's and Galápagos hawks is on average greater than that within either of them. There is overlap, however, in the ranges of the genetic distances; the maximum divergence among Swainson's hawk lineages and among Galápagos hawk lineages is greater than the minimum divergence between the two species (Fig. 1). It may be that if we sampled Swainson's hawks more broadly and included additional outgroups, we would find that Swainson's hawks are paraphyletic.

Although the genetic divergence between Galápagos and Swainson's hawks is minimal, their morphological differences are great enough to have prevented their earlier identification as sister species (e.g., Brown and Amadon, 1968; de Vries, 1973). Many studies have found significant morphological differentiation between species that show

little if any mitochondrial divergence (e.g., Seutin et al., 1995; Freeland and Boag, 1999; Piertney et al., 2001). In an analysis of Old World *Buteo* lineages, Kruckenhauser et al. (2004) also found little mitochondrial divergence among morphologically distinct species and subspecies. The life histories of Swainson's and Galápagos hawks (migratory vs. sedentary, prey base) differ greatly in ways that affect their morphology, especially their wings and talons. In addition to selection, the rapid morphological differentiation could be the result of genetic bottlenecks and ongoing drift in small island populations. Swainson's and Galápagos hawks are not necessarily less divergent than other *Buteo* sister species. Using Riesing et al.'s (2003) sequence data for the mitochondrial gene nd6, we calculated a *p*-distance of 0.008 between Swainson's and Galápagos hawks and an average *p*-distance of 0.010 ± 0.002 (SD) within five other well-supported (based on bootstrap values) pairs of *Buteo* sister species. There are few other raptor mitochondrial studies; however, Groombridge et al. (2002) found similarly low levels of divergence between some kestrel species.

The extremely low level of divergence between the Galápagos and Swainson's hawks indicates that they separated only very recently (less than 300,000 years ago). Of the native Galápagos fauna studied to date, Galápagos hawks appear to be the most recently arrived lineage. Some taxa predate the current islands. The endemic land (*Conolophus*) and marine (*Amblyrhynchus*) iguanas are sister taxa, likely having diverged 10 to 20 million years ago (MYA) on the now sunken islands (Wyles & Sarich 1983; Rassmann 1997). Lava lizards (*Microlophus* spp.) likely colonized the islands multiple times between 6 and 20 MYA (Wright, 1983; Lopez et al., 1992; Kizirian et al., 2004), and *Galapaganus* weevils separated from their mainland relatives approximately 11

MYA (Sequeira et al., 2000). Other lineages arrived in Galápagos more recently, colonizing the current islands. The oldest divergence among the 11 extant Galápagos tortoise (*Geochelone nigra*) subspecies occurred 1.5 to 2 MYA (Caccone et al., 1999, 2002). Sato et al. (2001) estimated that Darwin's finches diverged from their closest mainland relative around 2.3 MYA, likely arriving in Galápagos from the Caribbean (Burns et al. 2002). The yellow warbler (*Dendroica petechia aureola*) diverged from the mainland form approximately 2.5 MYA (Collins, 2003).

4.2. Galápagos hawk phylogeography

Most Galápagos lineages underwent further differentiation as they colonized multiple islands, and, in many taxa, older lineages occur on the older eastern islands (San Cristóbal, Española, and Floreana) and younger lineages on the western islands (e.g., Rassmann et al., 1997; Sequeira et al., 2000; Beheregaray et al., 2004). For example, six of the 11 tortoise subspecies occur on different islands (the rest inhabiting the five volcanoes of Isabela), and mitochondrial and microsatellite data indicate significant genetic differentiation among them (Caccone et al., 2002; Ciofi et al., 2002). There should be greater genetic divergence among the older lineages due to a longer period of isolation. In the tortoises, differences among populations explain 97% of mitochondrial molecular variance for older islands and only 60% for younger islands (Beheregaray et al., 2004). Within geckos (*Phyllodactylus* spp.) and lava lizards, Wright (1983) found that the populations on the central and western islands tended to have higher allozyme similarities than the more divergent populations to the east.

The Galápagos hawk haplotype network shows a striking pattern of genetic monomorphism within populations and short genetic distances among populations at the mitochondrial loci. Four different populations (Santa Fe, Santiago, Marchena, and Pinta) comprising 58 sampled individuals were fixed for a single haplotype. Fernandina, Pinzón, and Española were also fixed but for different haplotypes. Only the populations on Isabela and Santa Cruz had any variability. Española hawks in the east have the highest mean genetic distance from the other populations; however, Española is not necessarily the oldest population, but instead may have become the first population to be isolated from the rest. The paucity of different haplotypes and the small genetic distances among them suggests the hawks spread across the archipelago relatively quickly, with subsequent lineage sorting resulting in different haplotypes on different islands. The pattern on Isabela, with haplotypes that are not most closely related to each other, and the presence of the same haplotype on San Cristóbal as on Fernandina (at opposite ends of the archipelago) further supports this. It is difficult to say from which direction the initial hawk colonization of the archipelago occurred; the Swainson's hawks were most closely related to the Fernandina/Isabela/San Cristóbal haplotype that was located on the far eastern and western islands. Limitations due to lineage sorting and possible homoplasy prevent a more definitive determination of the colonization pattern. Our understanding is also hindered by the missing information from the extirpated Floreana population, and our four samples from Santa Cruz (the most central island) are likely not representative of the former population there (see next section).

The role of genetic drift in these island populations was also demonstrated by the finding that the majority of nucleotide substitutions in the 3′ end of COI within

Galápagos hawks were nonsynonymous. This finding is unsurprising from a theoretical perspective, given that slightly deleterious mutations with respect to fitness are expected to drift to fixation at a higher rate within small populations relative to larger populations (reviewed in Johnson and Seger, 2001). This qualitative interpretation is supported further by Johnson and Seger's (2001) empirical study, which found elevated rates of nonsynonymous substitutions on lineages of island bird taxa compared to their mainland relatives. Finally, the fact that Galápagos hawks have very small island populations, the majority of which are genetically isolated (Bollmer et al., 2005) also lends support for the role of drift in generating these patterns.

4.3. Mitochondrial vs. nuclear differentiation among populations

Mitochondrial and nuclear markers can often be used in conjunction to draw more accurate conclusions about genetic structure. The eastern population on Española was clearly genetically isolated at both mitochondrial and minisatellite loci. The central and northern populations (Santa Fe, Santiago, Marchena, and Pinta) share a common mitochondrial haplotype even though our pairwise F_{ST} estimates show significant differentiation among them at the more rapidly evolving minisatellite loci (Bollmer et al., 2005). The western populations of Fernandina and Isabela, less than 5 km apart, were statistically indistinguishable at minisatellite loci (Bollmer et al., 2005) and shared a mitochondrial haplotype; moreover, one female hawk banded as a juvenile on Isabela (Volcan Alcedo) in 1998 was observed in a territorial group on Fernandina in 2003, though we do not know which is its natal island (Bollmer et al., 2005). The presence of

other haplotypes on Isabela, however, resulted in a significant F_{ST} value between them for the mitochondrial data. This discrepancy between the nuclear and mitochondrial data could be due to male-biased gene flow, though we have no other evidence that this occurs. Another explanation is that it is due to the differing natures of the two markers. Santiago, Isabela, and Fernandina are the largest of the hawk populations and have retained the most genetic variability. The fact that they are more distinguishable at mitochondrial loci than at minisatellite loci could be attributed to the shorter coalescent time of the mitochondrial loci, thus allowing significant genetic structuring to arise more quickly.

The combined mitochondrial and nuclear data can also be used to determine the populations of origin of dispersers, which is of potential conservation importance, both from the perspective of disease transmission and population management. Given the apparent absence of a breeding population on Santa Cruz, both the mitochondrial and the minisatellite data suggest that the four Santa Cruz juveniles are likely dispersers from different islands. One was very likely born on Pinzón and one on Santa Fe; both their minisatellite and mitochondrial profiles are consistent with that. The origin of the other two individuals is less clear. Neither of them is closely associated with any of the more inbred populations at the minisatellite loci, leaving Fernandina, Isabela, and Santiago as possible source populations. One shares the same haplotype as Santiago; the other has a unique haplotype that is most closely related to the one shared by Isabela and Pinzón. Given the genetic monomorphism on Pinzón, the latter bird more likely originated on Isabela.

Taking both the nuclear and mitochondrial data into account, the overall pattern among Galápagos hawk populations is one of genetic isolation. The Santa Cruz population is certainly an exception in that juveniles appear to be dispersing there, and there may be gene flow between Fernandina and Isabela, since they are indistinguishable at the nuclear loci (though not at the mitochondrial loci). All the other populations show statistically significant divergence at nuclear or mitochondrial loci or both. This, combined with the morphological differentiation among populations and the recentness of its arrival, may mean that the Galápagos hawk is in the very early stages of speciation. The much older finch colonization of the archipelago resulted in fourteen morphological species; however, mitochondrial data only distinguished four groups (Sato et al., 1999), and interspecific genetic distances at microsatellite loci were generally lower among sympatric populations than among allopatric populations, likely due to introgressive hybridization (Grant et al., 2005). Galápagos hawks are less vagile, and most of their populations, like those of other sedentary species in the archipelago (e.g., tortoises, lava lizards), appear to be on separate evolutionary trajectories. Although the colonization history of the Galápagos hawk remains unclear, reconstructing the genealogies of its parasites (de Vries, 1975; Whiteman and Parker, 2005) may yield insight into the hosts' movements within the archipelago.

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Figure Legends

Fig. 1 Mitochondrial haplotype network of Galápagos and Swainson's hawks. Within the Galápagos hawks, each haplotype is represented by a different symbol (corresponding to symbols in Table 4 and Figure 2), and the Swainson's hawks haplotypes are represented by different letters (corresponding to those in Table 4). Only one haplotype was found in each Galápagos hawk population except for Isabela (four haplotypes) and Santa Cruz (three haplotypes). The number of individuals with each haplotype is listed next to the corresponding symbol. It should be noted that while the Swainson's hawk haplotypes are drawn connecting to the Fernandina/Isabela haplotype, that same haplotype is also present on San Cristóbal, though it is based on fewer sequenced sites.

Fig. 2 A midpoint rooted neighbor-joining tree of Galápagos hawk populations based on minisatellite distances (1–similarity). Populations are identified with abbreviations: E = Española, F = Fernandina, I = Isabela, M = Marchena, PT = Pinta, PZ = Pinzón, SA = Santiago, SC = Santa Cruz, and SF = Santa Fe. The symbols following the population abbreviations represent mitochondrial haplotypes and correspond to those on the haplotype network (Fig. 1). The four Santa Cruz individuals are in boxes.

Table 1

Summary of terrestrial vertebrate taxa of Galápagos, including the number of linea ges that evolved on the archipelago, the number of colonizing species from which they evolved, and whether they are endemic

Class	Taxa	Number of lineages	Number of founding taxa	Endemic
Reptilia	giant tortoises (*Geochelone nigra*)	11 subspecies	1 (Caccone et al., 1999)	yes
	marine (*Amblyrhynchus cristatus*) and land (*Conolophus subcristatus, C. pallidus*) iguanas	7 subspecies (marine), 2 species (land)	1 (Rassmann, 1997)	yes
	lava lizards (*Microlophus* spp.)	7 species	2 (Kizirian et al., 2004)	yes
	geckos (*Phyllodactylus* spp.)	6 species	2 (Wright, 1983)	yes
	snakes (*Philodryas hoodensis*, *Antillophis slevini*, *A. steindachmeri*, *Alsophis biseralis* subspp.)	3 species, 3 subspecies	at most 4	yes
	Total	**40**	**10**	
Mammalia	rice rats (*Oryzomys* spp., *Nesoryzomys* spp., *Megaoryzomys curiori*)	at least 8 species	3	yes
	bats (*Lasiurus brachyotis*, *L. cinerius*)	2 species	2	yes (*L.*

				brachyotis)
	Total	**10**	**5**	
Aves	Darwin's finches (*Geospiza* spp., *Camarhynchus* spp., *Cactospiza* spp., *Platyspiza crassirostris*, *Certhidea olivacea*	13 species	1 (Sato et al., 1999; Burns et al., 2002)	yes
	Galápagos mockingbirds (*Nesomimus* spp.)	4 species	1	yes
	Galápagos dove (*Zenaida galapagoensis*)	2 subspecies	1	yes
	Galápagos hawk (*Buteo galapagoensis*)	1 species	1 (this study)	yes
	Barn owl (*Tyto alba punctatissima*)	1 subspecies	1	subspecies
	Short-eared owl (*Asio flammeus galapagoensis*)	1 subspecies	1	subspecies
	Galápagos martin (*Progne modesta*)	1 species	1	yes
	Yellow warbler (*Dendroica petechia aureola*)	1 subspecies	1 (Collins, 2003)	subspecies
	Galápagos flycatcher (*Myiarchus magnirostris*)	1 species	1	yes
	Vermilion flycatcher (*Pyrocephalus rubinus*)	1 species	1	no
	Dark-billed cuckoo (*Coccyzus melacoryphus*)	1 species	1	no

Galápagos rail (*Laterallus spilonotus*)	1 species	1	yes
Paint-billed crake (*Neocrex erythrops*)	1 species	1	no
Common gallinule (*Gallinula chloropus*)	1 species	1	no
Total	**30**	**14**	

Only native, resident taxa are listed (i.e., no introduced species or seasonal migrants), and lineages that arose in Galápagos but have since gone extinct are included. There are references listed where genetic studies have determined the likely number of founding events; otherwise, the numbers reflect what is believed based on morphological characters.

Table 2

Sample sizes of Galápagos and Swainson's hawks sequenced at mitochondrial loci and fingerprinted at minisatellite loci

Species	Population	No. sequenced at all regions	No. sequenced at variable regions	No. fingerprinted at minisatellite loci
Galápagos hawk	Española	2	10	10
	Santa Fe	2	9	9
	Santa Cruz	4	4	4
	Santiago	2	21	20
	Pinzón	2	10	10
	Marchena	2	15	15
	Pinta	2	13	12
	Isabela	4	20	19
	Fernandina	2	20	20
	San Cristóbal	0	1	0
Swainson's hawk		4	29	0
Total		26	152	119

A total of 26 hawks were sequenced at all four mitochondrial regions (CYB, CR, COI, and ND2). An additional 126 hawks were then sequenced at the two variable regions (COI 3′ and CR) for a total of 152 hawks sequenced at those regions, though the San Cristóbal hawk sequence is incomplete.

Table 3

Primers used in this study to amplify and sequence three hawk mitochondrial regions

Region	Primer	Source	Sequence (5′ to 3′)	T_M (°C)
CYB-CR	L15662	Kimball et al., 1999	CTAGGCGACCCAGAAAACTT	54°,
	H15414	this study	CAAGTAGTGCTAGGGGTTTAGG	30 sec
	L15004	this study	CACATATCATGAACTATTATGGG	Seq. only
	H16065	Kimball et al., 1999	TTCAGTTTTTGGTTTACAAGAC	Seq. only
COI	L6615	modified from	TCTGTAAAAAGGACTACAGCC	52°,
		Sorenson et al., 1999		30 sec
	H7539	Sorenson et al., 1999	GATGTAAAGTAGGCCGGGTGTCTAC	
	H7181	this study	TACGAATAGGGGTGTTTGG	Seq. only
	L7201	this study	ACCAAACACCCCTATTCGTATG	54°,
	H8214	this study	ATGCRGYTGGCTTGAAACC	30 sec
	L7651	this study	GGAACTATCAAATGAGACCC	Seq. only
ND2	L5216	Sorenson et al., 1999	GCCCATACCCCRAAAATG	52°,

H6313	Sorenson et al., 1999	CCTTATTTAAGGCTTTGAAGGC	30 sec
L5716	this study	CCCTACTYACCYTCCTAGCAAT	Seq. only
H5766	modified from Sorenson et al., 1999	GATGARAAGGCTAGGATYTTTCG	Seq. only

Table 4

The polymorphic sites within the variable COI 3′ and CR regions of the Galápagos and Swainson's hawk mitochondrial DNA

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		2 1 1 7 3	3 0 2 6 8	6 8 7 7 8	9 2 4 9 0	1 4 7 1 4 4 0
Galápagos hawks	▼	CTGAT	CACCA	TGTCT	TGAGA	CGTTTAC
	■	TTGGT	CACCA	TGTCT	TGAGA	CGTTTAC
	Δ	TTGGT	CGTCA	TGTCT	TGAGA	CGTTTAC
	□	TTAGT	CGCCA	TGTCT	TGAGT	TGTTTAC
	●	TTAGT	CGCCA	TGTCT	TGAGA	CGTTTAC
	▲	TTGGT	TGCCA	TGTCT	TGAGA	CGTTTAC
	+	TTGGC	TGCCA	TGTCT	TGAGA	CGTTTAC
Swainson's hawks	A	TTGGC	CACCA	TGTCT	TAGGA	CATCTGT
	B	TTGGC	CACTG	TGTCT	TGGGA	TATTTGT
	C	TTGGC	CACCA	TGTCT	TAAGA	CATTTGT
	D	TCGGC	CACCA	TGTTT	CAAGA	CATTTGT

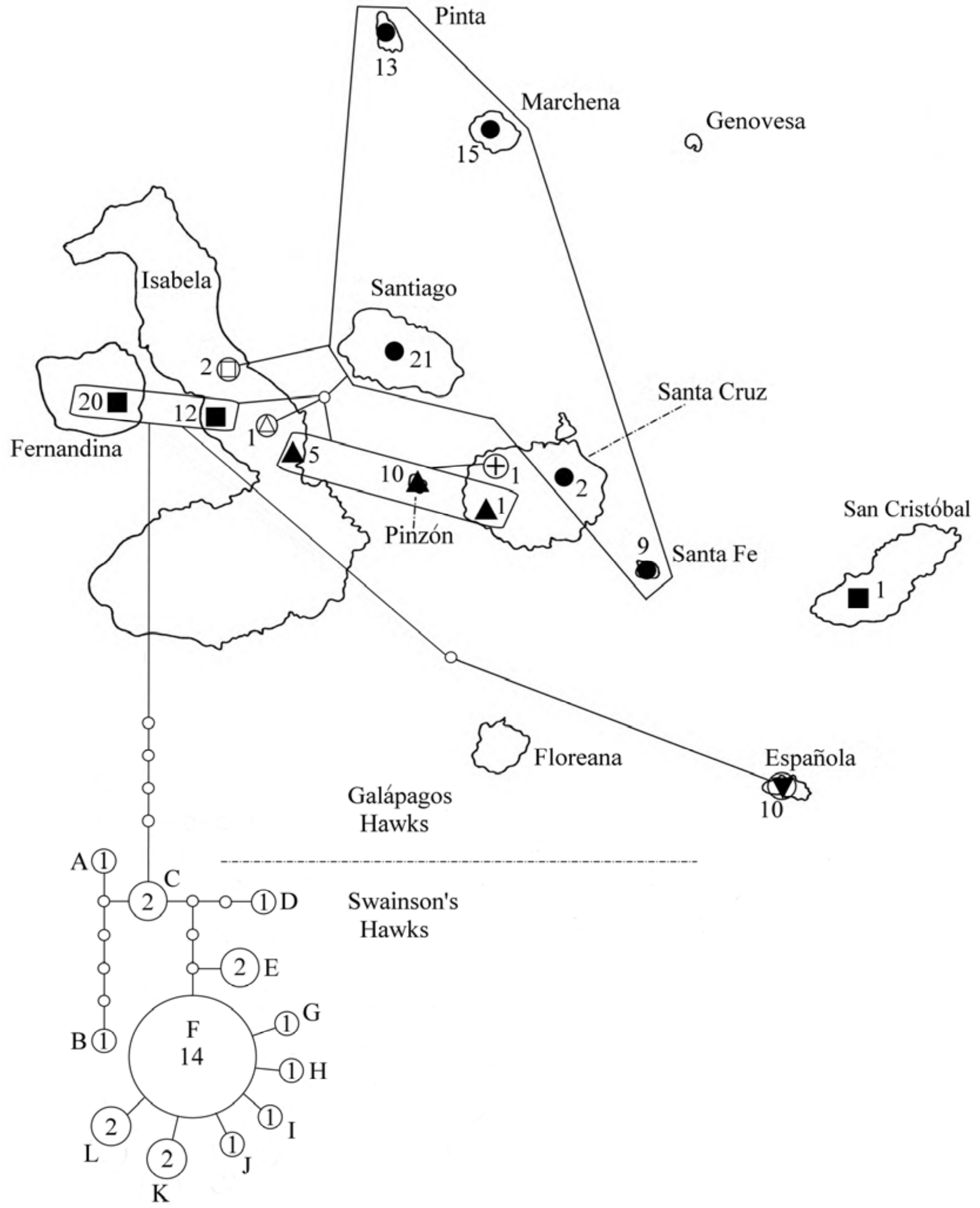
E	TTGGC	CACCA	TATTC	TAAGA	CATTCGT
F	TTGGC	CACCA	TGCTC	TAAGA	CATTCGT
G	TTGGC	CACCA	TGCTC	TAAGA	CACTCGT
H	TTGGC	CACCA	CGCTC	TAAGA	CATTCGT
I	TTGGC	CACCA	TGCTC	TAAGT	CATTCGT
J	TTGGC	CACCA	TGCTC	TAAAA	CATTCGT
K	TTGGC	TACCA	TGCTC	TAAGA	CATTCGT
L	TTGGC	CACCA	TGCTC	TAAGA	CGTTCGT

Of the 911 bp sequenced at the COI 3′ and CR regions, there were 27 variable sites. The sites are numbered according to their position within our combined COI and CR dataset; positions 1-496 are COI sites and positions 497-911 are CR sites. Each Galápagos hawk haplotype is labeled with a symbol corresponding to the symbols in Figures 2 and 3. Each Swainson's hawk haplotype is labeled with a letter corresponding to the letters in Figure 2.

Table 5

Genetic variability at five mitochondrial regions within Galápagos (*N* = 122; excluding the San Cristóbal hawk) and Swainson's (*N* = 29) hawks

		CYB, ND2, COI 5′ (1949 bp)	COI 3′ (496 bp)	CR (415 bp)	COI 3′/CR combined (911 bp)
B. galapagoensis	No. of polymorphic sites	0	4	5	9
	Nucleotide diversity	0	0.0017	0.0019	0.0018
	No. of haplotypes	1	4	5	7
	Haplotype diversity (±SD)	0	0.578 ± 0.023	0.625 ± 0.025	0.671 ± 0.030
B. swainsoni	No. of polymorphic sites	0	1	18	19
	Nucleotide diversity	0	0.0001	0.0059	0.0028
	No. of haplotypes	1	2	12	12
	Haplotype diversity (±SD)	0	0.069 ± 0.063	0.766 ± 0.081	0.766 ± 0.081


Pinta
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Marchena
15
Genovesa
Isabela
Santiago
21
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20
12
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Fernandina
Santa Cruz
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10
1
2
1
Pinzón
9
Santa Fe
San Cristóbal
1
Floreana
Española
10
Galápagos
Hawks
Swainson's
Hawks
A 1
C 2
D 1
E 2
B 1
F 14
G 1
H 1
I 1
J 1
K 2
L 2

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Chapter 3

Disease ecology in the Galápagos hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies

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ABSTRACT

An increased susceptibility to disease is one hypothesis explaining how inbreeding hastens extinction in island endemics and threatened species. Experimental studies show that disease resistance declines as inbreeding increases, but data from in situ wildlife systems are scarce. Genetic diversity increases with island size across the entire range of an extremely inbred Galápagos endemic bird, providing the context for a natural experiment examining the effects of inbreeding on disease susceptibility. Extremely inbred populations of Galápagos hawks had higher parasite abundances than relatively outbred populations. We found a significant island effect on constitutively produced natural antibody (NAb) levels and inbred populations generally harboured lower average and less variable NAb levels than relatively outbred populations. Furthermore, NAb levels explained abundance of amblyceran lice, which encounter the host immune system. This is the first study linking inbreeding, innate immunity and parasite load in an endemic, in situ wildlife population and provides a clear framework for assessment of disease risk in a Galápagos endemic.

Key words: disease; Galápagos Islands; genetic diversity; immune function; natural antibodies

1. INTRODUCTION

Extinctions of island endemics account for 75% of animal extinctions and 90% of bird extinctions (Myers 1979; Reid & Miller 1989). Several synergistic key factors may be responsible for this high extinction rate, including introduction of exotic animal and human predators (Blackburn et al. 2004), habitat destruction (Rolett & Diamond 2004), demographic stochasticity (Drake 2005), and inbreeding in island endemics and threatened species (Frankham 1998; Spielman et al. 2004a). The interaction of disease agents with genetically depauperate (Pearman & Garner 2005) and isolated populations is one hypothesis explaining how inbreeding facilitates extinction in small populations (de Castro & Bolker 2005). Parasites evolve more quickly than hosts, so host antiparasite adaptations are perpetually obsolete (Hamilton et al. 1990; Lively & Apanius 1995). Consequently, genetically uniform host individuals (Acevedo-Whitehouse et al. 2003) and populations (Spielman et al. 2004b) are more susceptible to parasitism than genetically diverse hosts. Studies of model laboratory systems (Arkush et al. 2002), captive wildlife (Cassinello et al. 2001), and free-ranging domesticated animal populations (Coltman et al. 1999) support this claim, although other studies do not (Trouvé et al. 2003). Scant evidence of this phenomenon exists from *in situ* native wildlife populations (Meagher 1999), and no study has examined the effects of inbreeding on parasite load and innate, humoral immunity across bird populations in the

wild (Keller & Waller 2002). The intact endemic avifauna of the Galápagos Islands provides a unique opportunity to examine disease ecology and will provide insight into the impact of invasive disease agents that may enter the ecosystem (Lindström et al. 2004; Thiel et al. 2005).

The Galápagos hawk (*Buteo galapagoensis*), an endemic raptor threatened with extinction (2004 IUCN Red List), breeds on eight islands within the Galápagos National Park, and has been extirpated from several others (figure 1). Island size and genetic diversity are positively related and between-island population structure is high, rendering it an appealing model system in which to examine the effects of inbreeding on disease severity (Bollmer et al. in press a). The basic biology of its two chewing louse species (Insecta: Phthiraptera), an amblyceran (*Colpocephalum turbinatum*) and an ischnoceran (*Degeeriella regalis*), has been described (Whiteman & Parker 2004a,b). Thus, we examined the response of each parasite lineage to variance in host inbreeding, using population-level heterozygosity values from the eight island populations of *B. galapagoensis* and one population of the sister species (*Buteo swainsoni*; Riesing et al. 2003).

We also examined the relationship between immunological host defences, island-level inbreeding effects, and parasite abundance. To assess immunological host defences, we quantified non-specific natural antibody (NAb) titres within seven populations of *B. galapagoensis*. Quantification of NAbs has several conceptual and methodological advantages over other methods used to assess immune response of wild vertebrates (Matson et al. 2005). NAbs are a product of the innate, humoral immune system and their production is constitutive (stable over time and generally not induced by external

antigenic stimulation). Encoded by the germ-line genome, NAbs are present in antigenically naive vertebrates (Ochsenbein & Zinkernagel 2000), form a large percentage of the serum immunoglobulin (Kohler et al. 2003), are capable of recognizing any antigen, and prime the adaptive immune response (Adelman et al. 2004). In chickens, NAbs reacting to ectoparasite-derived antigens have been identified (Wikel et al. 1989) and in lines artificially selected for either high or low levels of specific antibodies, specific and NAb levels covary (Parmentier et al. 2004). NAb response is hypothesized to predict the strength of the adaptive immune response (Kohler et al. 2003). Thus, NAbs form a functional link between the innate and acquired parts of the humoral immune system (Lammers et al. 2004).

Inbreeding may negatively impact phytohaemagglutinin (PHA) induced swelling within wild bird populations (Reid et al. 2003), and reductions in population size reduce overall within-population genetic variation, including variation at loci of immunological import in vertebrates (Miller & Lambert 2004). Since variation in NAb levels responds to artificial selection in chickens (Parmentier et al. 2004), it is reasonable to predict that variation in NAb levels will covary with variation in wild bird population genetic diversity. However, the impact of natural microevolutionary processes on circulating levels of NAbs is unknown in wild vertebrates.

Amblyceran lice (e.g. *C. turbinatum*) directly encounter host immune defences because they feed on blood and living skin (Marshall 1981). Conversely, bird ischnocerans (e.g. *D. regalis*) generally feed on the keratin of feathers and dead skin (Marshall 1981) and mainly encounter the mechanical host defences (e.g. preening). Feeding by ectoparasites on skin and blood elicits immune responses (Wikel 1982) that

vary from cell-mediated (Prelezov et al. 2002) to humoral (i.e. antibodies; Pfeffer et al. 1997) and from innate (Wikel et al. 1989) to acquired (Ben-yakir et al. 1994). Host antibodies reduce louse fecundity and survivorship, and regulate population growth rate (Ben-yakir et al. 1994). Across bird species, variation in PHA-induced swelling was directly related to amblyceran but not ischnoceran species richness (Møller & Rózsa 2005). However, whether NAbs regulate ectoparasites populations, and louse populations in particular, is unknown.

We measured host inbreeding, parasite abundance and NAb response, and made three predictions: (i) at the island-level, higher inbreeding results in lower average humoral immune response relative to outbred populations; (ii) also at the island-level, higher inbreeding results in reduced variation in humoral immune response relative to outbred populations and (iii) birds with high humoral immune responses harbour fewer parasites (amblyceran lice) relative to birds with lower immune responses.

2. MATERIAL AND METHODS

(a) *Host sampling*

We live-captured a total of 211 *Buteo* hawk individuals on eight of the Galápagos Islands (*n*=202 *B. galapagoensis*; figure 1) and near Las Varillas, Córdoba, Argentina (*n*=9 *B. swainsoni*; Whiteman & Parker 2004a), from May–August 2001 (Islas Española, *n*=8; Isabela, *n*=25; Marchena, *n*= 26; Santa Fe, *n*=13), May–July 2002 (Isla Santiago, *n*=58), January 2003 (Argentina, *n*=9), and May–July 2003 (Islas Fernandina, *n*=28; Pinta, *n*=31; Pinzón, *n*=10). Birds were sampled following Bollmer et al. (in press a) from multiple

locations throughout each island. The University of Missouri- St Louis Animal Care Committee and the appropriate governmental authorities approved all procedures and permits.

(b) ***Parasite sampling***

We quantitatively sampled parasites from birds via dust ruffling with pyrethroid insecticide (non-toxic to birds; Zema Z3 Flea and Tick Powder for Dogs, St John Laboratories, Harbor City, California; Whiteman & Parker 2004a,b). Dustruffling provides excellent measures of relative louse intensity (Clayton & Drown 2001).

(c) ***Blood collection***

From each bird, we collected two 50 ml blood samples via venipuncture of the brachial vein for genetic analyses. Samples were immediately stored in 500 μl of lysis buffer (Longmire et al. 1988). For immune assay, whole blood samples were collected from a subsample of birds (*n*=46) in heparinized tubes, centrifuged in the field and plasma was stored in liquid nitrogen. Due to logistical constraints, no plasma was collected from the Pinzón population of *B. galapagoensis* or from *B. swainsoni*.

(d) ***Innate humoral immunity***

We used the general haemolysis–haemagglutination assay protocol (Matson et al. 2005) with two minor modifications (we used plates from Corning Costar #3798, instead of #3795 and Dulbecco's PBS, #D8662, Sigma, St Louis, MO). Sample sizes from Galápagos hawk island populations were as follows: Española, *n*=3; Fernandina, *n*=15;

Isabela, *n*=3; Marchena, *n*=5; Pinta, *n*=7; Santa Fe, *n*=5; Santiago, *n*=8. In each plate, we ran the assay on six hawk samples and two positive controls (pooled chicken plasma, #ES1032P, Biomeda, Foster City, CA). Using digitized images of the assay plates, all samples were blindly scored twice to individual, plate number and position. To demonstrate positive standard reliability, assay variation never exceeded 6.8 and 5.6% coefficient of variation (in all cases, CV was calculated using the sample size correction; Sokal & Rohlf 1995) for agglutination titres among and within plates, respectively. Mean NAb agglutination titres and CV were then calculated for each island population from which plasma was collected. CV is a useful measure in studies such as these, since island population means varied widely and CV is dimensionless and relatively stable compared to standard deviation (Snedecor & Cochran 1989).

(e) ***DNA fingerprinting***

To determine island-level population genetic diversity, we performed phenol–chloroform DNA extraction on a subset of hawks from each population comprising a total of 118 individuals (Galápagos hawks: Española, *n*=7; Fernandina, *n*=20; Isabela, *n*=10; Marchena, *n*=20; Pinta, *n*=10; Pinzón, *n*=10; Santa Fe, *n*=10; Santiago, *n*=23; Swainson's hawks: *n*=8), followed by multi-locus minisatellite (VNTR) fingerprinting using the restriction endonuclease Hae III and Jeffreys' probe 33.15 (Jeffreys et al. 1985) and following procedures described elsewhere for birds generally (Parker et al. 1995) and Galápagos hawks (Bollmer et al. in press a). Estimates of island-level population genetic diversity were obtained by calculating multilocus VNTR heterozygosity values (referred

to as *H*; Stephens et al. 1992) for each island population and for the population of Swainson's hawks using GELSTATS v. 2.6 (Rogstad & Pelikan 1996). These markers yield an excellent measure of relative genetic diversity in small, isolated vertebrate populations (Gilbert et al. 1990; Stephens et al. 1992; Parker et al. 1998; Bollmer et al. in press a) but do not measure individual heterozygosity values.

A large study on Galápagos hawk population genetics (Bollmer et al. in press a) used the same multilocus minisatellite markers to estimate population genetic diversity (and included all of the individuals genotyped here). Bollmer et al. (in press a) strongly support the pattern of genetic diversity that we found among these hawk populations. Nearly 90% of the variation in hawk population genetic diversity was explained by island area, and the latter correlates with hawk population size (Bollmer et al. in press a). The four smallest islands with hawk populations had the highest reported levels of minisatellite uniformity of any wild, relatively unperturbed bird species.

As in Bollmer et al. (in press a), we randomly selected individuals sampled within each population to assess the relative amount of genetic diversity within each population. We prioritized samples from adults in territorial breeding groups (groups are comprised of unrelated adults; Faaborg et al. 1995). On Isla Pinzón, we sampled only from nonterritorial birds from multiple geographic locales because we were unable to capture adults there. However, these birds were likely offspring of multiple breeding groups given that many were of the same age cohort (based on plumage characteristics), and that hawks usually produce only one offspring per breeding attempt. Moreover, marked, nonterritorial birds disperse from the natal territory following fledging and roam over their entire natal islands (de Vries 1975; Faaborg 1986; Bollmer et al. in press a). To

ensure that our sampling of birds was not biased by the possible presence of within-island population genetic structure, we sampled and multilocus genotyped birds from multiple geographic locales. For example, on Islas Española and Santiago (which harbour hawk populations with among the lowest and highest genetic diversity, respectively), we sampled territorial birds from the extreme eastern and western portions of the islands (figure 1). On the smaller islands, we sampled birds from a greater proportion of island area than on the larger islands (figure 1). Due to the low genetic diversity within the four smallest hawk populations (Española, Santa Fe, Pinzón, and Marchena), sampling from relatively fewer individuals on the smallest islands was sufficient to characterize their population genetic diversity (Bollmer et al. in press a). Bollmer et al. (in press a) found only four multilocus genotypes within Isla Santa Fe in the 15 birds sampled from both multiple years and geographic locations throughout the island (the entire population of hawks on Santa Fe is likely to be ~30 birds). Bollmer et al. (in press a) further found that populations from Islas Santa Fe, Española, Pinzón, and Marchena were all relatively inbred compared to more variable (but still inbred) populations from Islas Pinta, Fernandina, Isabela and Santiago. Our samples from Swainson's hawks (n=8) and from Isla Isabela (n=10) were small relative to the larger Galápagos hawk population sample sizes, yet both were relatively outbred based on H estimated from the minisatellites. Given this, our estimation of relative genetic diversity within each hawk population sampled is representative of the standing genetic diversity within each population and is not an artifact of sampling bias or within-population genetic structure.

(f) ***Statistical analyses***

For all statistical analyses except the overall comparison of prevalence between louse species which utilized QUANTITATIVE PARASITOLOGY v. 2.0 (Reiczigel & Ro´zsa 2001), louse abundance data were ln + 1 transformed and Stephen's heterozygosity values were arcsine square root transformed to meet assumptions of normality.

We performed a Pearson's correlation analysis in SPSS v. 11.0 (2004) to assess the strength of the relationship between host population genetic diversity (*H*) and average host population parasite abundance from nine hawk populations (eight *B. galapagoensis* and one *B. swainsoni*). The correlation analyses were one-tailed given our *a priori* predictions about the direction of the relationship between the variables. We then examined the relationship between average louse abundance and *H* for the eight Galápagos hawk populations to determine if the relationship was being driven by the relatively outbred Swainson's hawks.

Next, we examined the relationship between innate humoral immunity (NAb agglutination titres) and *H* on the entire subset of individuals (nZ46) for which plasma was collected. The relationship between average island Nab agglutination titres and *H* was not linear. Thus, we used the GLM procedure in SPSS to determine if there was a significant effect of island-level *H* (a fixed factor) on NAb agglutination titres (the dependent variable) instead (Española, n=3; Fernandina, n=15; Isabela, n=3; Marchena, n=5; Pinta, n=7; Santa Fe, n=5; Santiago, n=8).

Finally, we performed a GLM analysis in SPSS using a subset of data that included all 43 birds sampled for both plasma and parasites to determine if antibodies and louse abundances were correlated. In order to control for the effect of island inbreeding

we used the GLM procedure as in the preceding analysis (NAb agglutination titres of the 43 hawks dependent on island as a fixed factor) except that louse abundance for each of the 43 individuals was included as a covariate in the model (Española *n*=3; Fernandina *n*=14; Isabela *n*=3; Marchena *n*=5; Pinta *n*=7; Santa Fe *n*=4; Santiago *n*=7). One analysis was performed for each louse species. A scatterplot of the louse abundance data and NAb agglutination titres was created to show the relationships between the two variables before the analyses and individuals were labelled as either inhabiting a relatively inbred (Española, Marchena or Santa Fe) or outbred (Fernandina, Isabela, Pinta or Santiago) island (see figure 3).

3. RESULTS

(a) *Parasite collections*

We collected a total of 14 843 individuals of the louse *C. turbinatum* and 2858 individuals of the louse *D. regalis* from 199 Galápagos hawks sampled for lice. These lice typically occur on no other birds in the Galápagos, but have been reported from mainland *B. swainsoni* (Whiteman & Parker 2004a). Overall prevalence (across islands) of *C. turbinatum* (97.5%) was higher than that of *D. regalis* (85.4%; Fisher's exact test, $p<0.001$); both louse species occurred in all eight host populations.

We collected a total of 17 individuals of *C. turbinatum*, 22 individuals of *Laemobothrion maximum* and 11 individuals of a *Kurodaia* sp. from the nine Swainson's hawks. These three species abundances were pooled and constitute the amblyceran lice from Swainson's hawks; *C. turbinatum* was the only amblyceran collected from

Galápagos hawks. No *Degeeriella* were collected from the nine Swainson's hawks.

(b) ***Assessment of population genetic diversity***

Untransformed values of *H* for each host population are shown in figure 1. Individuals from the smallest island populations of the Galápagos hawk had the highest reported levels of minisatellite uniformity of any wild, unperturbed bird species and these results are consistent with those of Bollmer et al. (in press a). As in Bollmer et al. (in press a), we found >50% of all bands were fixed within these populations (Santa Fe, 13/16 bands fixed; Española, 10/16 bands fixed; Pinzón, 11/20 bands fixed; Marchena, 11/18 bands fixed). The four most inbred populations contained multiple individuals or sets of individuals that were genetically identical at all loci, whereas no identical individuals were found within the four larger islands populations or within Swainson's hawks (Bollmer et al. in press a).

(c) ***Effects of genetic diversity and other host factors on parasite load***

Among *Buteo* populations (*n*=208 total individuals sampled for lice by population: Española, *n*=8; Fernandina, *n*=28; Isabela, *n*=25; Marchena, *n*=26; Pinta, *n*=31; Pinzón, *n*=10; Santa Fe, *n*=13; Santiago, *n*=58; Swainson's hawks n=9), average amblyceran louse abundance within populations and *H* were significantly and negatively related across populations (figure 2a; *C. turbinatum*; Pearson's r = -0.949, *n*=9, $p<0.0001$; *D. regalis*; r = -0.854, n=9, $p<0.01$). When limited to the eight Galápagos hawk island populations only, similar negative relationships were found for *C. turbinatum* (r = -0.875, *n*=8, $p<0.01$) and *D. regalis* (r = -0.69, *n*=8, $p<0.05$).

(d) ***Innate antibody levels, genetic diversity and parasite load***

We found a significant (and nonlinear) effect of island on average NAb agglutination titres (figure 2b; one-way ANOVA; n=46, $F_{6,39}$=3.41, p<0.01). The Marchena population, the third most inbred population, exhibited the highest average titre and Española and Santa Fe, the most inbred populations, exhibited the lowest (figure 2b). The more outbred island populations had intermediate NAb titres. The variance in NAb titres was lower within the inbred populations than the more outbred populations (figure 2b). The CV of the inbred populations (Santa Fe, Española, Marchena) was 12% within and 25.5% among islands, whereas the CV of the more outbred islands (Fernandina, Isabela, Pinta, Santiago) was 17.8% within and 4.7% among islands. Furthermore, *C. turbinatum* abundance was negatively related to NAb agglutination titres (marginally significant) when individual birds were considered (controlling for the effects of island in a GLM; corrected model $F_{7,35}$=4.05, p<0.01; island effect F=2.50, p<0.05, *C. turbinatum* abundance parameter estimate β = -0.342, F=4.10, p=0.05; figure 3). The scatterplot yielded a triangular pattern whereby birds with low NAb titres consistently harboured high *C. turbinatum* abundances, but birds with high NAb titres harboured both low and high louse abundances. As predicted, no significant relationship was found between the ischnoceran, feather-feeding *D. regalis* and NAb agglutination titres (controlling for the effects of island in a GLM; corrected model $F_{7,35}$=3.01, p<0.05; island effect F=2.60, p<0.05, *D. regalis* abundance parameter estimate β = -0.259, F=1.68, p>0.05).

4. DISCUSSION

We have shown that variation in host population genetic diversity is correlated negatively with average parasite load and positively with variation in NAb levels across populations of the Galápagos Hawk. Smaller, more inbred host populations had higher parasite loads, lower average immune responses (generally) and lower variation in within-population immune response than more outbred populations. NAb levels were negatively correlated with the abundance of a skin and blood feeding amblyceran louse, further linking inbreeding, immune response and parasite burden.

As a result of lower within-population genetic variability and lower and less variable within-population Nab levels, most of the peripheral, inbred and highly differentiated island populations of the Galápagos hawk are vulnerable to disease agents. This result may not be surprising, but few studies have evaluated this relationship in wildlife populations. These populations contained more among-island variability in NAb levels than the larger island-populations, possibly due to the strong effects of genetic drift (Spielman et al. 2004b; Pearman & Garner 2005) or local coevolutionary dynamics (Thompson 1999). Protection of the highly differentiated peripheral hawk populations should be prioritized as the variation they contain is essential for the long-term viability of this species (Lesica & Allendorf 1995). Conversely, the large amount of within-population genetic and immunological variation within the largest hawk island populations is also important from a conservation perspective. Since tradeoffs exist between the humoral and cellular immune response (Lindström et al. 2004), these populations may be better able to respond to multiple invasions of pathogens than

the smaller, more isolated populations. Notably, breeding populations within three large islands (Islas Floreana, San Cristóbal and Santa Cruz) are now likely extinct (Bollmer et al. in press a,b) and each of these is geographically proximal to one or several of the most inbred island populations. Thus, if metapopulation dynamics were operating in this system (Thompson 1999; Templeton et al. 2001), the potential for the introduction of novel alleles (e.g. resistance alleles) by recurrent gene flow among populations has now been reduced given that only 8 out of 11 island populations remain intact. Thus, managers of the Galápagos National Park may consider restricting travel to the smallest island populations of the hawk, given that invasive avian disease vectors have established within several human-inhabited islands that serve as a base of operations for the tourism industry (Wikelski et al. 2004; Whiteman et al. 2005).

As a potential mechanism underlying the relationship between host genetic diversity and average parasite load, we showed that NAb agglutination titres were negatively related to abundance of native parasites that feed on skin and blood (*C. turbinatum*), although the correlational nature of this analysis and its marginal significance, after correcting for the effects of island, indicate that this result be accepted with caution and requires confirmation. However, strength of the PHA-induced immune response in birds was directly related to amblyceran species richness, indicating that amblycerans and their avian hosts are engaged in coevolutionary arms races (Møller & Rózsa 2005). Thus, our finding of a potential relationship between host immune response and amblyceran but not ischnoceran abundance at the individual host level is in accord with this macroevolutionary trend.

The influence of another unmeasured factor correlating with population genetic diversity may also explain the results, although we know of no such factor. Nearly 90% of the variation in hawk genetic diversity is explained by island size, and these hawk populations are genetically isolated from one another (Bollmer et al. in press a,b). Given that larger island populations typically had lower parasite loads, a simple relationship between host population size and parasite load is unlikely here (Lindström et al. 2004). Specific mechanisms underlying the relationship between *H* and disease susceptibility may include the exposure of deleterious recessive alleles (Keller & Waller 2002), the fixation of slightly deleterious alleles through genetic drift (Johnson & Seger 2001), other microevolutionary processes associated with founder events and maintenance of small population sizes over time, or a combination of these. Generalized inbreeding depression may also lead to physical and behavioural changes that affect preening efficiency and this may be particularly germane for *D. regalis*, which mainly encounters mechanical host defences (Clayton et al. 1999; Whiteman & Parker 2004b).

Extinction and disease ecology are 'by their nature cryptic and difficult to study in natural communities' (de Castro & Bolker 2005). Clearly, however, this information is of basic biological interest and offers insight into how populations will respond to invasions of alien pathogens, which is underway in most previously isolated ecosystems. Future studies examining host immunogenetics, parasite population genetics and transmission dynamics are necessary for fully assessing the threat of pathogens to this island endemic.

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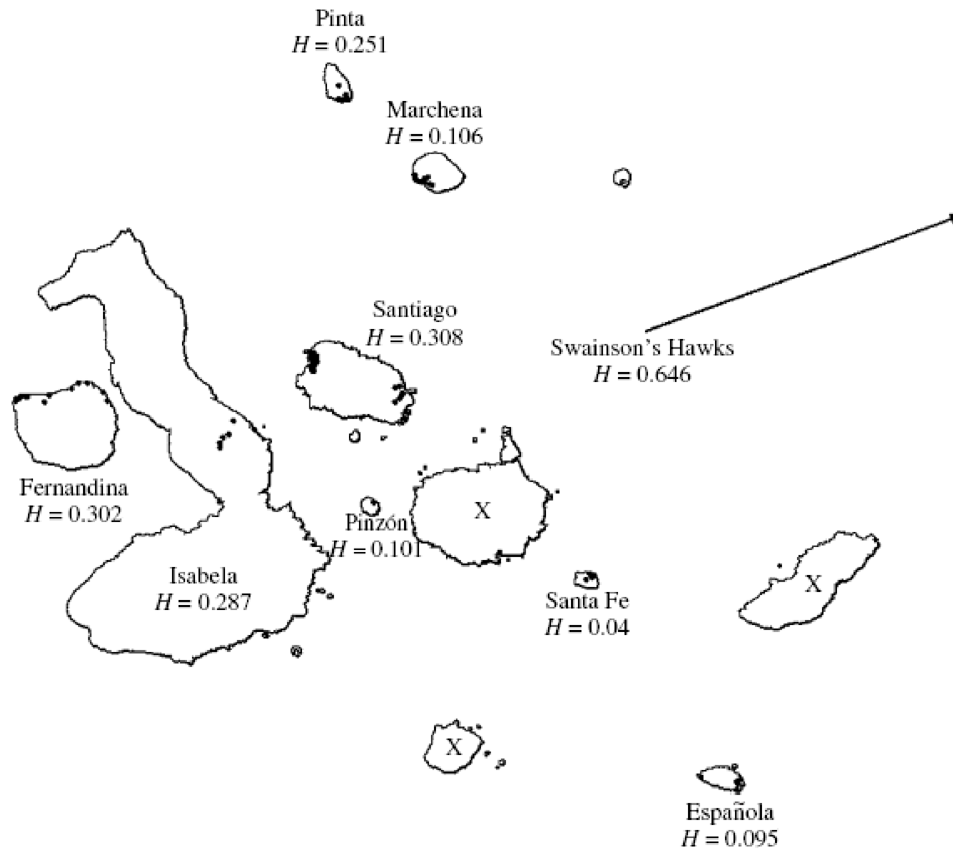
Figure legends

Figure 1 Map of the Galápagos Archipelago, located approximately 1000 km west of mainland Ecuador, South America. Extant breeding island populations of the Galápagos hawk (*Buteo galapagoensis*) are named, followed by estimates of island population genetic diversity (*H*; Stephens heterozygosity values) calculated from multilocus minisatellite data. Small black dots within islands indicate sampling localities. An estimation of *H* from the mainland Swainson's hawk (the putative sibling species of *B. galapagoensis*) was included for comparative purposes. Extinct island populations of *B. galapagoensis* are indicated by an 'X' (there is no evidence indicating hawks have ever inhabited Isla Genovesa located in the northeastern part of the archipelago).

Figure 2 Scatterplot of two disease susceptibility variables versus estimated host population genetic diversity (heterozygosity) values. (a) Louse abundance versus host population genetic diversity. Closed circles, average amblyceran abundance ±95% confidence intervals (*Colpocephalum turbinatum*, *Laemobothrion maximum*, and *Kurodaia* sp.; $r = -0.949$, $n=9$, $p<0.0001$); open circles, average ischnoceran abundance ±95% confidence intervals (*Degeeriella regalis*; $r = -0.854$, $n=9$, $p<0.01$). Dyads with heterozygosity values greater than 0.9 represent a mainland *B. swainsoni* population and the remaining values represent eight island populations of *B. galapagoensis*. Island populations reading left to right are as follows: Santa Fe, Española, Pinzón, Marchena, Pinta, Isabela, Fernandina, Santiago; (b) average agglutination titres (NAbs) ± SDM from 46 *B. galapagoensis* individuals versus

estimated host population genetic diversity (the relationship between NAb agglutination titres and genetic diversity was not linear, although significant differences existed in average NAb agglutination titres among island-populations, one-way ANOVA: $F_{6,39}$, $p<0.01$). Island populations reading left to right are as follows: Santa Fe, Española, Marchena, Pinta, Isabela, Fernandina, Santiago.

Figure 3 Negative linear relationship between *Colpocephalum turbinatum* abundance and natural antibody (NAb) titres. The regression line through the raw data (uncorrected for island) is shown ($\beta = -0.355$, $p<0.01$). The relationship was marginally significant after controlling for the effects of island and other host factors ($\beta = -0.342$, $p=0.05$). Open circles, individuals from more inbred island populations (Española, Marchena, Santa Fe); solid circles, individuals from more outbred island populations (Fernandina, Isabela, Pinta, Santiago).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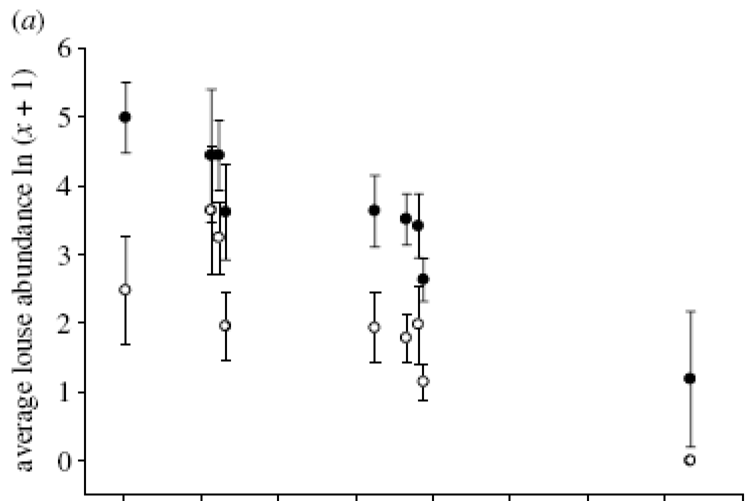
Marchena
H = 0.106
Santiago
H = 0.308
Swainson's Hawks
H = 0.646
Fernandina
H = 0.302
Pinzón
H = 0.101
X
Isabela
H = 0.287
Santa Fe
H = 0.04
X
X
Española
H = 0.095


(a)
average louse abundance ln (x + 1)
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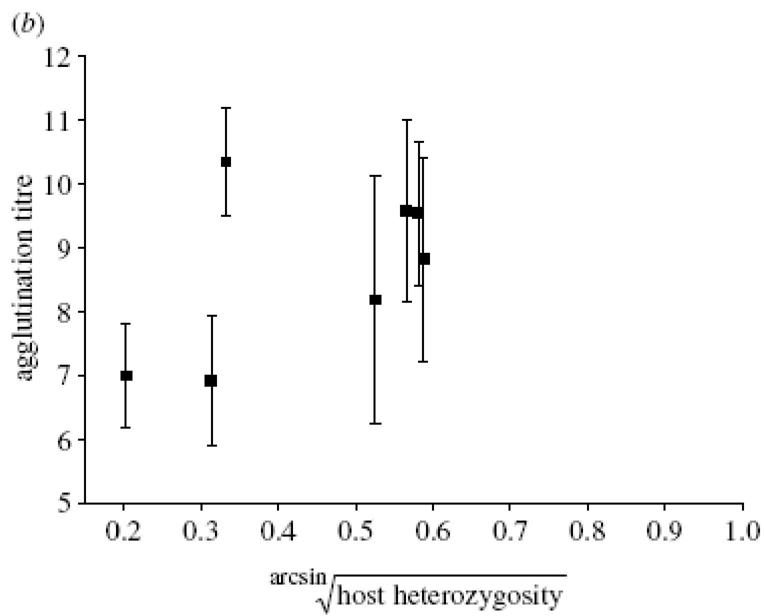
(b)
agglutination titre
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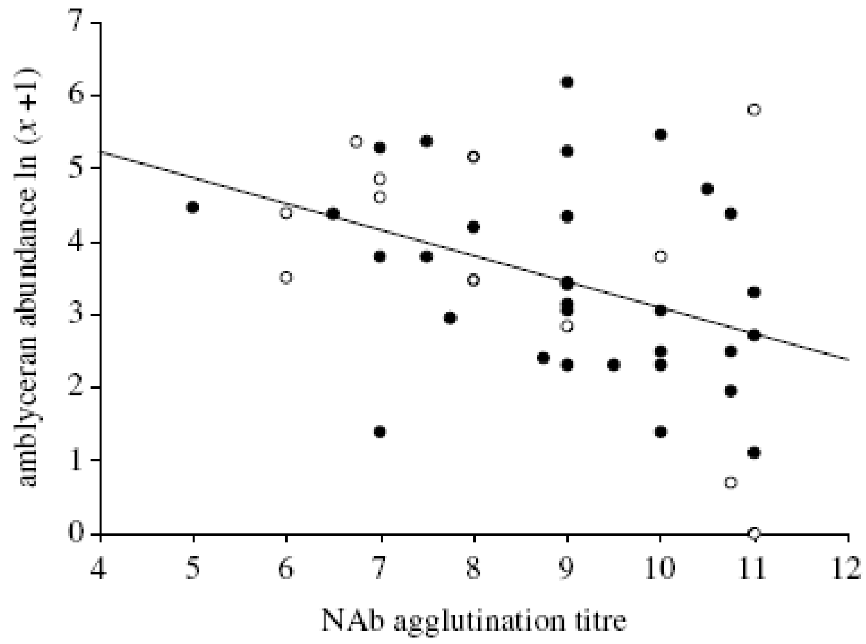
0.9
1.0
arcsin √host heterozygosity



amblyceran abundance ln (x +1)
NAb agglutination titre
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Chapter 4

Low MHC variation in the endangered Galápagos penguin (*Spheniscus mendiculus*)

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ABSTRACT

The major histocompatibility complex (MHC) is one of the most polymorphic regions of the genome, likely due to balancing selection acting to maintain alleles over time. Lack of MHC variability has been attributed to factors such as genetic drift in small populations and relaxed selection pressure. The Galápagos penguin (*Spheniscus mendiculus*), endemic to the Galápagos Islands, is the only penguin that occurs on the equator. It relies upon cold, nutrient-rich upwellings and experiences severe population declines when ocean temperatures rise during El Niño events. These bottlenecks, occurring in an already small population, have likely resulted in reduced genetic diversity in this species. In this study, we used MHC class II exon 2 sequence data from a *DRB1*-like gene to characterize the amount of genetic variation at the MHC in 30 Galápagos penguins, as well as one Magellanic penguin (*S. magellanicus*) and two king penguins (*Aptenodytes patagonicus*), and compared it to that in five other penguin species for which published data exist. We found that the Galápagos penguin had the lowest MHC diversity (as measured by number of polymorphic sites and average divergence among alleles) of the eight penguin species studied. A phylogenetic analysis showed that Galápagos penguin MHC sequences are most closely related to Humboldt penguin

(*Spheniscus humboldti*) sequences, its putative sister species based on other loci. An excess of nonsynonymous mutations and a pattern of trans-specific evolution in the neighbor-joining tree suggest that selection is acting on the penguin MHC.

KEYWORDS Galápagos penguin, *Spheniscus mendiculus*, major histocompatibility complex, genetic bottleneck, trans-species evolution

INTRODUCTION

The genes at the major histocompatibility complex (MHC) are among the most polymorphic known, having unusually large numbers of alleles as well as higher nucleotide diversity than other loci (Parham and Ohta 1996, Gaudieri et al. 2000, Garrigan and Hedrick 2003). MHC molecules play a central role in the immune system by recognizing foreign peptides, binding to them, and presenting them to T-cells, thus initiating the immune response (Klein 1986). It is generally believed that MHC variability is the result of alleles being maintained in populations by some form of balancing selection, with the two most likely mechanisms being selection for resistance to parasites (either through overdominance or negative frequency-dependent selection) or sexual selection via mate choice (Doherty and Zinkernagel 1975, Takahata and Nei 1990, Penn and Potts 1999; reviewed in Bernatchez and Landry 2003, Piertney and Oliver 2006). The long-term maintenance of allelic lineages in populations due to balancing selection may result in trans-specific evolution, where the coalescent times of MHC alleles found in different species predate speciation events (Takahata 1990, Klein et al. 1993).

In birds, there is a growing body of data describing MHC structure in non-model species. Earlier work on chickens found a very simplified MHC structure compared to that in mammals (Kaufman et al. 1999), but subsequent research on other species has shown that the simple chicken MHC is not representative of all birds, and there is much variation among species in number and organization of MHC genes (Hess and Edwards 2002). Many studies of natural populations of birds have found the large numbers of divergent alleles expected at these loci (e.g., Ekblom et al. 2003, Bonneaud et al. 2004, Westerdahl et al. 2004). In some cases, though, the effects of genetic drift appear to outweigh balancing selection, resulting in reduced MHC polymorphism (e.g., Richardson and Westerdahl 2003, Miller and Lambert 2004).

Galápagos penguins (*Spheniscus mendiculus*) are endemic to the Galápagos Islands (Fig. 1) and are the only tropical penguin species. They are able to persist at the equator due to the cold, nutrient-rich upwellings from the Cromwell current (Boersma 1977, 1978). About 95% of Galápagos penguins are distributed around the westernmost islands of Fernandina and Isabela where the upwelling is greatest, while the other 5% occur in small, isolated populations around three other islands (Bartolomé, Santiago, and Floreana; Boersma 1977, 1978). The Galápagos penguin undergoes dramatic population fluctuations in response to El Niño events, when warmer water temperatures reduce food available to penguins and other species dependent on the normally cold, productive waters (Boersma 1998; Vargas et al. 2005a, 2006). Using a capture-mark-resight method, Vargas et al. (2005a) estimated that the population has fluctuated between 699 and 3386 penguins since the first penguin census in 1970, and the population was estimated to have dropped by 77% during the 1982-83 El Niño (Valle and Coulter 1987).

In 2005, there were approximately 1900 penguins (Vargas et al. 2005b). Because of its limited distribution and population crashes, the Galápagos penguin is listed as endangered (BirdLife International 2005).

While censuses indicate that the Galápagos penguin population has been fluctuating since at least the 1970s (Vargas et al. 2005a, 2006), it is likely that the penguin population has fluctuated for much longer. Riedinger et al. (2002) estimated that El Niño events have been occurring in Galápagos for at least the last 6000 years, and the penguins are estimated to have arrived in the archipelago probably much earlier than that, about 4 million years ago (Baker et al. 2006). Small populations experience increased genetic drift and are thus expected to lose genetic variation more quickly than larger ones; furthermore, populations that undergo fluctuations in size are also expected to lose variation to drift (Wright 1931, Nei et al. 1975, Frankham 1996). Because of its small population size and repeated bottlenecks, the Galápagos penguin likely has less genetic variability than other penguin species and other outbred, mainland species. In the only published genetic study of the Galápagos penguin, Akst et al. (2002) found a low level of heterozygosity (3%) at five microsatellite loci in the Galápagos penguin, which contrasted sharply with the 46% heterozygosity present in the Magellanic penguin (*S. magellanicus*), a species numbering over one million individuals (Gandini et al. 1996).

In this study, we present the first description of the MHC in the endangered Galápagos penguin. While the Galápagos penguin appears to have low genetic variability at neutral microsatellite loci, MHC genes are under balancing selection, so they may show variability equivalent to that in more outbred species, unless the effect of genetic drift has been too strong or selection has been relaxed. MHC variation has been

characterized in several penguin species (Tsuda et al. 2001), but it has been particularly well described in the Humboldt penguin (*Spheniscus humboldti*; Kikkawa et al. 2005), a temperate species that is sister to the Galápagos penguin (Baker et al. 2006). It is also affected by El Niño events; however, its population is much larger than the Galápagos penguin's (at least 10,000 individuals; Ellis et al. 1998, Luna-Jorquera et al. 2000). We compared MHC variability in the Galápagos penguin to that in its sister the Humboldt penguin and other penguin species for which published data exist. In addition, we incorporated our Galápagos penguin class II sequences into a phylogeny of previously published sequences from other penguin species, as well as preliminary sequences from Magellanic (*S. magellanicus*) and king (*Aptenodytes patagonicus*) penguins, in order to compare interspecific relationships based on selected MHC genes with those based on nuclear and mitochondrial genes (Baker et al. 2006).

MATERIALS AND METHODS

Sampling

Blood samples were collected from Galápagos penguins during four separate trips from 2003 to 2005. Penguins were sampled from multiple sites on the islands of Floreana, Fernandina, Santiago, Bartolomé, and Isabela, covering the entire range of this species. In order to characterize Galápagos penguin MHC, we chose a random subset of 30 individuals from 8 sites spread throughout the species' range (Fig. 1): Islote Las Bayas Pequeña by Floreana (*N*=2), Santiago/Bartolomé (*N*=4), Punta Espinosa on Fernandina

(*N*=4), and 5 sites on Isabela (El Muñeco, Villamil, Punta Moreno, Las Marielas, and Caleta Iguana; *N*=4 individuals each for a total of 20). All birds used were adults.

We also used three blood samples taken by the Saint Louis Zoo from their penguin collection in Saint Louis, Missouri: one Magellanic and two king penguins. MHC sequences from these two species have not been previously published, and the primary purpose of these samples was for incorporation of more species (including a new genus, *Aptenodytes*) into our phylogeny.

MHC genotyping

We used the primers pen1 and pen4 (Tsuda et al. 2001; Kikkawa et al. 2005) to amplify a 198 bp fragment (primers included) of exon 2 of a class II MHC *DRB1*-like gene. Tsuda et al. (2001) found that this primer set amplified no more than two alleles per individual in the four penguin species they screened, suggesting that it was amplifying only one locus.

We genotyped 12 Galápagos penguin individuals using a combination of cloning and sequencing. First, the MHC was amplified using the pen1/pen4 primer set in 40 µl reactions: 1 mM $MgCl_2$, 0.7X PCR buffer, 0.2 µM dNTPs, 0.4 µM each primer, 0.3 units of *Taq* polymerase, and 80 ng genomic DNA. The PCR was run for 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min. Fragments were gel purified using QIAquick Gel Extraction Kits (QIAGEN) and then cloned using the pGEM-T Easy Vector cloning kit (Promega). Positive colonies were picked and suspended in 10 µl dH_2O. They were screened for inserts of the correct size using M13 primers and then sequenced on an ABI 3100 using the primers SP6 and T7. We amplified and cloned each

individual at least twice, and we sequenced at least 10 positive clones from each individual. All sequences were double-stranded with 100% overlap.

We confirmed the genotypes of these 12 individuals and screened the other 18 Galápagos penguins using denaturing gradient gel electrophoresis (DGGE; Westerdahl et al. 2004, Knapp 2005). We used the same primers to amplify the MHC fragment; however, we added a GC-clamp to the 5′ end of pen4 (Sheffield et al. 1989). We ran the reactions using the same temperature cycle as above, but reactions were in a volume of 48 μl: 1.25 mM $MgCl_2$, 0.85X PCR buffer, 0.25 μM dNTPs, 0.3 μM each primer, 1.0 units of *Taq* polymerase, and 70 ng genomic DNA. PCR amplicons were run on 8% 19:1 acrylamide/bisacrylamide gels using a 40 to 60% denaturing gradient of formamide and urea. Gels ran at 160V for 5 hours at 60°C, and then we stained them using SYBR© gold (Promega) and visualized them on a Kodak IS440CF imaging system.

The Magellanic and king penguins were also genotyped using a combination of cloning, sequencing, and DGGE. All sequences were deposited into GenBank (accession numbers EF212007 to EF212014).

Data analysis

We assembled and edited sequences using Seqman v. 6.1 (DNASTAR, Inc.) and aligned them manually in BioEdit v. 7.0.5.2 (Hall 1999). We calculated nucleotide diversity (π) using the program DnaSP v. 4.0 (Rozas et al. 2003), and we measured the rates of synonymous (d_S) and nonsynonymous (d_N) substitutions using the Nei and Gojobori (1986) method with the Jukes-Cantor correction in MEGA v. 2.1 (Kumar et al. 2001). We calculated d_S and d_N separately for peptide-binding codons and non-peptide-binding codons as determined by Brown et al. (1993). We tested for positive selection

($d_N > d_S$) using a Z-test in MEGA. In order to study the phylogenetic relationships among the MHC alleles, we constructed a neighbor-joining tree (Saitou and Nei 1987) from Jukes-Cantor distances in MEGA. It is important to note that, while we refer to our sequences as alleles, they do not encompass the entire length of exon 2 and are thus only partial allelic sequences.

RESULTS

Identification of alleles

In the 30 Galápagos penguins screened, we confirmed the presence of three sequences (Spme1, 2, and 3). Two of them (Spme1 and 2) occurred in homozygous and heterozygous form in multiple individuals, while Spme3 was present in only one individual (a heterozygote). During the cloning and sequencing process, we obtained other apparent alleles as well. Those differing by a single mutation from the confirmed ones were attributed to *Taq* error. Two alleles, though, arose multiple times in the Spme1/2 heterozygotes. When amplifying multiple sequences in one reaction, spurious alleles can form either through *in vitro* recombination when an incompletely amplified sequence pairs with the template of another or as heteroduplexes that form during the last PCR cycle when two completed alleles with different sequences anneal to each other (Jansen and Ledley 1990, L'Abbe et al. 1992, Longeri et al. 2002). The sequences of both of the suspect alleles could be explained by Spme1 and 2; one was identical to the 5′ end of Spme1 and 3′ end of Spme2, and the other was the reverse. When these individuals were run on the DGGE gels, they clearly had only the Spme1 and 2 alleles.

Spme3 is a combination of Spme1 and 2, matching the 5′ end of Spme2 and the 3′ end of Spme1; however, we confirmed it as a true allele in one individual. That individual was run multiple times on DGGE gels, and it consistently showed the Spme1 allele and another unique allele that ran slightly differently from Spme2. Cloning and sequencing of the individual produced the Spme3 allele. We also ran a clone of the Spme3 allele adjacent to a direct PCR of the individual on a DGGE gel to verify that the clone comigrated with the unique allele.

The Magellanic penguin yielded two alleles after cloning and sequencing (Spma1 and 2), and we confirmed the sequences of three alleles from the two king penguins (Appa1, 2, and 3), though there was at least one other allele we did not confirm.

Because we only sequenced from genomic DNA, we cannot be sure that these confirmed alleles are expressed. However, we did not find any frameshift mutations or stop codons within them. Also, Tsuda et al. (2001) found that these primers amplified the same alleles from both genomic DNA and DNA from RT-PCR in an Adelie and chinstrap penguin, indicating that they amplified expressed alleles in those species.

Sequence variability

Among the three Galápagos penguin alleles, there were only three polymorphic sites in the 157 bp sequenced (after removing the primers). Spme1 and Spme2 differed from each other at three sites, while Spme3 differed from Spme2 at only one site and from Spme1 at two. MHC diversity was low in the Galápagos penguin compared to the other penguin species studied, in terms of both number of alleles and degree of divergence among alleles (Table 1). In the other penguin species, there were many more polymorphic sites and consequently greater nucleotide diversity than what was present in

the Galápagos penguin (Table 1, Fig. 2). While we sampled only one Magellanic and two king penguins, it appears that their variability (Table 1) may be comparable to that of the species studied by Tsuda et al. (2001) and Kikkawa et al. (2005).

All three Galápagos penguin substitutions were nonsynonymous, and two of them occurred at probable antigen-binding sites (ABS; Fig. 2). We compared the nonsynonymous and synonymous substitution rates at probable antigen-binding codons and at the codons that are non-antigen-binding sites (non-ABS) for the penguin species studied (Table 2). Nonsynonymous rates were higher at the ABS than at the non-ABS for all the species. At the non-ABS, d_N was not significantly greater than d_S for any species, while at the ABS d_N was significantly greater than d_S for six of the eight species. The results for the Galápagos penguin ($Z = 1.47$, $p = 0.07$) and the gentoo penguin ($Z = 1.24$, $p = 0.11$) were not significant.

Phylogenetic analysis

A neighbor-joining analysis showed that all of the penguin sequences formed a monophyletic group separate from the fowl and passerine outgroups (Fig. 3). Within the penguin group, alleles from the Adelie, chinstrap, and Galápagos penguins formed single clusters by species. The little blue penguins separated into two clusters, one of which was closely related to the king penguins. The gentoo penguins fell into two clusters, while the Humboldt penguins fell into three. One of the Magellanic alleles was identical to a previously published Humboldt allele, at least at the 157 bp for which we have data, while the other fell within a cluster of Humboldt alleles. At the generic level, the three *Pygoscelis* species grouped into a large cluster, though the one *Eudyptula* and one *Aptenodytes* species fell within them. The three *Spheniscus* species also formed clusters

together. Most of these relationships have to be considered cautiously, though, as the bootstrap support for many of the nodes is very low.

DISCUSSION

MHC loci are known for their large numbers of divergent alleles. Contrary to what has been found in most other species, the Galápagos penguin had very little genetic diversity at the *DRB1*-like MHC class II locus we studied. We found only three alleles in the 30 individuals we genotyped, and one of those alleles was present in only one individual. Because these are only partial allelic sequences from exon 2, there could be a greater number of alleles when the entire length of the exon is taken into account. While the number of sequences we found in the Galápagos penguin at these 157 bp is comparable to the number found in some other penguin species, the sample sizes of individuals genotyped in those species were small, likely missing other alleles. In addition, the Galápagos penguin was less variable than all the other species in number of polymorphic sites and divergence among alleles. The other well sampled species is the Humboldt penguin, the Galápagos penguin's closest living relative. The Humboldt showed much higher diversity than the Galápagos penguin at this gene (20 polymorphic sites versus 3 and a nucleotide diversity of 0.06 versus 0.01 in 157 bp of the exon).

Genetic drift is the most likely explanation for the reduced genetic diversity in the Galápagos penguin given its demographic history in which there was probably an initial founder effect when the population established itself in the archipelago followed by repeated population bottlenecks caused by El Niño events over thousands of years (in an

already small population). There are other cases of low MHC diversity, especially in bottlenecked or naturally small populations (e.g., island populations). For example, Miller and Lambert (2004a,b) found that the Chatham Island black robin (*Petroica traversi*) of New Zealand was fixed for 3 of 4 MHC class II alleles (probably from four loci), and the endemic Seychelles warbler (*Acrocephalus sechellensis*) has substantially reduced MHC diversity compared to the widespread great reed warbler (*A. arundinaceus*; Richardson and Westerdahl 2003). In mammals, small island populations of the Australian bush rat (*Rattus fuscipes greyii*) were mostly fixed for different MHC alleles (Seddon and Baverstock 1999), as were populations of the bottlenecked Eurasian beaver (*Castor fiber*; Babik et al. 2005). In these cases it is thought that the effect of genetic drift was particularly strong and overwhelmed the effect of balancing selection. Hedrick et al. (2000) noted that in some species with reduced numbers of MHC alleles, the remaining alleles are highly divergent, which might allow for the recognition of a wider range of pathogens. The amino acid sequences of the four black robin alleles differed by an average of 25% (Miller and Lambert 2004b), whereas in the Galápagos penguin there was an average of only 4% amino acid divergence among alleles.

An alternative explanation for lower MHC diversity is reduced selection on the MHC due to reduced exposure to parasites. Slade (1992) hypothesized that the lower MHC variation in some whales (Trowsdale et al. 1989, Murray and White 1998) and seals (Slade 1992, Lehman et al. 2004) compared to terrestrial mammals is a result of their exposure to a more limited suite of pathogens. Penguins evolved in cold, marine habitats where there are few other bird species to act as disease reservoirs and where pathogen and vector diversity is low (Jones and Shellam 1999, Clarke and Kerry 2000).

There have been no records of haematozoa from Antarctic or sub-Antarctic penguin populations, and haematozoa are found at low prevalences and intensities in those more northern populations that are infected (Jones and Shellam 1999). Parasite and vector diversity should be higher in the tropics, so it might be expected that the Galápagos penguin should be under greater selection pressure than other penguin species; however, the Galápagos Islands are isolated and likely have reduced parasite diversity compared to tropical mainland habitats. In a baseline health survey of the Galápagos penguin, Travis et al. (2006) found that while 89% of the tested penguins were seropositive for *Chlamydophila psittaci*, all were seronegative for 14 common avian viruses (e.g., paramyxovirus, infectious bursal disease, Marek's disease, and adenovirus). Also, blood smears revealed the presence of microfilarid nematodes, though at low intensities and at an overall prevalence of 13.8% (Merkel et al. in press). While relaxed selection on the MHC might be partially responsible for the reduced genetic diversity, it is likely that demographic factors are having a stronger effect.

There are species where MHC variation exists despite reduced neutral genetic variation due to genetic drift. Aguilar et al. (2004) found variation at the DRB MHC locus and three microsatellite loci linked to the MHC in a population of Channel Island foxes (*Urocyon littoralis dicheyi*) that was previously shown to be completely monomorphic at neutral minisatellite (Gilbert et al. 1990) and microsatellite (Goldstein et al. 1999) loci. Aguilar et al. (2004) concluded that periodic balancing selection may have preserved this variation despite genetic drift (but see Hedrick 2004). Jarvi et al. (2004) suggested that balancing selection might explain why a species of honeycreeper (*Vestiaria coccinea*) that was monomorphic at the mitochondrial control region had MHC

variability comparable to that of more outbred species. In the Galápagos penguin, though, the level of neutral genetic variability appears to be consistent with MHC variability. Akst et al. (2001) found reduced heterozygosity at microsatellite loci in the Galápagos penguin compared to Magellanic penguins, and a microsatellite study performed in our lab shows that Galápagos penguins have a small number of alleles per locus compared to other species (B. Nims et al., unpubl. data).

This primer set amplified transcribed alleles in two other penguin species (Tsuda et al. 2001), making it unlikely that the low MHC variability seen here was due to the amplification of a pseudogene or nonclassical locus (Hess et al. 2000, Aguilar et al. 2006). Furthermore, we found higher nonsynonymous substitution rates at antigen-binding sites than at non-antigen-binding sites in all penguins, and higher nonsynonymous substitution rates than synonymous substitution rates at antigen-binding sites in six of eight species, which also suggests that this locus is under selection.

Trans-specific evolution of MHC alleles

Baker et al.'s (2006) penguin phylogeny (based on 5691bp of nuclear and mitochondrial DNA) indicated that the *Aptenodytes* penguins (king and emperor) were the most basal, followed by the *Pygoscelis* species, chinstraps and gentoos being more closely related to each other than either was to the Adelies. *Eudyptula* and *Spheniscus* were sister genera, and within *Spheniscus*, Galápagos and Humboldt penguins were sister, as were Magellanic and African penguins.

Our neighbor-joining tree based on 157bp of MHC sequence data did not match these relationships. Instead of the king sequences being most basal followed by *Pygoscelis* and *Spheniscus*, it was the reverse with *Spheniscus* sequences being most

basal. The king penguin sequences clustered with two *Eudyptula* sequences within a larger cluster of *Pygoscelis* species. Gentoo sequences clustered with both Adelie and chinstrap sequences rather than more closely with the chinstraps. The *Eudyptula* sequences were more closely related to *Pygoscelis* and *Aptenodytes* than *Spheniscus*. Based on Baker et al.'s (2006) data, we had expected the close relationship we found between the Humboldt and Galápagos sequences since they are likely sister species. We had expected the Magellanic sequences, however, to be more divergent from the Humboldt and Galápagos sequences, but they clustered with the Humboldts, one of them being identical to a Humboldt allele at the 157 bp fragment sequenced. It will be interesting to see if this relationship holds true with a larger sampling of Magellanic penguins. The Magellanic and Humboldt penguin distributions overlap in the wild, and our sampled Magellanic penguin's close relationship with the Humboldt penguins in the phylogenetic tree suggests a hybridization event in its ancestry.

This lack of concordance between the two phylogenies could be due to sampling error, either from few individuals being genotyped for some species or a lack of resolution due to the relatively short fragment size sequenced. There was low bootstrap support for many of the nodes. Alternatively, the differences in topology and lack of stronger structure could be due to the effect of selection acting on the MHC. Balancing selection on MHC alleles may result in trans-species evolution, where alleles have long coalescent times (often predating speciation events) and show less divergence among species than what is found at neutral markers. Alleles from related species are sometimes interdigitated on trees as has been found within honeycreepers (Jarvi et al. 2004), Darwin's finches (Vincek et al. 1997), and warblers (Richardson and Westerdahl 2003).

There was evidence of this within the penguins as well. Only three of the eight species formed monophyletic clusters, and the Magellanic penguin shared an allele with the Humboldt penguins.

There is growing concern about the threat introduced diseases may pose to native bird species in the Galápagos Islands (Wikelski et al. 2004, Parker et al. 2006). Mosquito vectors are present in the archipelago, including the species known to vector avian malaria (*Plasmodium*) elsewhere (Whiteman et al. 2005). Both in the wild and in captivity, penguins are highly susceptible to exotic pathogens (Clarke and Kerry 1993). Other *Spheniscus* species have been found to be very susceptible to *Plasmodium* in captivity (Fix et al. 1988, Cranfield et al. 1991). There has not yet been any evidence of *Plasmodium* in Galápagos penguins (Miller et al. 2001), though other blood parasites (*Haemoproteus* sp. and microfilariae) have been reported (Parker et al. 2006, Travis et al. 2006). The Galápagos penguin is already at risk due to demographic factors (small population size and periodic bottlenecks), but it appears that genetic monomorphism at these immunological loci that are instrumental in disease resistance may put this species at even further risk.

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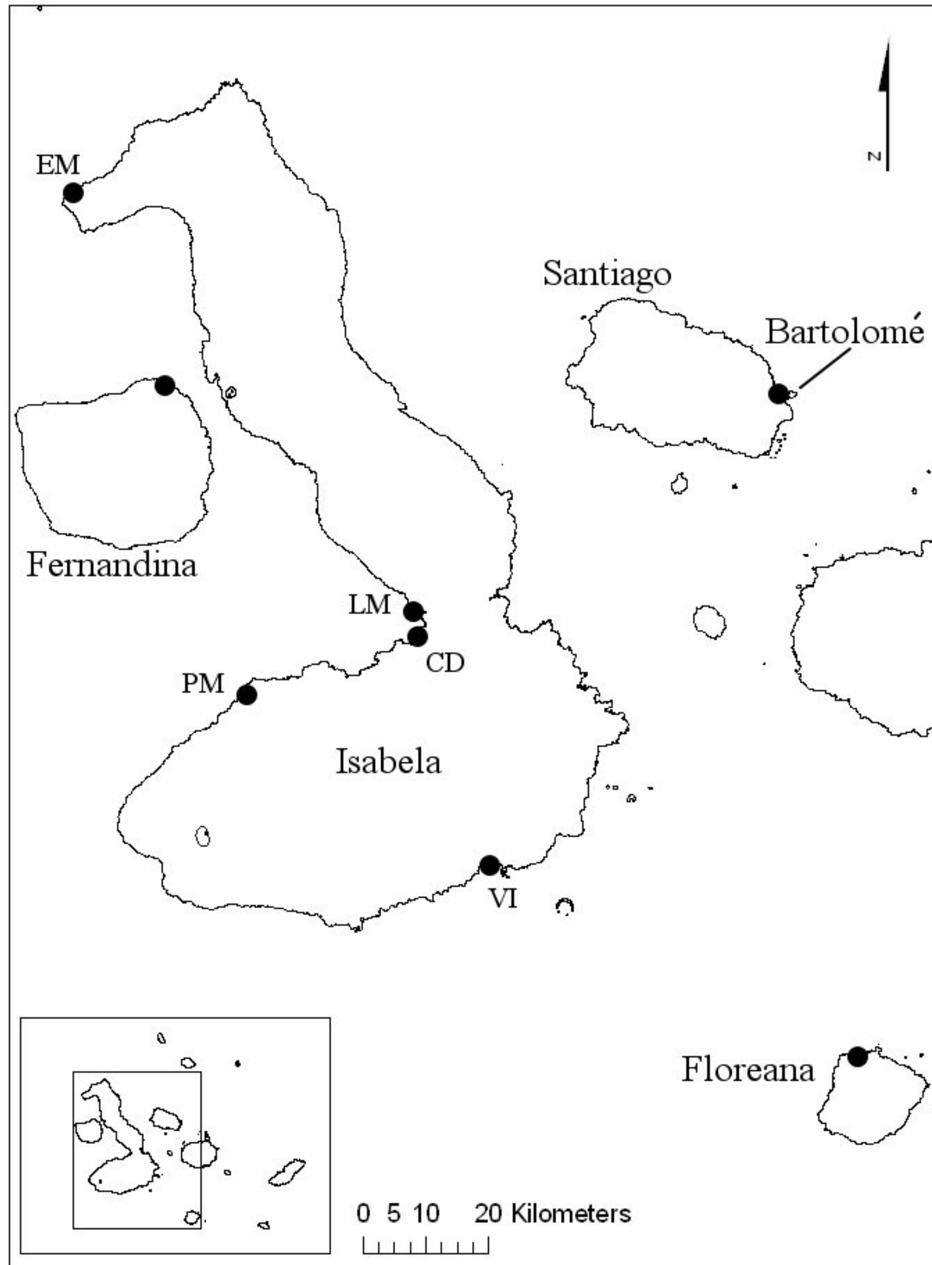
FIGURE LEGENDS

Fig. 1 Map of the western islands of the Galápagos archipelago (the inset shows the entire archipelago). The Galápagos Islands are located 1000 km off the west coast of South America and overlap the equator. The black dots represent sites where Galápagos penguins (*Spheniscus mendiculus*) used in this study were sampled. EM = El Muñeco, LM = Las Marielas, CD = Caleta Derek, PM = Punta Moreno, CI = Caleta Iguana, and VI = Villamil.

Fig. 2 Alignment of partial MHC class II exon 2 amino acid sequences from eight species of penguin. The asterisks indicate likely antigen-binding sites based on Brown et al. (1993). Spme = Galápagos penguin (*Spheniscus mendiculus*), Sphu = Humboldt penguin (*S. humboldti*), Spma = Magellanic penguin (*S. magellanicus*), Eumi = little blue penguin (*Eudyptula minor*), Pyad = Adelie penguin (*Pygoscelis adeliae*), Pyan = chinstrap penguin (*P. antarctica*), Pypa = gentoo penguin (*P. papua*), and Appa = king penguin (*Aptenodytes patagonicus*).

Fig. 3 Neighbor-joining tree constructed from Jukes-Cantor distances of partial MHC class II exon 2 sequences from eight penguin species. Bootstrap percentages (based on 1000 repetitions) below 50 are not shown. Sequences from a snipe (Game, *Gallinago media*; AF485407, duck (Anpl, *Anas platyrhynchus*; AF390589), chicken (Gaga, *Gallus gallus*; M29763), sparrow (Pado, *Passer domesticus*; AY518182), blackbird (Agph, *Agelaius phoeniceus*; AF328737) and finch (Gesc, *Geospiza scandens*; Z74412) were

used as outgroups. Spme = Galápagos penguin (*Spheniscus mendiculus*), Sphu = Humboldt penguin (*S. humboldti*), Spma = Magellanic penguin (*S. magellanicus*), Eumi = little blue penguin (*Eudyptula minor*), Pyad = Adelie penguin (*Pygoscelis adeliae*), Pyan = chinstrap penguin (*P. antarctica*), Pypa = gentoo penguin (*P. papua*), and Appa = king penguin (*Aptenodytes patagonicus*).



EM
Santiago
Bartolomé
Fernandina
LM
CD
PM
Isabela
VI
Floreana
N
0 5 10 20 Kilometers

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                   * * *     **         *         *   **   *   * **  *    *
Spme1 (EF212007) FVVRDIYNRQ QDVHFDSDVG YYVADTPLGE PDAKYWNSQT DILEQRRAEV DTY
Spme2 (EF212008) Y.E....... .......... .......... .......... ........A. ...
Spme3 (EF212009) Y.E....... .......... .......... .......... .......... ...
Sphu1 (AB154393) Y.E....... .......... QF........ .......... .L........ ...
Sphu2 (AB162144) .......... .N........ QF........ .......... ....DE..A. ...
Sphu3 (AB154395) Y.E....... .......... QF........ .......... .F...K.... ..V
Sphu4 (AB154397) .......... .......... QF........ .......... .F...S..A. ..I
Sphu5 (AB154398) ..E.Y..... .N........ .......... .......... .F...K.... ...
Sphu6 (AB154399) L.E....... .Y........ .......... .S........ ........A. ...
Spma1 (EF212010) .......... .......... QF........ .......... .F...S..A. ..I
Spma2 (EF212011) ..D.Y..... EY........ QF........ .S........ ....DE..A. ...
Eumi1 (AB060946) ....N..... .YA....... H......... .S........ ....RK.... ..V
Eumi2 (AB060949) .......... .......... H......... .......... ....RK.... ..V
Eumi3 (AB060948) ....K..... EYA....... .......... .I...L.... .V..DA..A. ...
Eumi4 (AB060947) ....K..... EYA....... H......... .S..HL.... .......... ...
Pyad1 (AB043601) Y......... .F........ RH........ .I..D...R. ....R...A. ..I
Pyad2 (AB043605) Y......... .F........ R......... ....D..... .F......A. ...
Pyad3 (AB029998) Y...A..... ..L....... LFE....... .......... .F...K..A. ..I
Pyad4 (AB029994) Y......... ..L....... R......... ....D..... .F........ ..F
Pyan1 (AB043556) Y.E.Y..... .YA....... L......... .........P .L..R..... ..V
Pyan2 (AB043559) .L..N.H... .L........ L......... .I..DF..R. ....R..... ..V
Pyan3 (AB043558) .L..N.H... .F........ L......... .........P .L..R...Q. ..V
Pypa1 (AB043590) Y.E...H... .Y........ H......... .......... .L..R..... ...
Pypa2 (AB043600) Y.E.Y..... .Y....I.M. H......... .S........ .L...K..A. ..F
Pypa3 (AB043599) .......... .Y........ H......... ....D..... ....R.L... ...
Pypa4 (AB043598) Y.E.Y.H... .Y....I.M. H......... .......... .L......A. ..F
Pypa5 (AB043597) .......... .Y........ H......... ....D..... ....R..... ...
Pypa6 (AB043595) Y.E.Y.H... .Y....I.M. H......... .S........ .L...K..A. ..F
Pypa7 (AB043594) ..E.Y.H... .F........ H......... ....D...R. .L......A. ...
Pypa8 (AB043591) ..E.Y..... MF........ H......... ....D...R. .L......A. ..F
```

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Appa1 (EF212012) ..E.YF.... ELL....... HF........ .I..D..... ........A. ...
Appa2 (EF212013) Y...N..... EY........ HH........ .I..D..... .......... ...
Appa3 (EF212014) ..D.YF.... EY........ HF........ .S........ .F...K.... ...
```

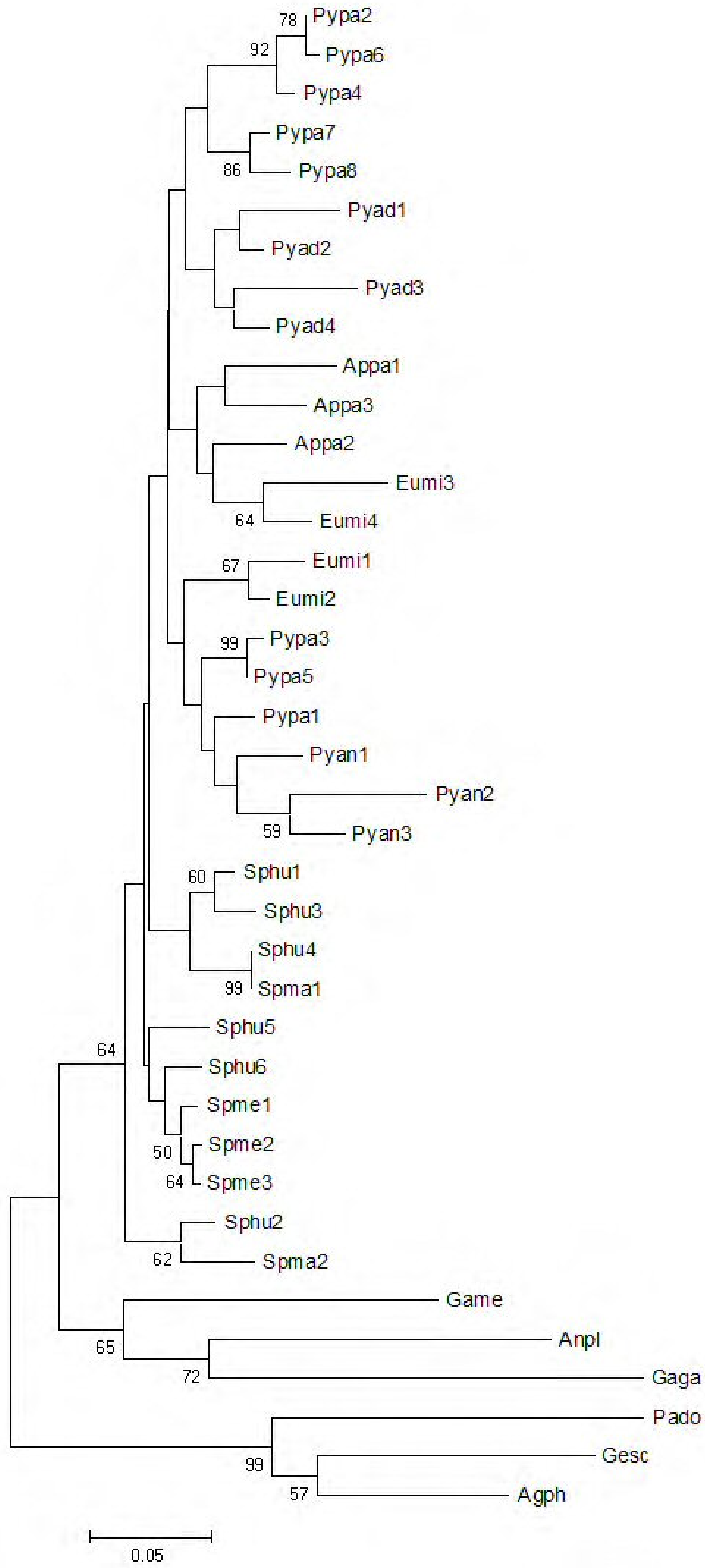
78
Pypa2
92
Pypa6
Pypa4
Pypa7
86
Pypa8
Pyad1
Pyad2
Pyad3
Pyad4
Appa1
Appa3
Appa2
Eumi3
64
Eumi4
67
Eumi1
Eumi2
99
Pypa3
Pypa5
Pypa1
Pyan1
Pyan2
59
Pyan3
60
Sphu1
Sphu3
Sphu4
99
Spma1
Sphu5
64
Sphu6
Spme1
50
Spme2
64
Spme3
Sphu2
62
Spma2
Game
65
Anpl
72
Gaga
Pado
99
Gesc
57
Agph
0.05

Table 1 Galápagos penguin MHC class II exon 2 sequence polymorphism compared to that in seven other penguin species (data are based on a 157 bp fragment). The number of individuals sampled (n), the number of alleles found, the number of polymorphic sites, the average number of differences between alleles, and nucleotide diversity (π) are presented.

Species	n	No. of alleles	No. of variable sites	Avg. no differences (± SE)	π
Adelie (*Pygoscelis adeliae*)[a]	4	4	20	10.7 ± 2.3	0.068
Chinstrap (*P. antarctica*)[a]	3	3	19	12.7 ± 2.7	0.081
Gentoo (*P. papua*)[a]	6	8	23	10.8 ± 2.1	0.068
Little blue (*Eudyptula minor*)[a]	4	4	21	12.7 ± 2.6	0.081
Humboldt (*Spheniscus humboldti*)[b]	20	6	20	9.7 ± 2.1	0.062
Galápagos (*S. mendiculus*)	30	3	3	2.0 ± 1.2	0.013
Magellanic (*S. magellanicus*)	1	2	16	16.0 ± 3.9	0.102
King (*Aptenodytes patagonicus*)	2	3	19	12.7 ± 2.7	0.081

[a]Data from Tsuda et al. (2001)

[b]Data from Kikkawa et al. (2005)

Table 2 Comparison of rates of synonymous (d_S) and nonsynonymous (d_N) substitutions among eight penguin species. Rates were calculated separately for the 38 codons making up the non-antigen binding sites (ABS) and the 15 codons making up the ABS.

Species	No. of	Non-ABS			ABS		
	alleles	$d_N \pm SE$	$d_S \pm SE$	d_N/d_S	$d_N \pm SE$	$d_S \pm SE$	d_N/d_S
Adelie (*Pygoscelis adeliae*)[a]	4	0.035 ± 0.017	0.020 ± 0.020	1.75	0.244 ± 0.068	0.023 ± 0.026	10.61*
Chinstrap (*P. antarctica*)[a]	3	0.052 ± 0.025	0.039 ± 0.030	1.33	0.270 ± 0.079	0.000 ± 0.000	n/a*
Gentoo (*P. papua*)[a]	8	0.051 ± 0.018	0.031 ± 0.027	1.65	0.168 ± 0.043	0.076 ± 0.066	2.21
Little blue (*Eudyptula minor*)[a]	4	0.039 ± 0.021	0.027 ± 0.027	1.44	0.327 ± 0.117	0.034 ± 0.024	9.62*
Humboldt (*Spheniscus humboldti*)[b]	6	0.041 ± 0.020	0.030 ± 0.026	1.37	0.195 ± 0.065	0.001 ± 0.001	195.00*
Galápagos (*S. mendiculus*)	3	0.008 ± 0.008	0.000 ± 0.000	n/a	0.041 ± 0.027	0.000 ± 0.000	n/a
Magellanic (*S. magellanicus*)	2	0.052 ± 0.031	0.020 ± 0.022	2.6	0.392 ± 0.198	0.062 ± 0.053	6.32*
King (*Aptenodytes patagonicus*)	3	0.039 ± 0.020	0.027 ± 0.028	1.44	0.289 ± 0.089	0.047 ± 0.039	6.15*

[a]Calculated using sequences from Tsuda et al. (2001)

[b]Calculated using sequences from Kikkawa et al. (2005)

*One-tailed test indicated $d_N > d_S$ with p-value < 0.03

Chapter 5

Evolution of MHC genes in two recently diverged species: the island endemic Galápagos hawk and the mainland Swainson's hawk

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ABSTRACT

Genes at the major histocompatibility complex (MHC) are known for their high levels of polymorphism maintained by balancing selection. In some cases, such as in small, bottlenecked populations, genetic drift may be strong enough to overwhelm the effect of balancing selection, resulting in reduced MHC variability. In this study we investigated MHC evolution in two recently diverged bird species with differing demography, the endemic Galápagos hawk (*Buteo galapagoensis*) and its widespread mainland relative the Swainson's hawk (*B. swainsoni*). We genotyped individuals at class II B genes, and we amplified at least two loci in each species. We recovered only three alleles from 32 Galápagos hawks; whereas, we found 20 alleles in 20 Swainson's hawks. No alleles were shared between species. The alleles clustered into two groups, with alleles in one group being much more divergent from each other than alleles in the other group. Both species had alleles in both groups, indicating that homologous loci are likely present. Our results show that genetic drift has had a strong effect on MHC variability in the Galápagos hawk, outweighing any positive effect of natural selection. The mechanisms controlling evolution at avian MHC genes are not well understood, and so we discuss how our results compare to patterns found in other studies.

Introduction

Genes at the major histocompatibility complex (MHC) are known for their high levels of polymorphism (Gaudieri et al. 2000, Robinson et al. 2003), as well as for their importance in initiating the immune response by recognizing and binding to foreign peptides and presenting them to T cells (Klein 1986). Their variability is thought to be maintained primarily through balancing selection, with parasite-mediated selection and MHC-dependent sexual selection being the two most likely mechanisms (Doherty and Zinkernagel 1975, Penn and Potts 1999, Piertney and Oliver 2006). A number of lines of evidence indicate that MHC genes are under selection (reviewed in Bernatchez and Landry 2003, Garrigan and Hedrick 2003, Piertney and Oliver 2006): an excess of nonsynonymous mutations at antigen-binding regions (Hughes and Nei 1988, 1989), the retention of alleles for long periods of time (trans-species polymorphism; Klein 1980), and discrepancies between population genetic structure at MHC and neutral loci (*e.g.*, Westerdahl et al. 2004a, Dionne et al. 2007) among others.

Many natural populations have the high level of variability expected at MHC loci (*e.g.*, Langefors et al. 1998, Westerdahl et al. 2004b, Harf and Sommer 2005), but a number of studies have described populations with reduced MHC variability. Population bottlenecks are predicted to result in a loss of genetic variability (Nei et al. 1975); however, loci under balancing selection are predicted to retain more variability as selection counteracts the effects of genetic drift (Maruyama and Nei 1981, Nevo et al. 1997, Takahata and Nei 1990). Nevertheless, reduced MHC diversity has been documented in small populations like those on islands (*e.g.*, Seddon and Baverstock

1999, Hedrick et al. 2001, Bollmer et al. 2007) and mainland populations that have gone through severe bottlenecks (*e.g*., Mikko and Andersson 1995, Hedrick et al. 2000, Babik et al. 2005). Most of these studies concluded that genetic drift had been strong enough to overwhelm balancing selection, thus resulting in low MHC diversity. In contrast, a few studies have found relatively high variability at MHC genes in bottlenecked species with low variability at neutral loci (*e.g*., Hambuch and Lacey 2002, Aguilar et al. 2004, Jarvi et al. 2004).

While much work has been done on the domestic chicken, the characterization of the MHC in natural bird populations has lagged behind that of other taxa (Hess and Edwards 2002). In contrast to the very minimal chicken MHC (Kaufman et al. 1999), work in non-model birds is showing their MHC regions to be more complex. Species differ in number of loci due to duplication events, and a number of studies have identified probable pseudogenes (Edwards et al. 1998, 2000; Hess et al. 2000; Ekblöm et al. 2003; Aguilar et al. 2006). Evidence indicates that bird MHC genes are evolving differently from mammalian genes. On phylogenetic trees, mammalian MHC class II alleles tend to cluster into orthologous gene groups (even alleles from distantly related species), and within loci, alleles from different species may be intermixed (e.g., Gutierrez-Espeleta et al. 2001, Van Den Bussche et al. 2002). This suggests that many loci and allelic lineages predate speciation events. The presence of gene conversion, though, may bias estimates of divergence times of alleles, making alleles appear older than they actually are, so care must be taken in their interpretation (Bergström et al. 1998, Martinsohn et al. 1999).

In contrast, MHC alleles in birds tend not to cluster into orthologous loci either within or across species, suggesting that many avian MHC genes have been duplicated

more recently (post-speciation) or that birds experience increased gene conversion between loci, thus homogenizing them in a process called concerted evolution (Edwards et al. 1995, 1999; Wittzell et al. 1999; Hess and Edwards 2002). Nevertheless, orthologous loci have been identified in some closely related species. In the galliforms, Wittzell et al. (1999) described two loci in the ring-necked pheasant (Phco-DAB1 and DAB2) that are orthologous to two chicken genes (BLBI and BLBII), and Strand et al. (2007) recently described black grouse alleles that are orthologous to the chicken BLB and YLB complexes. In the passerines, alleles from four Hawaiian honeycreeper species cluster into two groups on a tree with alleles from three of the species in both clusters (Jarvi et al. 2004), and alleles from Darwin's finch species cluster into five groups in a similar manner (Vincek et al. 1997, Sato et al. 2001).

In this study we investigate the distribution of MHC variation in an island endemic, the Galápagos hawk (*Buteo galapagoensis*), and its closest mainland relative, the Swainson's hawk (*B. swainsoni*). The Galápagos hawk is endemic to the Galápagos Archipelago (Fig. 1), and it breeds on eight of the islands. Previous genetic work on this species showed low within-population variability and significant between population differentiation at VNTR (variable number of tandem repeats) and mitochondrial loci (Bollmer et al. 2005, 2006). The Swainson's hawk breeds in western North America but migrates annually to southern South America (Fuller et al. 1998; Fig. 1). With their broader distribution and larger population sizes, Swainson's hawks are genetically more variable than Galápagos hawks (Bollmer et al. 2006; Hull et al. 2008), and they have limited population genetic structuring across their North American breeding range (Hull et al. 2008). In a *Buteo* phylogeny, Riesing et al. (2003) identified Galápagos and

Swainson's hawks as sister species, and Bollmer et al. (2006) estimated that the split between them occurred relatively recently, likely around 126,000 years ago (95% confidence interval of 51,000–254,000 years ago). In a more thorough analysis using a broader sampling of Swainson's hawks, Hull et al. (accepted) found that Galápagos hawk haplotypes formed a monophyletic clade that fell within a clade of Swainson's hawk haplotypes, making Swainson's hawks paraphyletic with respect to Galápagos hawks.

The main objective of this study was to describe MHC variability in the Galápagos hawk, an island endemic and a species for which we have neutral nuclear and mitochondrial genetic data, and compare it with MHC variability in its close relative the Swainson's hawk, a widespread mainland species. Galápagos hawks exhibit reduced genetic variability at other loci; however, balancing selection may be acting to retain ancestral variability at MHC loci. We also explore the relationships among the alleles both within and between species, identifying possible loci within species and comparing allelic composition between species. By studying two very recently diverged bird species with different population histories, we hope to gain a better understanding of how MHC genes evolve in birds, as well as gain a better understanding of the effect of demography on MHC variability.

Methods

Sampling

We sampled Galápagos hawks from eight islands encompassing the entire breeding range of the species, and we sampled overwintering Swainson's hawks near Las Varillas, in Córdoba province, Argentina (see Bollmer et al. [2003, 2005] and Whiteman

and Parker [2004a,b] for more details about sampling methods). For this study, we genotyped four Galápagos hawks from each of the eight breeding populations (using only territorial adults) for a total of 32 individuals, and we genotyped 20 Swainson's hawk individuals. We preferentially chose individuals that had been used in previous population genetic studies (Bollmer et al. 2005, 2006).

MHC genotyping

Laboratory protocols were identical for both species. We primarily used denaturing gradient gel electrophoresis (DGGE) to genotype individuals, and in a small number of cases we also used bacterial cloning. In order to amplify exon 2 of MHC class II loci in the Galápagos and Swainson's hawks, we first used the primers Acc2FC and Acc2RC developed by Alcaide et al. (2007) from other Accipiters. This PCR amplification was carried out in 40 μl reactions using 5 μl of 10X buffer, 0.025 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 μM of each primer, 0.5 μl of *Taq* DNA polymerase, and 100 ng of genomic DNA. Reaction conditions were as follows: 94°C for 4 min, then 35 cycles of 94°C for 40 sec, 56°C for 40 sec, and 72°C for 1 min, and then a final extension of 72°C for 5 min. We used QIAquick gel extraction kits (QIAGEN) to gel-purify the PCR products, and then we cloned them using the pGEM-T easy vector cloning kit (Promega). Positive clones were sequenced on an ABI 3100.

For DGGE genotyping, we used the primers Acc2FC and a new reverse primer ButeoR (5′-TTC TGG CAC RCA CTC ACC TC-3′) developed from the Galápagos and Swainson's hawk sequences obtained from the above cloning. We added a GC-clamp to the 5′ end of ButeoR to facilitate the separation of alleles on the gel (Sheffield et al. 1989). The reactions using Acc2FC and ButeoR were run using the same conditions as

above. PCR products were run on 8% 19:1 acrylamide/bisacrylamide gels using a 25 to 35% denaturing gradient of formamide and urea. We ran gels for 4.5 h at 160 V at a constant temperature of 60°C. The gels were then stained with SYBR© gold (Promega) and visualized on a Kodak IS440CF imaging system. In order to obtain the sequences of the alleles, we cut the bands out of the gels, suspended them in 50 µl of dH_2O, re-amplified them using the Acc2FC/ButeoR primer set, and then sequenced them using those same primers. Because spurious alleles may form when amplifying multiple sequences in one reaction (Jansen and Ledley 1990, L'Abbe et al. 1992), we only considered alleles to be confirmed if they were amplified in at least two independent reactions.

Data analysis

We assembled and edited the sequences using SeqMan Pro v. 7.1 (DNASTAR, Inc.) and then aligned them by eye using BioEdit (Hall 1999). The forward primer straddles the intron and the beginning of exon 2, extending 7 bp into the exon. Of those seven bases in the exon, only the third base is variable, with sequences having either a C or T. This site was unresolved for a number of the Swainson's hawk sequences, so to be conservative we removed the codons in the primer region (the first three of the 89 codons in the exon) from the analyses. Those codons, however, do appear in the amino acid alignment (Fig. 2). The unresolved site is a synonymous substitution, and thus the amino acid is the same regardless of the base.

We calculated genetic diversity measures within and between species in the program DnaSP (Rozas et al. 2003). We constructed neighbor-joining trees (Saitou and Nei 1987) using Kimura 2-parameter distances in the program MEGA2 (Kumar et al.

2001). We also tested for the presence of gene conversion among sequences using the program GENECONV v. 1.81 (Sawyer 1999). GENECONV compares sequences in a pairwise fashion and searches for segments that are unusually similar for a given pair of sequences. The program permutes the data and calculates global *P*-values (corrected for multiple comparisons) that compare each segment with all possible segments for the entire alignment. We ran 10,000 permutations and allowed zero mismatches.

Selection at the codon level can be measured as the ratio of non-synonymous/synonymous substitutions (d_N/d_S). A ratio of $d_N/d_S > 1$ is attributed to the effect of positive selection, whereas $d_N/d_S = 1$ indicates neutrality and $d_N/d_S < 1$ indicates purifying selection. First, we calculated d_N and d_S using the Nei and Gojobori (1986) method with the Jukes-Cantor correction for both peptide-binding and non-peptide-binding codons as determined by Brown et al. (1993). We then tested for positive selection using a *Z*-test. These analyses were also done in MEGA.

Results

From the 52 Galápagos and Swainson's hawks we recovered 23 unique sequences (GenBank accession numbers XXXXXX – XXXXXX), which each yielded a different amino acid sequence (Fig. 2). No frameshift mutations or stop codons were present. Kaufman et al. (1994) identified 19 evolutionarily conserved β domain residues believed to be important to the structural formation of a functioning MHC class II molecule. These residues are involved in glycosylation, salt bonds, and disulfide bonds among other things. The hawk sequences were completely conserved at 17 of these residues. At the

remaining two residues, T21 and rk72 (the 16th and 67th codons in Figure 2, respectively), the majority of the sequences had the conserved amino acids.

Within-species genetic diversity

Within the Galápagos hawk (*N* = 32), we found three different alleles, with each individual having at least two of them. One allele, *Buga*01*, was present in all individuals across all eight islands; all individuals also had one or both of the other two alleles, *Buga*02* and *Buga*03*. We interpreted this to mean that the primer set amplified two loci: one that is fixed for allele *Buga*01* and one that has two alleles, with individuals being homozygous or heterozygous. Alleles *Buga*02* and *Buga*03* had a one codon deletion not present in *Buga*01*, and they differed from each other by only one base pair (π = 0.004, not counting the three sites involved in the deletion or the primer region; Fig. 2). In contrast, *Buga*02* and *Buga*03* differed from *Buga*01* by an average of 30.5 bp (π = 0.118). Across all three sequences, 31 of the 255 sites included in the analyses were polymorphic. In the 32 individuals sampled, *Buga*02* and *Buga*03* had allele frequencies of 0.45 and 0.55, respectively. We sampled only four birds per island, so our characterization of the distribution of these two alleles is preliminary; however, each of them was present on at least six of the eight islands: Santa Fe, Pinta, Santiago, and Fernandina had both alleles; Pinzón and Marchena had only *Buga*02*; and Española and Isabela had only *Buga*03*.

Within the more variable Swainson's hawks (*N* = 20), we found 20 different alleles, confirming 3 or 4 alleles from each individual. We sequenced a fifth allele from three of the individuals, though we were unable to confirm these because in each case the fifth allele only amplified in one reaction or did not sequence cleanly. So, every

individual appeared to have at least two loci, and a third locus may be present in at least some individuals. In the 20 birds sampled, we found 18 different MHC genotypes (three birds each had the same three alleles). The most common allele (*Busw*08*) was recovered from 11 different birds, while 11 of the alleles were recovered from only one or two birds. Four of the 20 sequences had a 3 bp deletion at the same codon as the two Galápagos hawk sequences. Of the 255 sites considered, 72 were variable, and sequences differed by an average of 26.0 bp ($\pi = 0.102$).

Allelic relationships

A neighbor-joining tree of the Galápagos and Swainson's hawk sequences showed that most of the sequences fell into two clusters (Fig. 3). This division among the sequences is also apparent in the amino acid alignment (Fig. 2). The two Galápagos hawk alleles differing by 1 bp (*Buga*02* and **03*) fell into Cluster 1, whereas the fixed Galápagos hawk allele (*Buga*01*) fell into Cluster 2. Ten of the 20 Swainson's hawk alleles fell into Cluster 1, nine fell into Cluster 2, and one allele (*Busw*12*, which was present in only one individual) did not fall into either cluster. All six sequences with the codon deletion fell into Cluster 1. Cluster 2 has reduced variability compared to Cluster 1. Sequences in Cluster 1 had 53 variable sites and differed by an average of 23.0 bases ($\pi = 0.090 \pm 0.007$), whereas sequences in Cluster 2 had only 16 variable sites and differed by an average of 6.1 bases ($\pi = 0.023 \pm 0.004$). The two species did not share any sequences. We constructed a neighbor-joining tree using our alleles and sequences from more distantly related avian taxa for which multiple loci have been identified (Fig. 4). The *Buteo* sequences from the two clusters were more similar to each other than they were to sequences from other species.

Gene conversion

The program GENECONV found evidence of putative gene conversion events involving the Swainson's and Galápagos hawk sequences (Table 1). It identified 25 possible inner fragments (fragments resulting from gene conversion between ancestors of sequences within the alignment) that were globally significant and one possible outer fragment (a conversion event that may have involved a sequence outside the alignment). The outer fragment (14 bp in length beginning at site 171 and ending at 184 in our 258 bp alignment and corresponding to amino acids 60 through 65 in Figure 2; $P = 0.028$) involved sequence *Busw*12*, which was the most divergent of the sequences and fell outside Clusters 1 and 2. Three of the 25 inner fragments involved gene conversion between sequences within Cluster 1, while the 22 other fragments involved conversion between Cluster 1 and Cluster 2. We did not find any significant fragments between sequences within Cluster 2; however, the high similarity among sequences within that cluster makes it unlikely that a conversion event would be detected.

Positive selection

We found evidence for positive selection acting on codons likely involved in antigen-binding (Table 2). Of the 85 codons analyzed, we treated 23 as antigen-binding following Brown et al. (1993). Analyzing the two species separately, rates of nonsynonymous substitutions were significantly greater than synonymous substitutions at antigen-binding sites (ABS) but not at the remaining codons. The same was true when Cluster 1 and Cluster 2 were analyzed separately; however, substitution rates were an order of magnitude lower at the ABS in Cluster 2 than in Cluster 1.

Discussion

Major histocompatibility complex genes are well known for their high levels of variability due in large part to the effects of balancing selection. Some studies, though, have found that demography can overwhelm the effects of selection, leading to lower MHC variability. Our results showed greatly reduced variability at MHC class II loci in an island endemic compared to its closest mainland relative. We amplified alleles from at least two loci in the Galápagos and Swainson's hawks, and most of the alleles fell into two clusters on a phylogenetic tree, one of which had lower interallelic divergence than the other. The clusters may correspond to loci but that is unconfirmed. Alleles from both hawk species were present in both clusters, indicating that alleles from different Galápagos hawk loci are not more similar to each other than to Swainson's hawk alleles.

Low diversity in the Galápagos hawk

Polymorphic sites are needed first, though, for recombination to be effective. We recovered only three MHC alleles from the Galápagos hawk. All birds were fixed for *Buga*01*, and all had one or both of alleles *Buga*02* and *Buga*03*. While MHC loci generally have high levels of polymorphism, the low level of variability we found is instead similar to the reduced genetic variability we found at neutral nuclear and mitochondrial genes in the Galápagos hawk. At hypervariable VNTR (variable number of tandem repeats) loci, individuals within populations share an average of 69-96% of their alleles (Bollmer et al. 2005), whereas an average of 20-30% is more typical for large, outbred populations (Parker Rabenold et al. 1991). Bollmer et al. (2006) identified only seven mitochondrial haplotypes differing by an average of 3.1 bases out of almost 3

kb sequenced, and seven of the eight breeding populations were fixed for single haplotypes.

The geographic distribution of the MHC alleles suggests variability was lost soon after the hawks reached the archipelago. One allele (*Buga*01*) is fixed across all eight islands; the other two alleles are each present on at least six islands (four islands having both alleles), and it is possible that with further sampling (we sampled four individuals per island) we may find that more of the populations have both alleles. The most likely explanation for this distribution is that the Galápagos hawk's ancestral MHC polymorphism was reduced at or soon after founding the archipelago, and the hawks carried these alleles with them as they colonized the various islands. It is unlikely to be the result of recent gene flow, since there is substantial genetic differentiation among the hawk populations at other markers (Bollmer et al. 2005, 2006). The VNTR loci also hint at an early reduction in genetic variability because of the high background similarity among populations (Bollmer et al. 2005). In addition, four of the populations are fixed for the same mitochondrial haplotype (Bollmer et al. 2006).

In addition to drift, low variability at the MHC has been attributed to reduced selection pressures (Slade 1992). A number of studies have shown reduced parasite diversity on islands relative to the mainland (e.g. Fromont et al. 2001, Beadell et al. 2007), so island populations may experience lower parasite pressure. A health survey is currently underway in the Galápagos Islands with the goal of identifying parasites affecting native and introduced bird species (Parker et al. 2006). Three co-evolved louse species (Phthiraptera), one biting fly (Hippoboscidae), one mite (Epidermoptidae), and an undescribed *Trypanosoma* species (present in only one individual) have been identified

as parasites of the Galápagos hawk (Parker et al. 2006). The basic biology of two of the louse species has been well described (Whiteman and Parker 2004a,b). One of these (*Colpocephalum turbinatum*) feeds on skin and blood, thus interacting directly with the host's immune system. Whiteman et al. (2006) found that smaller, more inbred Galápagos hawk populations had higher louse loads and, in general, lower and less variable natural antibody titres than the larger, more genetically variable hawk populations. So, we do have evidence that parasites are exerting some selective pressure on Galápagos hawks; however, the diversity of both endo- and ectoparasites affecting mainland hawk species is likely greater. Swainson's hawks are migratory and are likely exposed to different sets of pathogens at their breeding and wintering grounds, whereas Galápagos hawks are not. This broader exposure to pathogens should lead to greater selection on the MHC genes of migratory species (Westerdahl et al. 2004a). Low MHC diversity has also been attributed to mating system, with monogamous species predicted to retain less diversity than more social species (Hambuch and Lacey 2002, Sommer et al. 2002). Galápagos hawks, however, are cooperative breeders on most islands (de Vries 1975, Bollmer et al. 2003), so their mating system should select for higher MHC diversity. Reduced selection pressure may be contributing to the lower MHC diversity seen in the Galápagos hawk; however, genetic drift has likely been the primary cause considering the hawk's small population sizes and probable bottlenecks at foundation.

MHC loci are characterized as having many alleles with high genetic distances between them. Bottlenecked populations typically have reduced numbers of alleles, but the remaining alleles are still divergent from each other (*e.g.*, Hedrick et al. 2000, Sommer 2005, Radwan et al. 2007 and references therein), possibly because selection

favors the retention of alleles that can recognize a broader range of antigens. The Galápagos hawk and the Galápagos penguin (Bollmer et al. 2007), though, both show a pattern of a few closely related alleles within loci. In the Galápagos hawk, alleles *Buga*02* and *Buga*03* differ by only one base, making them more closely related to each other than to any other allele sequenced in either *Buteo* species, which suggests that one of the alleles likely arose through mutation after the Galápagos hawks split from the Swainson's hawks. The Galápagos penguin shows a similar pattern with all three sequences at one locus differing by an average of only 2 bp out of 157 bp sequenced (having a total of 3 variable sites), while the same 157 bp sequences within other penguin species have an average of 20 variable sites and differ by an average of 12 bp (Bollmer et al. 2007). The pattern at the loci in these species could be the result of similar ancestral alleles being retained by chance, or these loci at one point became fixed and the similarity of the current alleles is due to the slow process of mutation building up new variation. Mutation rates at MHC loci do not appear to be elevated compared to other loci (Lundberg and DeVitt 1992, Satta et al. 1993). Instead, the evidence obtained thus far suggests that by shuffling sequence motifs at the antigen-binding region, recombination is more important than point mutation in generating MHC sequence variability (Richman et al. 2003, Reusch and Langefors 2005, Schaschl et al. 2006); however, starting variation from point mutation must first be present for recombination to be effective.

Evolution of avian MHC genes

MHC genes are prone to duplication events, and other studies have identified variation in number of loci both within species and between closely related species (*e.g.*, Málaga-Trillo et al. 1998, Doxiadis et al. 2001, Babik et al., 2005). In a survey of 26 bird of prey

species from five families, Alcaide et al. (2007) found between one and three loci per individual, including one to two loci among 14 Accipiter species. While we cannot be certain, in all likelihood the three alleles we recovered from the Galápagos hawks came from two loci. Most of the Swainson's hawks had three or four alleles, which also likely comprise at least two loci. Fifth alleles were unconfirmed in a subset of the Swainson's hawks suggesting that there may be a third locus in some birds, and some of the other birds with three or four alleles may have three loci as well if they are not heterozygous at every locus. Also, in preliminary trials using a degenerate primer set that amplifies an inner fragment of exon 2 (primers 326, 325; Ekblom et al. 2003), we recovered a fourth sequence in two Galápagos hawks that was not amplified by the primers we used in this study. A more thorough investigation of the class II architecture of these species is needed to determine the true number of genes.

As noted earlier, assignment of alleles to loci in birds has been difficult, with the differences among loci being blurred by more recent gene duplication events and/or higher rates of gene conversion. However, in a number of studies, authors were able to identify multiple loci or putative loci based on clustering of alleles. Interestingly, many of these cases involved one or more loci with highly divergent sequences and a locus with alleles with high sequence similarity (Figure 4; Vincek et al. 1997, Sato et al. 2001, Jarvi et al. 2004, Aguilar et al. 2006). The low variability loci could have multiple origins. Aguilar et al. (2006) concluded that the *Anvi*-DAB1 locus is likely a pseudogene, because it had a low d_N/d_S ratio at antigen-binding sites, a frameshift mutation in one allele, and none of the alleles at this locus were amplified from cDNA. In contrast, the low variability loci in the Hawaiian honeycreepers (Jarvi et al. 2004) and

Darwin's finches (Sato et al. 2001), do not have characteristics consistent with pseudogenes. Jarvi et al. (2004) suggested that the low variability Cluster 2 could be a locus akin to genes in the Y complex of the chicken. Two unlinked gene complexes (B and Y) have long been recognized within the chicken. Genes within the Y complex have much lower variability and a low rate of expression compared to B complex genes. Strand et al. (2007) recently identified homologous B and Y genes in the closely related black grouse, but the low variability loci in the passerines do not appear to be homologous to the fowl Y complex. The lack of variability at these loci could be due to purifying selection. Evidence suggests that MHC genes may evolve through a birth-and-death model, where new genes are formed through duplication and then may later be deleted or become inactive as pseudogenes (Parham and Ohta 1996, Nei et al. 1997). Newly formed genes are under diversifying selection to diverge in function, and may become specialized for a particular function at which time they would be under purifying selection (Axtner and Sommer 2007 and refs therein).

Our Swainson's and Galápagos hawk sequences also fell into distinct clusters with differing levels of variability. We did not confirm whether the loci we amplified are expressed; however, a number of lines of indirect evidence suggest that they are not pseudogenes. We did not find any frameshift mutations or stop codons, and these sequences have evolutionarily conserved amino acid residues that are known to be important for the structural integrity of class II molecules. Also, we found an excess of nonsynonymous substitutions, which is evidence that selection has acted on these loci, though not necessarily recently (Garrigan and Hedrick 2003). While the nucleotide substitution rate in Cluster 2 was much lower than in Cluster 1, Cluster 2 still had a

significant excess of nonsynonymous substitutions. So, Cluster 2 does not appear to be a pseudogene, but rather may be a locus similar to the ones found in the honeycreepers and finches.

According to the GENECONV results, gene conversion has taken place between alleles from different clusters, though the extent of gene conversion was not such that the alleles have been homogenized. If the two clusters do indeed represent two loci, then an orthologous relationship has been retained between these Galápagos and Swainson's hawk genes. This appears to be true for alleles within the closely related honeycreeper species (Jarvi et al. 2004), as well as for Darwin's finch species (Vincek et al. 1997, Sato et al. 2001). The lack of orthology among more distantly related species, though, suggests that this pattern may disappear with increasing divergence times. In a survey of Darwin's finches and their mainland relatives, Sato et al. (2001) found that the low variability locus was not present in all species and likely arose 2-3 million years ago. Alcaide et al. (2007) sampled 11 alleles from three wild cape vultures and 12 alleles from three white-backed vultures, which are in the same family (Accipitridae) as the *Buteo* hawks, though distantly related; however, we did not find a low variability allele cluster among the vulture sequences (unpubl. analysis).

While it has become clear that MHC structure in most bird species is complex, especially within the passerines, the frequency of duplication and recombination events and their impact on the evolution of avian MHC genes is poorly understood. More work is needed on species with varying degrees of relatedness to identify the forces at work in producing the observed patterns and the timescale at which they are acting. A better

understanding of these mechanisms will help to inform broader questions concerning MHC variability, parasite resistance, and population viability.

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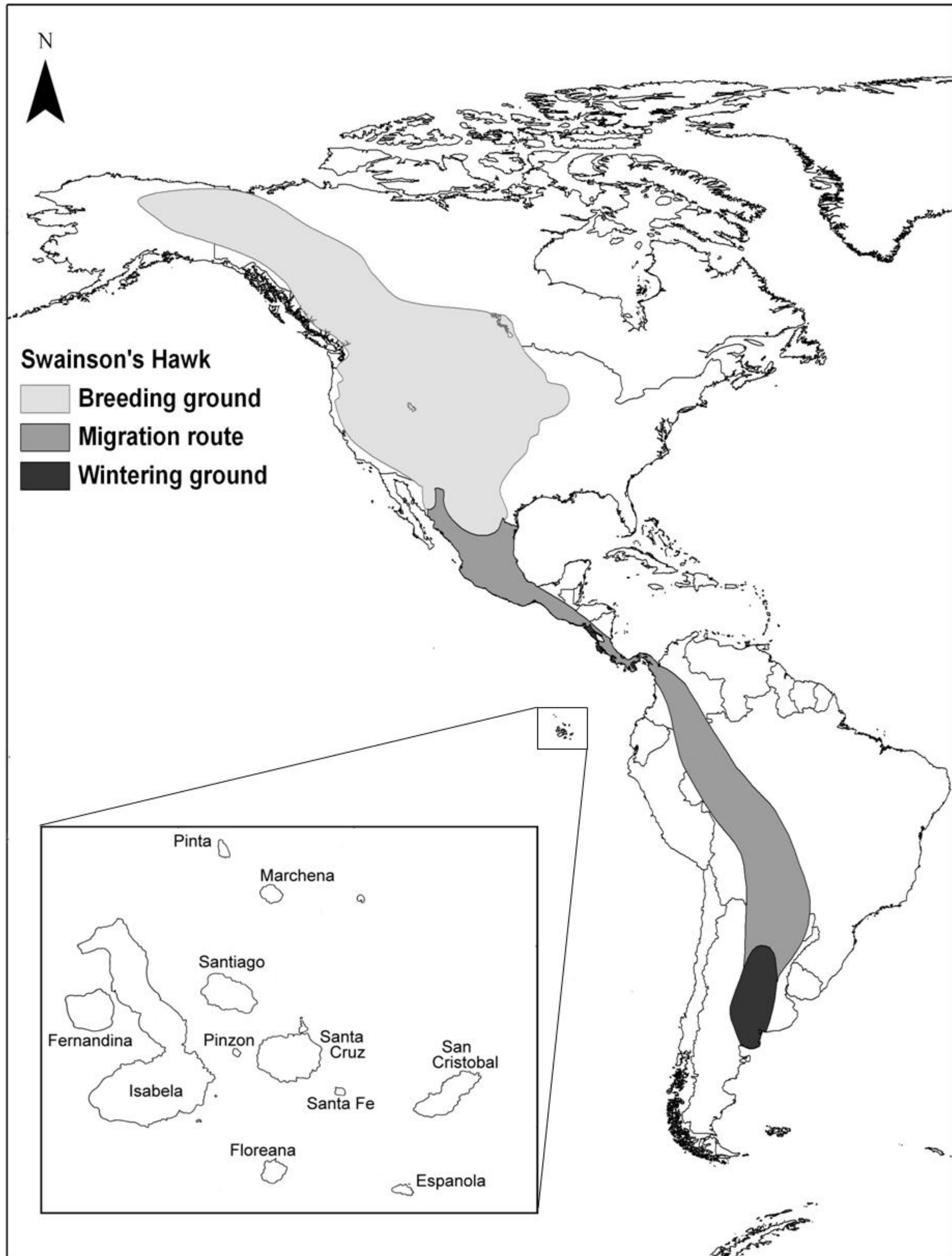
Figure Legends

Fig. 1 Distributions of the Galápagos and Swainson's hawks. The Galápagos Islands (inset) are located on the equator about 1000 km off the coast of South America. The archipelago is volcanic in origin and has never been connected to the mainland. The Galápagos hawk has breeding populations on all the labeled islands except Santa Cruz, San Cristóbal, and Floreana, where the populations have been extirpated. The Swainson's hawk distribution is from Ridgely et al. (2007).

Fig. 2 Alignment of MHC class II exon 2 amino acid sequences from two species of hawk: *Buteo galapagoensis* (*Buga*) and *B. swainsoni* (*Busw*). The asterisks indicate likely antigen-binding sites based on Brown et al. (1993). Dots indicate identity with sequence *Buga*01* and dashes indicate deletions. The first ten sequences listed (*Buga*01* through *Busw*18*) make up the less variable Cluster 2, the next 12 sequences (*Buga*02* through *Busw*20*) make up Cluster 1, and the last sequence (*Busw*12*) fell outside both clusters.

Fig. 3 Neighbor-joining tree of MHC class II exon 2 sequences from Galápagos (Buga, *Buteo galapagoensis*) and Swainson's (Busw, *B. swainsoni*) hawks. The tree was constructed using Kimura 2-parameter distances based on 255 bp of sequence data. Bootstrap values of 60 or greater are indicated on the tree. The sequences cluster into two main groups, one of which (Cluster 2) has much less genetic diversity than the other.

Fig. 4 Neighbor-joining tree using Kimura 2-parameter distances based on 135 bp of exon 2 from MHC class II genes. Bootstrap values of 60 or greater are indicated on the tree. The bird taxa used were species for which there appear to be multiple loci identified as clusters of sequences, and the sequences included are a subsample of the ones used in the original studies. Strand et al. (2007) recovered black grouse sequences that were orthologous to the chicken BLB and YLB complexes. Hawaiian honeycreeper sequences from four species fell into two clusters: Cluster 1 and Cluster 2, a set of sequences with reduced polymorphism. Darwin's finch sequences formed five clusters, four of which (1-4) had normal variability and one of which (5) had reduced variability (Vincek et al. 1997, Sato et al. 2001). Little greenbuls also have a locus with reduced variability (*Anvi*-DAB1) in addition to more variable sequences (Aguilar et al. 2006). Buga, *Buteo galapagoensis*; Busw, *Buteo swainsoni*; Gefu, *Geospiza fuliginosa*; Gefo, *G. fortis*; Plcr, *Platyspiza crassirostris*; Capa, *Cactospiza pallida*; Geco, *G. conirostris*; Gema, *G. magnirostris*; Ceol, *Certhidea olivacea*; Gesc, *G. scandens*; Anvi, *Andropadus virens*; Tete, *Tetrao tetrix*; Gaga, *Gallus gallus*; Hevi, *Hemignathus virens*; Hisa, *Himatione sanguinea*; Veco, *Vestiaria coccinea*; Loba, *Loxioides bailleui*


N
Swainson's Hawk
Breeding ground
Migration route
Wintering ground
Pinta
Marchena
Santiago
Fernandina
Pinzon
Santa
Cruz
San
Cristobal
Isabela
Santa Fe
Floreana
Espanola

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                  10        20        30        40        50        60        70        80
        ....  |....| ....  |....| ....  |....| ....  |....| ....  |....| ....  |....| ....  |....| ....  |....|
....  |....

            *  *  *                    *  *  *      **             *         *    **    *     *  **   *     *
 **   *  *  **
Buga*01 FFQEMTKFEC HHLNGNKNVR YLEKYIYNRE QRVHFDSDVG HYVADTPLGE PDAKYWNSQP DILERNRAEV DRLCRHNYEV VTPFTVERR
Busw*04 ...D.A.... .......... .......... .......... .......... R......... .......... .......... .........
Busw*06 .....A.... .......... .......... .......... .......... .......... .......... .......... S...L....
Busw*08 .......... .......... .......... .......... .......... .S........ .......... .......... A...L....
Busw*09 ...D.A.... .......... .......... .......... .......... .......... .....K.... .......... .........
Busw*10 .......... .......... .......... .......... .......... .S........ .......... .......... .........
Busw*11 .......... .......... .......... .......... .......... .......... .......... .......... A...L....
Busw*13 .....A.... .......... .......... .......... .......... .......... .......... .......... A...L....
Busw*14 .......... .......... .......... .T........ .......... .......... ....MRQ... .......... S...L....
Busw*18 ...D.A.... .......... .......... .......... .......... .......... .....K.... .......... S...L....
Buga*02 .....D.G.. QY...T.Q.K ..M....... .T........ .F........ .......... ....DAQ.A. .T-....... A...L....
Buga*03 .....D.G.. QY...T.Q.K ..M....... .T........ .F........ .......... ....DAQ.A. .T-....... S...L....
Busw*01 .....D.G.. QY...T.Q.K L.V...H... .I........ .F........ .E........ ....NAQ.A. .TY....... A...L....
Busw*02 ...YLF.... QY...T.Q.K L.V.WT.... .TL.Y..... .......... .I.ND..... ....MRQ... ..V....... ....L....
Busw*03 .....D.A.. QY...T.Q.K ..M....... .......... .F........ .......... .....K.... .T-....... S...L....
Busw*05 ...YLF.A.. QY...T.Q.K L.V.WT..Q. .Y..Y..... .F........ .......... .....K.... .T-....... S...L....
Busw*07 .....D.G.. QY...T.Q.K L.V...H... .I........ .F........ .E........ .....FQ... ..F.....DA FR..L....
Busw*15 .....D.A.. QY...T.Q.K L.V...H... .I........ .F........ .E........ ....NAQ.A. .TY....... A...L....
Busw*16 ...YLF.A.. QY...T.Q.K L.V.WT..Q. KY..Y..... .F........ .......... .....K.... .T-....... ....L....
Busw*17 ...YLF.... QY...T.Q.K L.V.WT.... .TL.Y..... .......... .I.ND..... ....MRQ... ..V....... A...L....
Busw*19 .....D.A.. QY...T.Q.K ..M....... .......... .F........ .......... ....DAQ.A. .T-....... S...L....
Busw*20 .....F.G.. QY...T.Q.K L.V.WT.... .Y........ .F...H.... .S.......L .....K.... .TV....... S...L....
Busw*12 .....F.A.. QY...T.Q.K ..K.....G. .......... .F........ .S......L. EE..YR.TG. ..F.....D. FR.......
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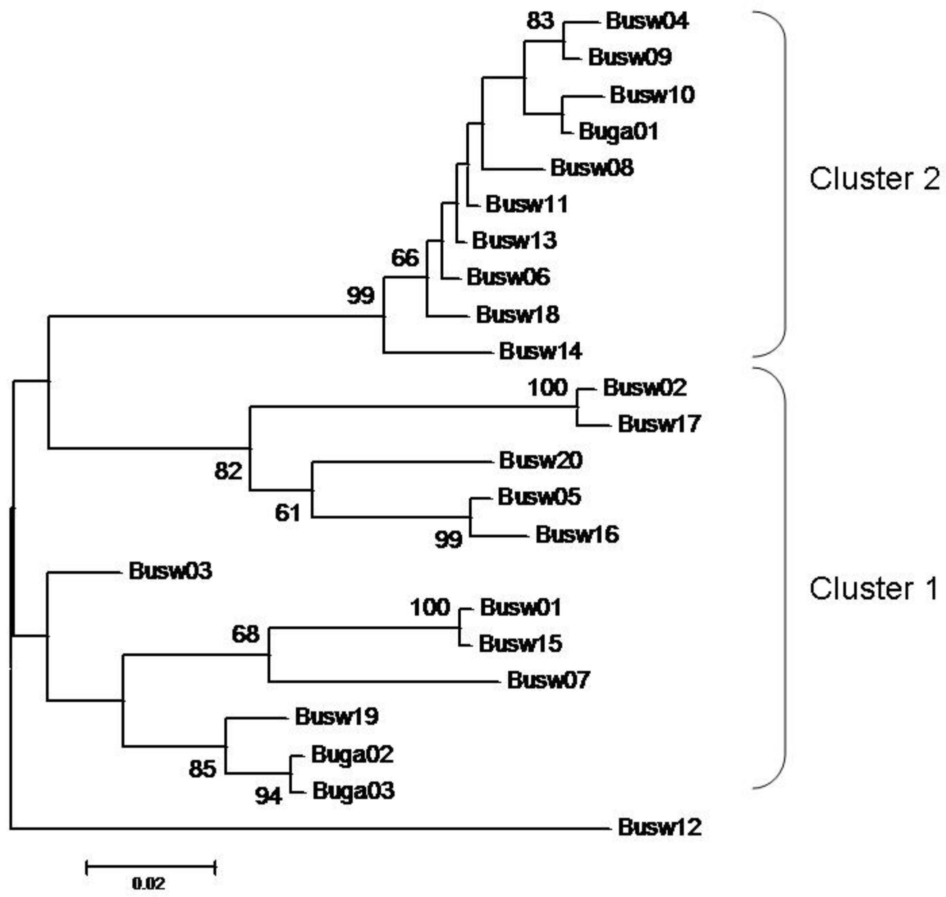
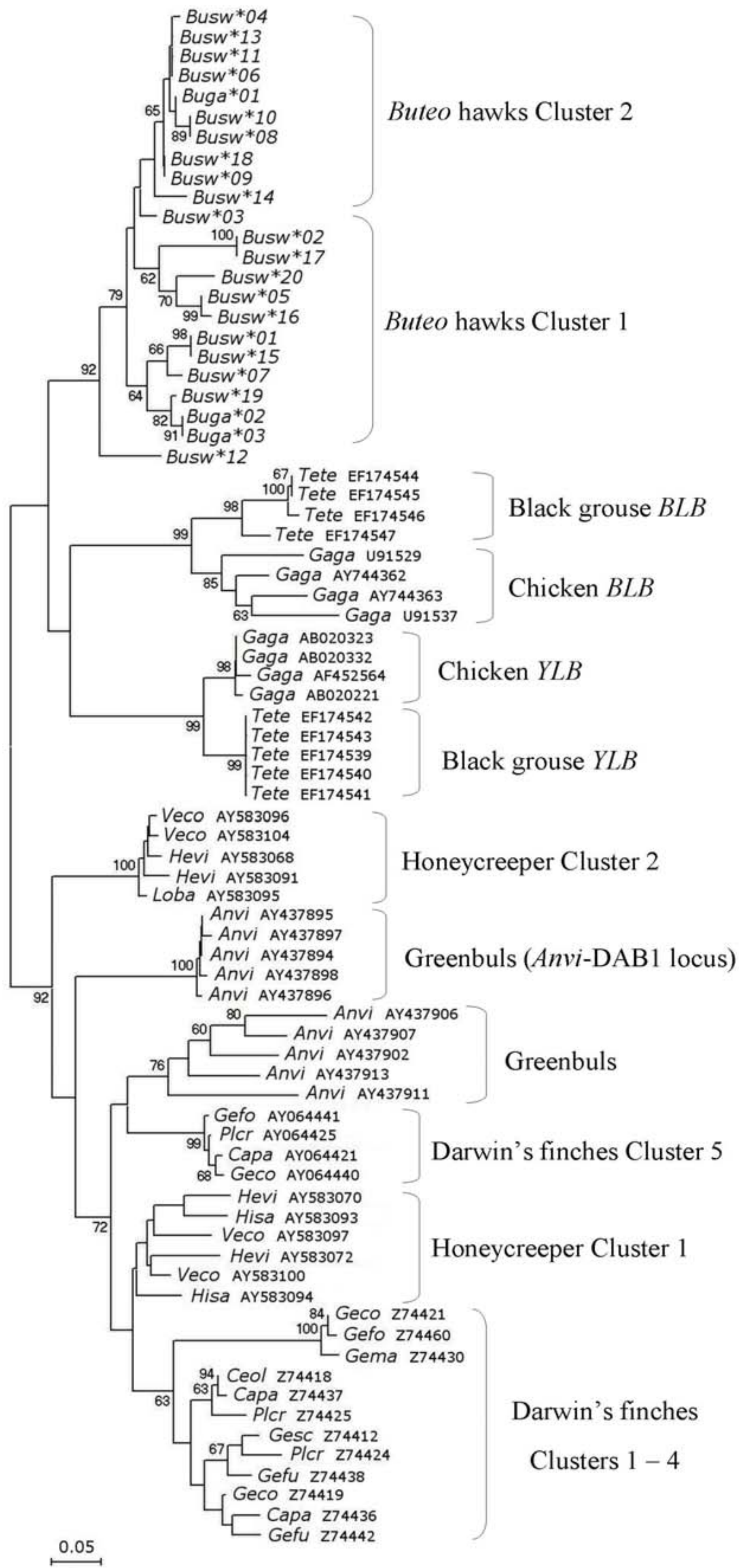

83
Busw04
Busw09
Busw10
Buga01
Busw08
Busw11
Busw13
66
Busw06
99
Busw18
Busw14
Cluster 2
100
Busw02
Busw17
Busw20
82
Busw05
61
99
Busw16
Busw03
Cluster 1
100
Busw01
68
Busw15
Busw07
Busw19
Buga02
85
94
Buga03
Busw12
0.02



Busw*04
Busw*13
Busw*11
Busw*06
Buga*01
Busw*10
Busw*08
Busw*18
Busw*09
Busw*14
Busw*03
Buteo hawks Cluster 2
Busw*02
Busw*17
Busw*20
Busw*05
Busw*16
Busw*01
Busw*15
Busw*07
Busw*19
Buga*02
Buga*03
Busw*12
Buteo hawks Cluster 1
Tete EF174544
Tete EF174545
Tete EF174546
Tete EF174547
Black grouse BLB
Gaga U91529
Gaga AY744362
Gaga AY744363
Gaga U91537
Chicken BLB
Gaga AB020323
Gaga AB020332
Gaga AF452564
Gaga AB020221
Chicken YLB
Tete EF174542
Tete EF174543
Tete EF174539
Tete EF174540
Tete EF174541
Black grouse YLB
Veco AY583096
Veco AY583104
Hevi AY583068
Hevi AY583091
Loba AY583095
Honeycreeper Cluster 2
Anvi AY437895
Anvi AY437897
Anvi AY437894
Anvi AY437898
Anvi AY437896
Greenbuls (Anvi-DAB1 locus)
Anvi AY437906
Anvi AY437907
Anvi AY437902
Anvi AY437913
Anvi AY437911
Greenbuls
Gefo AY064441
Plcr AY064425
Capa AY064421
Geco AY064440
Darwin's finches Cluster 5
Hevi AY583070
Hisa AY583093
Veco AY583097
Hevi AY583072
Veco AY583100
Hisa AY583094
Honeycreeper Cluster 1
Geco Z74421
Gefo Z74460
Gema Z74430
Ceol Z74418
Capa Z74437
Plcr Z74425
Gesc Z74412
Plcr Z74424
Gefu Z74438
Geco Z74419
Capa Z74436
Gefu Z74442
Darwin's finches
Clusters 1 – 4
0.05

Table 1 MHC class II exon 2 fragments from Galápagos and Swainson's hawk sequences indicative of past gene conversion events. The fragments listed are all globally significant inner fragments. Beginning and ending positions refer to the 258 bp sequence alignment. Num poly is the number of polymorphic sites in the overall alignment in the region the fragment spans, Num dif is the number of base pair differences between the two sequences within the fragment (0 because no mismatches were allowed), and Tot difs is the total number of mismatches between the two sequences.

Fragments	Seq Names	Sim *P*-val	Aligned Offsets			Num Poly	Num Dif	Tot Difs
			Begin	End	Length			
Within cluster 1	Busw*01/07	<0.0001	1	184	184	50	0	17
	Busw*15/07	<0.001	15	184	170	43	0	18
	Busw*03/16	0.021	142	224	83	26	0	22
Between clusters	Busw*05/09	<0.0001	124	205	82	24	0	34
	Busw*16/09	<0.0001	124	205	82	24	0	33
	Busw*05/18	<0.001	124	205	82	24	0	30
	Busw*16/18	<0.0001	124	205	82	24	0	33
	Busw*05/06	0.002	124	188	65	20	0	32
	Busw*16/06	<0.001	124	188	65	20	0	35
	Busw*05/13	<0.001	124	188	65	20	0	33
	Busw*16/13	<0.001	124	188	65	20	0	34
	Busw*05/11	<0.001	124	188	65	20	0	33
	Busw*16/11	<0.001	124	188	65	20	0	34
	Busw*05/10	0.007	147	188	42	14	0	39
	Busw*16/10	0.012	147	188	42	14	0	38
	Busw*05/04	0.005	144	188	45	16	0	36
	Busw*16/04	0.008	144	188	45	16	0	35
	Busw*05/08	0.029	147	188	42	14	0	36
	Busw*16/08	0.018	147	188	42	14	0	37
	Busw*05/14	0.022	124	184	61	16	0	33
	Busw*16/14	0.005	124	184	61	16	0	36
	Busw*02/14	0.049	169	207	39	15	0	33
	Busw*17/14	0.049	169	207	39	15	0	33
Between species	Busw*05/ Buga*01	<0.001	142	188	47	17	0	37
	Busw*16/ Buga*01	0.002	142	188	47	17	0	36

Table 2 Comparison of rates of non-synonymous (d_N) and synonymous (d_S) substitutions calculated separately for the codons making up the antigen-binding sites (ABS) and non-ABS within both *Buteo* species and within both sequence clusters.

	No. of	ABS				Non-ABS			
	alleles	$d_N \pm$ SE	$d_S \pm$ SE	d_N/d_S	P	$d_N \pm$ SE	$d_S \pm$ SE	d_N/d_S	P
B. galapagoensis	3	0.275 ± 0.107	0.050 ± 0.032	5.55	0.01	0.051 ± 0.019	0.043 ± 0.027	1.17	0.40
B. swainsoni	20	0.268 ± 0.076	0.027 ± 0.017	9.82	<0.001	0.073 ± 0.017	0.114 ± 0.042	0.64	1.00
Cluster 1	12	0.299 ± 0.085	0.036 ± 0.025	8.25	<0.001	0.045 ± 0.014	0.092 ± 0.034	0.49	1.00
Cluster 2	10	0.072 ± 0.029	0.007 ± 0.007	10.43	0.02	0.014 ± 0.009	0.010 ± 0.010	1.40	0.38

Chapter 6

Genetic and morphological differentiation among Galápagos mockingbird populations

Unpublished manuscript: Bollmer, J.L., M.E. McPhee, and P.G. Parker. Genetic and morphological differentiation among Galápagos mockingbird populations.

ABSTRACT

Island archipelagoes have played a critical role in the study of factors contributing to population differentiation and speciation. The presence of closely related lineages in multiple, isolated populations is ideal for the study of evolutionary mechanisms such as genetic drift and natural selection. We collected genetic and morphological data from six mockingbird populations comprising two species (*Mimus macdonaldi* and *M. parvulus*) in the Galápagos Islands. Microsatellite analyses showed a pattern of increasing genetic variability with increasing island area and a pattern of isolation by distance, both indicating the influence of genetic drift. Significant levels of genetic differentiation existed among all six populations. We found morphological differentiation among populations as well. Morphological distances were smaller between islands of similar area (supporting a previous finding); bill length in particular was correlated with island area. Morphological distances showed no pattern of isolation by distance after controlling for differences in island area. These patterns suggest that natural selection may be influencing morphological differentiation in these small island populations.

KEYWORDS: Galápagos Islands; genetic drift; microsatellites; *Mimus*; morphology; natural selection; population differentiation

Introduction

Studies of population differentiation and speciation on island archipelagoes have contributed much to our understanding of evolutionary processes (Grant, 1998). Island systems facilitate the study of evolution through their simple communities, the presence of multiple, closely related lineages, and clearly delimited population boundaries. Their isolation fosters divergence, both genetic and phenotypic. Founder effects and long-term genetic drift in small populations result in a pattern of decreased genetic diversity in island populations compared to mainland populations (Frankham, 1997). Also, water acts as an effective barrier to gene flow, resulting in significant inter-island population structuring in many species, including vagile taxa such as birds and bats (*e.g.*, Hille et al., 2003; Salgueiro et al., 2004), though there are exceptions (*e.g.*, Santiago-Alarcon et al., 2006).

A number of classic examples of adaptive morphological divergence come from island archipelagos (*e.g.*, Hawaiian honeycreepers, Darwin's finches, *Anolis* lizards). Evidence for the importance of natural selection in shaping phenotype is well established, including the repeated independent evolution of certain traits in response to similar environments and correlations between trait variation and variation in environmental characteristics (*e.g.*, Wainwright & Reilly, 1994; Losos et al., 1998; Clegg et al., 2002; Langerhans & DeWitt, 2004). However, experimental evidence suggests that morphological differentiation can arise due to genetic drift in bottlenecked populations (Bryant & Meffert, 1996; Saccheri et al., 2006), and drift has been invoked in the differentiation and speciation in allopatry of some wild taxa where adaptive explanations

for their differences were not evident (Gittenberger, 1991; Highton et al., 1989; Cameron et al., 1996; Bostwick & Brady, 2002). Also, genetic and morphological differentiation are not necessarily associated with each other. Morphological differentiation across habitat types can occur even with moderate amounts of gene flow, while populations in similar habitats that have been genetically isolated for long periods may show little morphological divergence, presumably due to similar selective pressures (*e.g.*, Smith et al., 1997, 2005; Schneider & Moritz, 1999; Schneider et al., 1999).

The Galápagos Islands have served as a natural laboratory for the study of evolutionary processes in a number of taxa (*e.g.*, Grant, 1986; Sequeira et al., 2000; Caccone, 2002). The islands are volcanic in origin and are located 1000 km west of mainland Ecuador. The endemic Galápagos mockingbirds (*Mimus* spp.) are widespread in the archipelago, occurring on almost all of the major islands (Fig. 1). The Galápagos mockingbirds were formerly in the genus *Nesomimus*; however, in 2007 the South American Classification Committee of the American Ornithologists' Union merged *Nesomimus* into *Mimus* based on mitochondrial data in Arbogast et al. (2006). Based on phenotypic traits, there are four recognized species (only one found per island): *M. macdonaldi* (Española and its satellite Gardner), *M. trifasciatus* (Champion and Gardner-by-Floreana), *M. melanotis* (San Cristóbal), and *M. parvulus* (most of the rest of the islands; Fig. 1). In a phylogeny based on ND2 (1041 bp) that included most populations, Arbogast et al. (2006) identified four distinct mitochondrial lineages: (1) *M. trifasciatus*; (2) *M. melanotis*, *M. macdonaldi*, and the Genovesa population of *M. parvulus*; (3) *M. parvulus* individuals from Isabela; and (4) *M. parvulus* individuals from Santa Fe, Santa Cruz, Rábida, Santiago, and Marchena. The first lineage supports the phenotypic species

designation but the others do not. The second lineage is particularly surprising because the low sequence divergence among the *Mimus* populations on San Cristóbal (*M. melanotis*), Española (*M. macdonaldi*), and Genovesa (*M. parvulus*) has resulted in the grouping of three morphological species.

Abbott and Abbott (1978) analyzed morphological data from all the Galápagos *Mimus* populations in a canonical variates analysis and found that they formed four clusters: (1) both *M. macdonaldi* populations; (2) both *M. trifasciatus* populations; (3) *M. parvulus* populations on larger islands and *M. melanotis*; and (4) *M. parvulus* populations on smaller islands. Abbott and Abbott (1978) further investigated the split within *M. parvulus* by correlating morphological divergence within clusters 3 and 4 with variation in inter-island geographic distance, island area, and island plant diversity (*i.e.*, number of species). They found no consistent patterns; morphological divergence was correlated with inter-island distance for males but not females from larger islands (neither was significant among smaller islands), and small islands that had similar plant diversities had mockingbird populations with more divergent morphologies, a counter-intuitive pattern that was not present among the large islands.

This morphological variation among populations (Abbott & Abbott, 1978), as well as the presence of different mitochondrial haplotypes on different islands (Arbogast et al., 2006), suggests that the *Mimus* populations are genetically isolated. The primary goal of this study was to use microsatellite markers to determine genetic structure of six *Mimus* populations (comprising the species *M. macdonaldi* and *M. parvulus*), describing within-population genetic variability and the degree of inter-population connectivity. In addition, we revisited the morphological differentiation, further investigating the patterns

found by Abbott and Abbott (1978) and interpreting them in light of the recent genetic data (both microsatellite and mitochondrial) that were unavailable thirty years ago.

Materials and methods

Field methods

We sampled individuals from five *M. parvulus* populations (Pinta, Santa Cruz, Fernandina, Isabela, and Genovesa) and one *M. macdonaldi* population (Española) for a total of six islands (Fig. 1). We sampled Pinta, Santa Cruz, Fernandina, and Genovesa from May to July of 2003; Isabela, Española, and Santa Cruz from February to April of 2004; and Genovesa and Santa Cruz again in June of 2004. Birds were captured using mist nets and Potter traps. We color-banded each individual and took the following four measurements: mass (g), unflattened wing chord (to the nearest mm), bill length (length of upper mandible to the nearest 0.1 mm), and tarsus (to the nearest 0.1 mm). We also took two 50 µl blood samples via puncture of the brachial vein and stored each of them in 500 µl of lysis buffer (Longmire et al., 1988). We then released the birds at the site of capture.

Sampling

We genotyped 28 birds from Pinta, 43 from Santa Cruz, 25 from Fernandina, 40 from Isabela, 62 from Española, and 34 from Genovesa for a total of 232 individuals. In the field, we tried to space our netting sites so that each site was situated in a different group's territory. Because Galápagos mockingbirds live in cooperative groups with retained young (Curry & Grant, 1990), some individuals caught at the same site were

likely first order relatives. In order to test whether this affected our results, we performed our analyses on the full sample as well as on a subsample: Pinta (n = 19), Santa Cruz (17), Fernandina (19), Isabela (30), Española (34), and Genovesa (19). For the subsample, we limited the number of birds included to two or fewer per site. If two birds were caught at a site, we included both in the subsample; if more than two birds were caught, we randomly picked two of them. This does not eliminate the possibility of close relatives being included, but it does minimize the number of those occurrences.

Microsatellite genotyping

We extracted DNA using standard phenol/chloroform procedures (Sambrook et al., 1989). We genotyped individuals at six microsatellite loci using primers designed from *Mimus polyglottos* (Northern mockingbird; Hughes & DeLoach, 1997): Mp18, Mp25, Mp26, Mp45, Mp83, and Mp84. Microsatellites were amplified in 10 µl reactions: 1X PCR buffer, 0.1 mM dNTPs, 5 mM $MgCl_2$, 1 µM each primer, 4 ng BSA, 0.1 units of *Taq* polymerase, and 40 ng of genomic DNA. For Mp84, we added only 20ng of DNA and 0.25 µM each primer. Reaction cycle conditions were the same for each primer set and followed Hughes and DeLoach (1997). We separated PCR products on non-denaturing 7.5% polyacrylamide gels using BioRad sequencing rigs. We stained the gels with ethidium bromide and visualized them using a Kodak IS440CF imaging system. We ran all homozygotes at least twice to check for allelic dropout.

Statistical analyses

We tested for Hardy-Weinberg equilibrium by locus and population using a randomization test that employs the F_{IS} statistic. We tested for linkage disequilibrium between all pairs of loci within each population via randomization tests employing the log-likelihood ratio G-statistic. Bonferroni tests were used to correct for multiple comparisons (Rice, 1989). We calculated allelic richness as the number of alleles per locus after controlling for differences in sample size using rarefaction analysis (El Mousadik & Petit, 1996; Petit et al., 1998). We performed the above tests using FSTAT version 2.9.3 (Goudet, 2001). We used the web version of GENEPOP (Raymond & Rousset, 1995) to calculate expected and observed heterozygosities for each population. We tested for a relationship between genetic variation and population size by performing linear regressions of genetic variability measured as expected heterozygosity and allelic richness on the log of island area, an index of population size. We did these analyses using the statistical package R (R Development Core Team, 2006). Island areas were calculated from GIS maps of the archipelago using ArcMap 9.0.

In order to assess population genetic structure, we first calculated F_{ST} values (Weir & Cockerham, 1984) for each pairwise combination of islands in FSTAT. We also tested for significant differences in allele frequencies across populations using a Fisher's exact test in GENEPOP. We constructed an unrooted majority rule consensus tree (based on 500 bootstraps) using the neighbor-joining method (Saitou & Nei, 1987). We used the Cavalli-Sforza and Edwards' (CSE; 1967) chord distance, which Takezaki and Nei (1996) found to be reliable in obtaining correct tree topology under various conditions tested. We generated the distances and tree using SEQBOOT, GENDIST, NEIGHBOR, and CONSENSE in PHYLIP v. 3.66 (Felsenstein, 2006), and we visualized the tree in

TreeView 1.6.6 (Page, 2001). Lastly, we tested for isolation by distance using two genetic distance measures: the CSE distance and Rousset's (1997) distance (F_{ST} / [1 – F_{ST}]), which is more standard for isolation by distance analyses. We used Mantel (1967) tests in Arlequin version 3.1 (Excoffier et al., 2005) for these analyses. Inter-island geographic distances were measured in kilometers from GIS maps using ArcMap 9.0.

For morphological analyses, we used 216 individuals from the six populations: 44 from Santa Cruz, 40 from Isabela, 25 from Fernandina, 28 from Pinta, 34 from Genovesa, and 45 from Española. After removing outlying measurements (data points falling more than 1.5 times the interquartile range either below the first quartile or above the third), we tested for normality of each variable using Shapiro-Wilks tests. Not all data were normally distributed, so we used Kruskal-Wallis tests to assess morphological differences among populations. Fligner-Killeen tests confirmed homogeneity of variances across groups, so we examined multiple comparisons using Tukey's HSD tests. All of these tests were performed in R. We calculated Euclidean distances following Smith et al. (1997, 2005) from the normalized values of the four traits we measured (mass, wing, bill, and tarsus) and from the expanded dataset published in Abbott and Abbott (1978) on three traits (wing, bill, and tarsus). We only used Abbott and Abbott's data for males (female measurements were tightly correlated to those of the males). Abbott and Abbott (1978) had found that *M. parvulus* populations segregated into two clusters in multivariate space: one from large islands and one from small islands. The island area effect could be confounded by the geographic positions of the islands, with the large islands being centrally located. So, we used a partial Mantel test in Arlequin to test for a relationship between morphological distance (Euclidean distances calculated

from Abbott and Abbott 1978) and similarity of island area while controlling for geographic distance. For this test, we classified each pairwise comparison as being between islands of similar or dissimilar size after first categorizing the islands as small (<150 km^2) or large (>550 km^2). We also used the Euclidean distances to construct a neighbor-joining tree using the programs NEIGHBOR in PHYLIP and TreeView. Finally, we tested whether individual traits vary in their relation to island area. In R, we performed linear regressions of population means from Abbott and Abbott (1978) for bill, tarsus, and wing on island areas calculated using ArcMap 9.0.

Results

Within-population genetic variability

In both the full sample and the subsample, all loci were in Hardy-Weinberg equilibrium, the randomization tests all having *P*-values greater than the Bonferroni corrected value of 0.001 (nominal level of 5%). All the *P*-values were greater than the corrected value (0.0006) for tests of genotypic disequilibrium also, indicating the loci were not linked in either sample.

In the full sample, we identified a total of 75 alleles across the six loci, with individual loci having between 10 and 16 alleles and individual populations having between one and 13 alleles per locus (Table 1). A total of 20 alleles were private (Table 1). None occurred in the Pinta and Genovesa populations, while 10 occurred in the more variable Santa Cruz (6), Isabela (2), and Fernandina (2) populations. In the *M. macdonaldi* population on Española, 10 of 17 alleles were private (59%), though eight of them were from a single locus (Mp18). The subsample showed the same general pattern

(Table 1), though the total number of alleles decreased by nine due to the smaller sample size of individuals.

Genetic diversity varied across islands (Table 2). In the full sample, the total number of alleles per population ranged from 16 to 49 and observed heterozygosity ranged from 0.298 to 0.741. Again, the results based on the subsample were very similar to those from the full sample. Genetic variability was greater in populations residing on larger islands. Using the full sample, we found a significant positive relationship between H_E and island area ($r = 0.816$, $F = 7.98$, $P = 0.048$), as well as between allelic richness and island area ($r = 0.921$, $F = 22.44$, $P = 0.009$).

Inter-island genetic structure

All six *Mimus* populations were strongly differentiated. Pairwise F_{ST} values ranged from 0.033 to 0.589 for the full sample and 0.030 to 0.590 for the subsample (Table 3). The lowest F_{ST} value for both samples was between the populations on Isabela and Fernandina. The comparisons between *M. parvulus* and *M. macdonaldi* had the highest values. Fisher's exact tests showed that allele frequencies were significantly different for each pair of populations ($P < 0.00001$) in both datasets, including between Isabela and Fernandina. The unrooted neighbor-joining tree showed that the *M. macdonaldi* population on Española was the most divergent of the six populations, while the populations on Fernandina and Isabela were the most similar (Fig. 2a). Within *M. parvulus*, the microsatellite data showed greater divergence between the two populations we sampled from small islands (Genovesa and Pinta) than between those on larger islands (Fig. 3a). Mitochondrial divergences calculated from Arbogast et al. (2006; Fig.

3b) showed no pattern for three small islands (Santa Fe, Marchena, and Genovesa) and three larger islands (Santa Cruz, Santiago, and Isabela). The microsatellite data showed a pattern of isolation by distance, with genetic divergence increasing with geographic distance for both CSE distances ($r = 0.789$, $Z = 401.6$ $P < 0.001$) and Rousset's distances ($r = 0.764$, Z = 1274.5, $P < 0.001$). The patterns were still true when only *M. parvulus* populations were considered (CSE: $r = 0.830$, $Z = 146.3$, $P = 0.008$; Rousset's: $r = 0.634$, $Z = 309.5$, $P = 0.017$).

Morphological differentiation

Kruskal-Wallis tests showed significant differentiation among island populations in the four morphological traits (bill: $\chi^2 = 174.5$, df = 5, $P < 0.001$; tarsus: $\chi^2 = 69.5$, df = 5, $P < 0.001$; wing: $\chi^2 = 124.9$, df = 5, $P < 0.001$; and mass: $\chi^2 = 128.9$, df = 5, $P < 0.001$). *Mimus macdonaldi* was significantly different from all five *M. parvulus* populations for all four traits. Within *M. parvulus*, all five populations were significantly different for bill length except Santa Cruz and Isabela, while none of them were different for tarsus length except Pinta and Isabela. For both wing length and mass, four of the 10 *M. parvulus* comparisons were non-significant. So, the four traits varied in their degree of divergence among populations: all were divergent between *M. macdonaldi* and *M. parvulus*, bill length was also very divergent within *M. parvulus*, tarsus length was not, and wing length and mass were intermediate.

An unrooted neighbor-joining tree of Euclidean distances again showed *M. macdonaldi* to be distant from *M. parvulus* (Fig. 2b). Within *M. parvulus*, though, the two populations on small islands (Pinta and Genovesa) were separate from the three

populations on larger islands (Santa Cruz, Isabela, and Fernandina). In their morphological analysis, Abbott and Abbott (1978) found that *M. parvulus* populations on small islands clustered separately from *M. parvulus* populations on larger islands. After grouping the pairwise comparisons into three categories (between small islands, between large islands, and between small and large islands), we found that Euclidean distances between small islands and between large islands were similar and smaller than those between small and large islands for our *M. parvulus* data (Fig. 3c; comparisons between *M. parvulus* and *M. macdonaldi* show more divergence). However, we sampled only two small (Genovesa and Pinta) and three large (Isabela, Fernandina, and Santa Cruz) *M. parvulus* populations. Using data from Abbott and Abbott (1978), a larger sampling of *M. parvulus* populations (small islands: Santa Fe, Pinta, Marchena, Genovesa; large islands: Isabela, Fernandina, Santiago, Santa Cruz) showed the same pattern of a similar degree of divergence between small and between large populations (Fig. 3d).

Using Euclidean distances calculated from our data, we found a pattern of increasing morphological divergence with increasing geographic distance between populations ($r = 0.621$, $Z = 532.5$, $P = 0.019$), including when only the five *M. parvulus* populations were considered ($r = 0.722$, $Z = 194.5$, $P = 0.022$). Using Abbott and Abbott's data for eight *M. parvulus* populations, the relationship was weaker ($r = 0.406$, $Z = 282$, $P = 0.029$). To investigate the interaction between geographic distance and island area, we used the Euclidean distances calculated from Abbott and Abbott's measurements of the eight *M. parvulus* populations in a partial Mantel test. We found that morphological distance was not significantly related to geographic distance after controlling for differences in island area ($r = 0.253$, $P = 0.100$); however, morphological

distance was significantly related to similarity in island area after controlling for geographic distance (r = -0.751, P = 0.028).

We also asked whether individual morphological traits co-varied with island area. To increase sample size of populations, we used data on bill, tarsus, and wing length from Abbott and Abbott (1978). Linear regressions on eight central *M. parvulus* populations showed that bill was significantly related to island area (r = -0.956, F = 66.20, P < 0.001; Fig. 3), while tarsus (r = 0.691, F = 5.48, P = 0.058) and wing (r = -0.419, F = 1.28, P = 0.301) were not (bill was still significant at the Bonferroni-corrected α-level of 0.017). The relationship between bill and island area was also significant when *M. macdonaldi*, *M. melanotis*, *M. trifasciatus*, and two very isolated *M. parvulus* populations (Wolf and Darwin) were included (r = -0.592, F = 6.46, P = 0.026; Fig. 4), though *M. macdonaldi* was clearly an outlier.

Discussion

While phenotypic variation among Galápagos mockingbird populations has long been recognized, in this study we have shown that significant genetic structuring exists among populations as well, both between *M. macdonaldi* and *M. parvulus* and within *M. parvulus*. The degree of differentiation among the six populations we studied suggests that most *Mimus* populations are evolving in isolation. The microsatellites appear to be strongly influenced by genetic drift, whereas further analysis of the morphological data supports the influence of a different factor, possibly natural selection.

Population genetic structure

Among the six populations we sampled, genetic variability (as measured by heterozygosity and allelic richness) was lower on smaller islands (presumably with smaller populations), which implicates genetic drift as an important force influencing variability at these microsatellite loci. The *M. macdonaldi* population had relatively few alleles (17) at the six loci, and 59% of them were unique to that island, whereas in the other populations only 11% or fewer of alleles were unique. Pairwise F_{ST} values were large (all but one were greater than 0.1), indicating a high degree of genetic isolation between islands. The highest values were for those comparisons between *M. parvulus* and *M. macdonaldi* (0.44 – 0.59); whereas, the lowest F_{ST} value (0.03) was between Fernandina and Isabela. The young ages and close proximity of these two islands suggests that these populations might be more recently separated or experiencing higher current gene flow. Also, their greater within-island genetic variability contributes to a lower F_{ST} value. The results for the full sample and for the subsample were qualitatively the same, with minor differences due to the loss of some rare alleles in the subsample. Any genetic signature caused by having related individuals in the sample is likely negligible compared to the strong inter-island structuring.

Several other population genetic studies of endemic Galápagos birds have shown a range in the degree of structuring among islands. Santiago-Alarcon et al. (2006) found substantial gene flow among *Zenaida galapagoensis* (Galápagos dove) populations at microsatellite loci, whereas the level of gene flow among populations of Darwin's finches is much lower (Petren et al., 2005). Similar to *Mimus*, *Buteo galapagoensis* (Galápagos hawk) populations have little genetic variability within them and exhibit

significant inter-island differentiation at nuclear minisatellite (Bollmer et al., 2005) and mitochondrial (Bollmer et al., 2006) loci, indicating little to no gene flow among islands.

Patterns in morphological differentiation

In their analysis of Galápagos *Mimus* morphology, Abbott and Abbott (1978) found that populations on small islands clustered separately from those on large islands in multivariate space. This pattern of morphological differentiation may be due to phylogenetic history, genetic drift, natural selection, environmental effects, or a combination of factors. Given the results from our microsatellite analysis, ongoing gene flow is unlikely to be important in explaining morphological similarities among many populations. If morphological differentiation was influenced primarily by phylogenetic history and drift, we would have expected a stronger pattern of isolation by distance (assuming islands are colonized by neighboring populations) such as we found at the neutral microsatellite loci. Instead, we found that morphological distance was not related to geographic distance after controlling for variation in island area, while it was related to island area after controlling for geographic distance. More genetic work, though, is needed to elucidate the order of island colonization to better understand the influence of phylogenetic history. Also, morphological divergence between small islands was the same magnitude as divergence between large islands. Under neutrality, drift is expected to be stronger in small populations, resulting in greater differentiation between them than between large populations, which was not true for morphology. The microsatellite data, however, do show the expected pattern of higher divergence among the two small *M.*

parvulus populations we sampled. Overall, the evidence suggests that genetic drift is not the primary force shaping morphology.

Instead, natural selection may be a more likely factor influencing mockingbird morphology. The distribution of similar phenotypes on similarly-sized islands despite the genetic differentiation among them may be the result of selection acting to maintain similar phenotypes in similar habitats on different islands. Petren et al. (2005) found that allopatric populations of two Galápagos warbler finch species (*Certhidea olivacea* and *C. fusca*) had very similar beak morphologies despite large genetic differences between them, suggesting that stabilizing selection was acting in these species as well. The mockingbird pattern was primarily driven by bill length, which was strongly negatively correlated with island area. We found a nonsignificant trend of increasing tarsus length with increasing island area, but wing length was not related. Two classic examples of island bird radiations, the Hawaiian honeycreepers (Amadon, 1950; Pratt, 2005) and the Galápagos finches (Lack, 1947; Grant, 1986), also involve strong selection on bill size and shape.

Abbott and Abbott (1978) noted the morphological similarity of populations on similarly sized islands and recognized that this likely reflected similar selective pressures but specifically which ecological factors those are remains unknown. Larger islands have higher elevation and thus have a wider range of vegetation zones, whereas smaller islands may only have plant species that occur in the low, arid zone. Tye et al. (2002) found that Galápagos plant and vertebrate diversity are closely correlated with island area. In the Marquesas archipelago, Cibois et al. (2007) found that *Acrocephalus mendanae* (reed-warbler) morphology was also correlated to plant diversity, and their data suggested that

ecology had a greater influence on morphology than did phylogeny. They also noted that, as in *Mimus*, the pattern was driven primarily by variation in bill length and not by wing length. *Mimus* bill length may be affected by the range of food resources available or the suite of competitors with which they co-occur. For Darwin's finches, long-term ecological studies have shown that the evolution of beak morphology is influenced by the type of resources available and the presence of competitors (character displacement), as well as hybridization (Grant & Grant, 2002, 2006). Similar studies will need to be performed to identify the specific factors influencing mockingbird morphology.

Evolution of mockingbird populations

Historically, the mockingbirds of the Galápagos Islands have been separated into four species (*M. trifasciatus*, *M. melanotis*, *M. macdonaldi*, and *M. parvulus*) based on phenotypic characters, whereas more recent mitochondrial data (Arbogast et al., 2006) suggest four different groupings (*M. trifasciatus*; *M. melanotis*/*M. macdonaldi*/Genovesa; Isabela; and the rest of the *M. parvulus* populations sampled), though this is based on a single gene. Arbogast et al.'s study sampled more broadly than ours, but they were not able to include Fernandina or Pinta. Based on our microsatellite results, we have no reason to believe Fernandina or Pinta would form a lineage distinct from those Arbogast et al. identified. One of the most surprising results of Arbogast et al.'s study was their finding that the Genovesa population of *M. parvulus* was more closely related to *M. macdonaldi* and *M. melanotis* than to other *M. parvulus* populations. Our neighbor-joining tree is unrooted, however, so we cannot speak to the evolutionary position of Genovesa.

Some island avian taxa like the Hawaiian honeycreepers and the Galápagos finches have undergone extensive radiations resulting in the coexistence of closely related species occupying different niches, while other taxa have not. In a study of the factors affecting the lack of radiation in passerines of the Lesser Antilles, Ricklefs and Bermingham (2007) reviewed the evidence for the four steps necessary for the radiation and secondary sympatry of related species to occur successfully: genetic differentiation in allopatry, long-term persistence of differentiated populations leading to reproductive incompatibility, secondary colonization, and ecological compatibility of the descendent sympatric taxa. They found that the first three steps were often met but not the fourth. Darwin's finches radiated into 15 species in a relatively short time period (within the last two to three million years; Sato et al., 1999), with differentiation strong enough to allow as many as 10 species to coexist on a single island (Grant, 1986). Galápagos mockingbird populations have undergone some degree of morphological differentiation in allopatry that has resulted in the recognition of four species. Our data confirm that, in addition to the morphological differences that have long been recognized, genetic differentiation among mockingbird populations exists as well, including among populations that are of the same morphological species (*M. parvulus*). Like the finches, the mockingbirds likely differentiated within the archipelago relatively recently, in the last five million years (Arbogast et al., 2006), but in contrast to the finches, no successful secondary sympatry has occurred. Arbogast et al. (2006) noted that the mockingbird's omnivorous diet probably limits their potential for successful coexistence, thus limiting their potential for further speciation. However, the genetic isolation of the mockingbird populations leaves open the possibility for further speciation in allopatry.

In conclusion, we found that there is little to no gene flow among Galápagos *Mimus* populations. The correlation between genetic variability and island area, as well as the pattern of isolation by distance, indicate the action of genetic drift at these microsatellite loci. Morphological differentiation, however, was more closely related to

island area, suggesting the influence of ecology and thus, selection. These data contribute to a growing body of work describing morphological and genetic patterns across populations of bird species from Galápagos. Future work comparing patterns across taxa will add to our understanding of how geography influences microevolution and speciation in island archipelagoes.

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Figure Legends

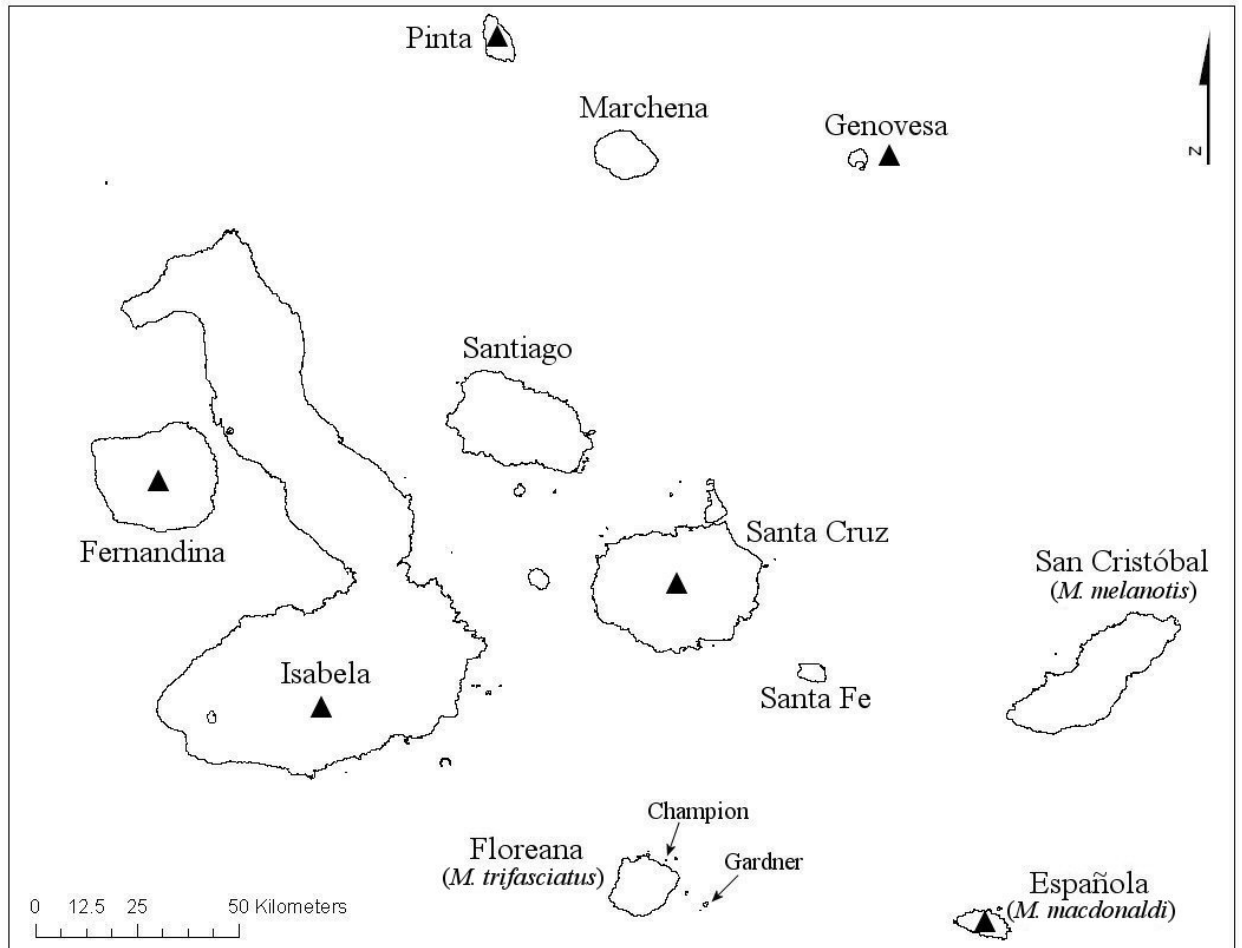
Fig. 1 Distribution of the four recognized mockingbird species in the Galápagos archipelago. *Mimus melanotis* occurs on San Cristóbal, *M. macdonaldi* occurs on Española and an offshore islet, and *M. trifasciatus* (extirpated from Floreana) is restricted to the islets of Champion and Gardner. Populations of *M. parvulus* inhabit the rest of the archipelago, including all of the labeled islands, as well as the islands of Wolf and Darwin (not shown) that are over 100 km northwest of the central archipelago. The six islands sampled for this study are labeled with black triangles.

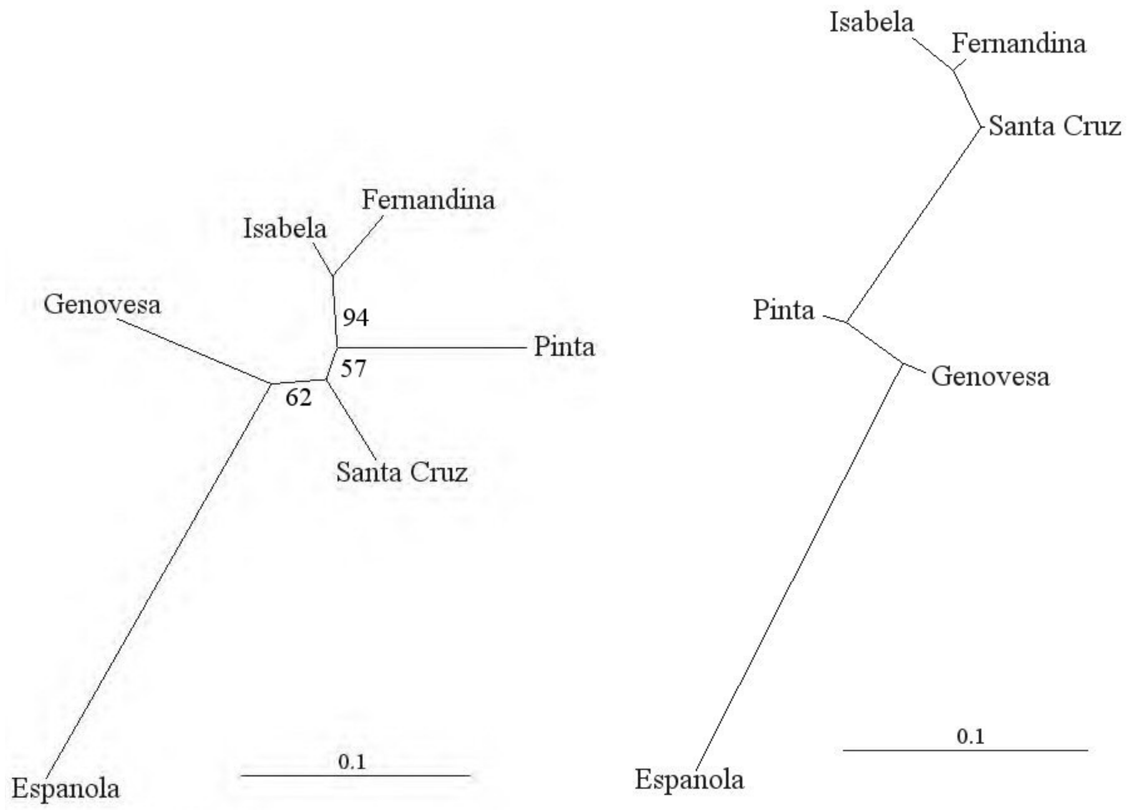
Fig. 2 Unrooted neighbor-joining trees based on (A) Cavalli-Sforza-Edwards distances calculated from the microsatellite data and (B) Euclidean distances calculated from the morphological data. Bootstrap values for tree A are shown. Both trees show that the *M. macdonaldi* population on Española is divergent from the *M. parvulus* populations. The tree based on morphological data also shows greater divergence between *M. parvulus* on small (Pinta, Genovesa) versus large (Santa Cruz, Isabela, Fernandina) islands.

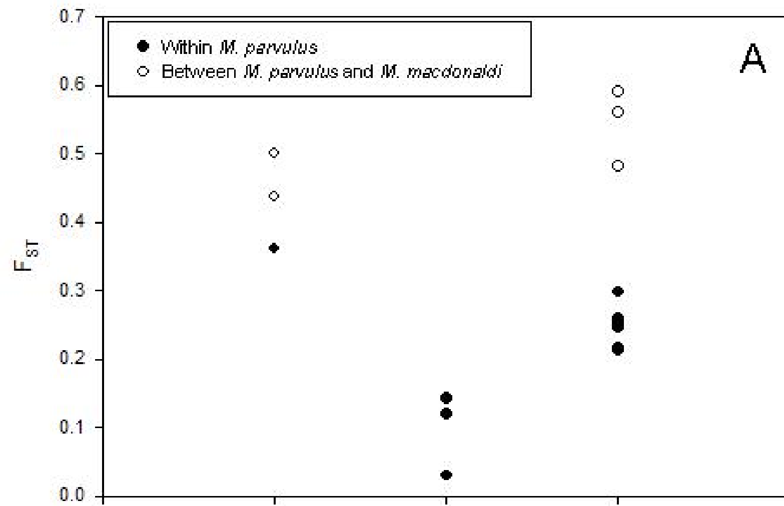
Fig. 3 Galápagos mockingbird morphological and genetic distances between small islands, between large islands, and between small and large islands. (A) The microsatellite F_{ST} value between the two small *M. parvulus* populations was larger than between populations on the larger islands, while small-large comparisons were intermediate. Comparisons involving *M. macdonaldi* again showed greater divergence. (B) There was no pattern related to island area among mitochondrial distances calculated

from ND2 sequence data from Arbogast et al. (2006). (C) Euclidean distances calculated from our data showed low and relatively equal divergence between *M. parvulus* populations on similarly sized islands compared with populations on differently sized islands. Comparisons between *M. parvulus* and *M. macdonaldi* showed greater divergence. (D) Euclidean distances calculated from an expanded sample of *M. parvulus* populations from Abbott and Abbott (1978) supported the pattern in our data.

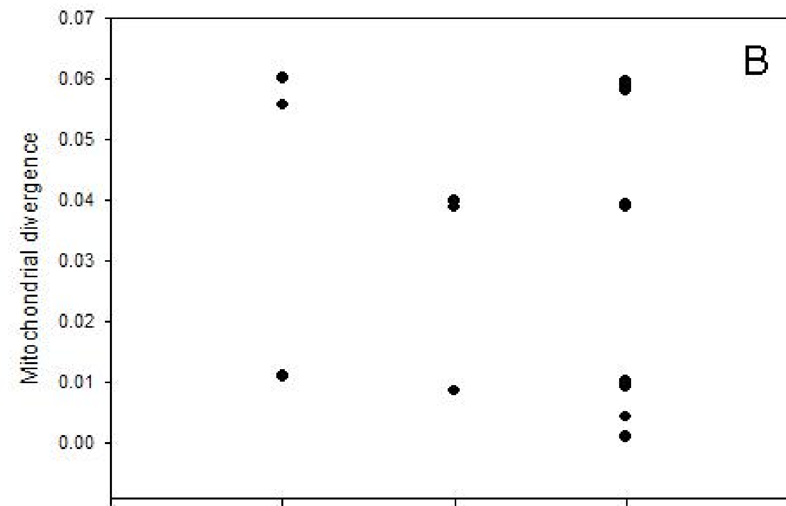
Fig. 4 Relationship between bill length (mm) and island area in Galápagos mockingbirds. Data from all four species are shown: *M. macdonaldi* from Española, *M. melanotis* from San Cristóbal, *M. trfasciatus* from Champion and Gardner, and *M. parvulus* from eight islands in the central archipelago and two isolated islands to the northwest of the main archipelago (Wolf and Darwin). There was a general pattern of decreasing bill size with increasing island area, except for one outlier, *M. macdonaldi*, which had a bill much longer than that of any of the other populations.


Pinta
Marchena
Genovesa
N
Santiago
Fernandina
Santa Cruz
San Cristóbal
(M. melanotis)
Isabela
Santa Fe
Champion
Floreana
(M. trifasciatus)
Gardner
Española
(M. macdonaldi)
0 12.5 25 50 Kilometers

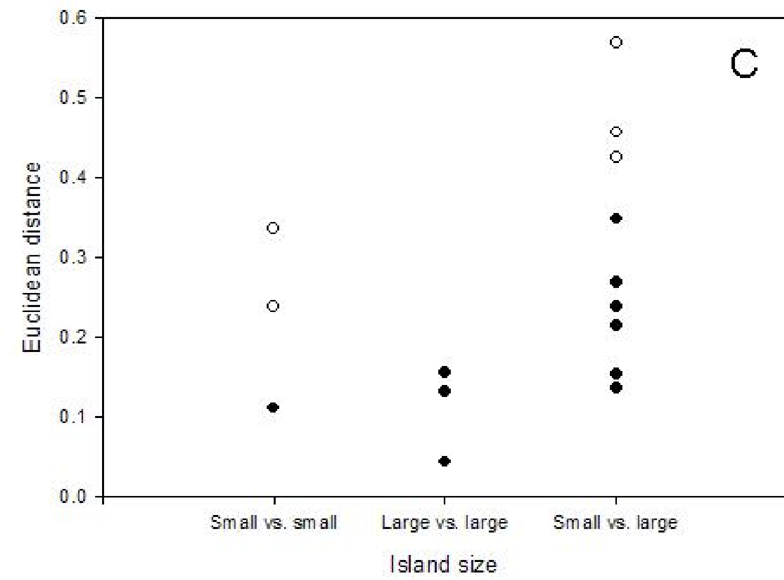

Fernandina
Isabela
Genovesa
94
Pinta
57
62
Santa Cruz
0.1
Espanola
Isabela
Fernandina
Santa Cruz
Pinta
Genovesa
0.1
Espanola



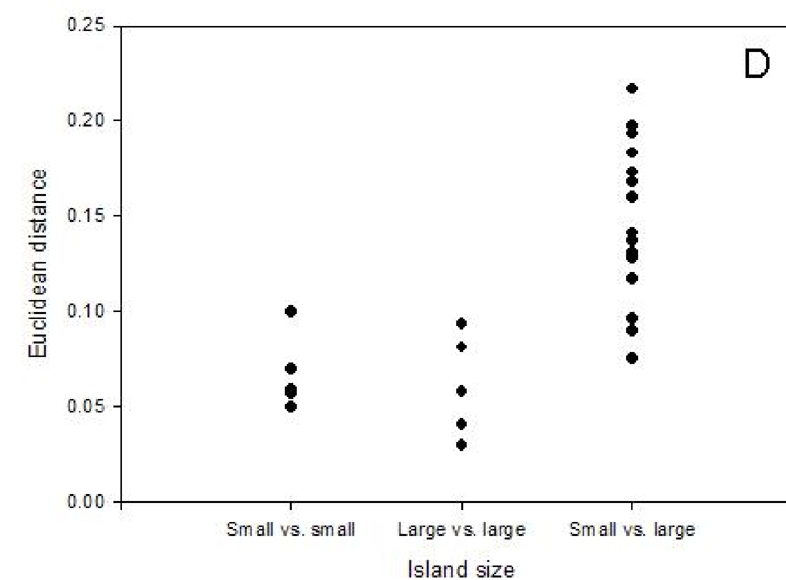
A
F_{ST}
Within *M. parvulus*
Between *M. parvulus* and *M. macdonaldi*
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7



B
Mitochondrial divergence
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07



C
Euclidean distance
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Small vs. small
Large vs. large
Small vs. large
Island size



D
Euclidean distance
0.00
0.05
0.10
0.15
0.20
0.25
Small vs. small
Large vs. large
Small vs. large
Island size

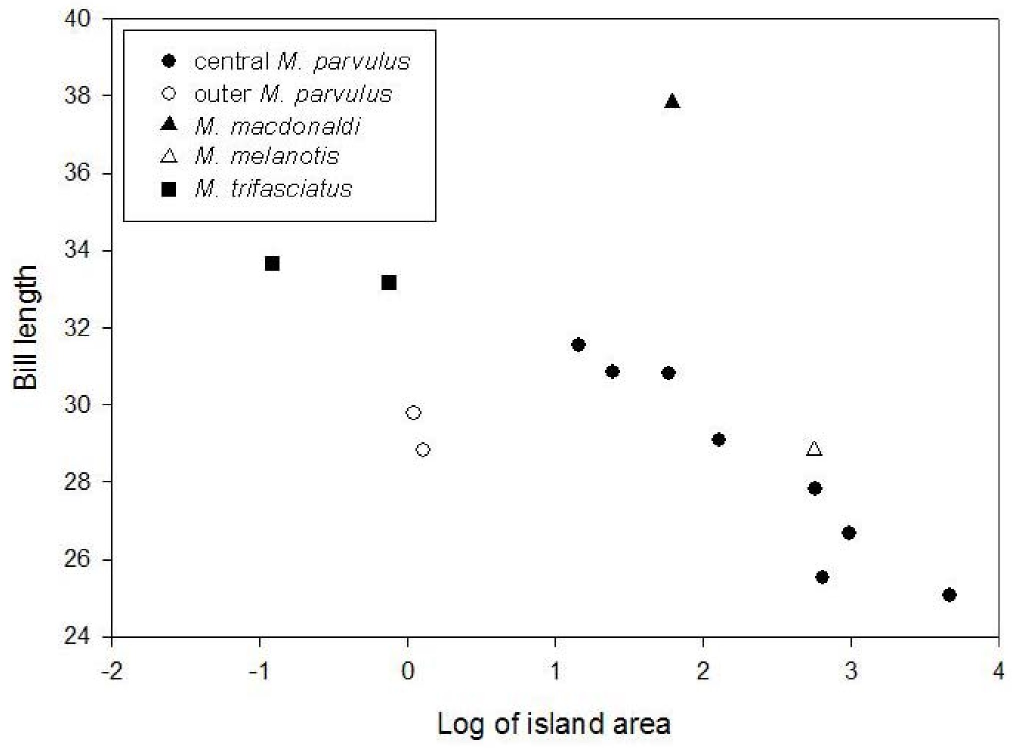

central *M. parvulus*
outer *M. parvulus*
M. macdonaldi
M. melanotis
M. trifasciatus
Bill length
Log of island area
40
38
36
34
32
30
28
26
24
-2
-1
0
1
2
3
4

Table 1 Number of microsatellite alleles per locus and per population (private alleles in parentheses) and the proportion of private alleles per population in Galápagos mockingbirds (*Mimus* spp.). Data from both the full sample and the subsample are presented.

		Mp18	Mp25	Mp26	Mp45	Mp83	Mp84	Total no. alleles	% Unique
Full sample	Santa Cruz	6(1)	9(2)	7	7(1)	8	9(1)	46	0.11
	Isabela	6	5	13(2)	8	9	8	49	0.04
	Fernandina	6	5	8	8	9(1)	7(1)	44	0.05
	Pinta	3	4	7	3	6	4	27	0.00
	Genovesa	2	2	3	3	4	2	16	0.00
	Española	8(8)	2(1)	1	2(1)	3	1	17	0.59
	Total no. alleles	16	11	14	12	12	10		
Subsample	Santa Cruz	6(1)	9(2)	7	6(1)	8	8	44	0.09
	Isabela	5	5	13(3)	8	8	7	46	0.07
	Fernandina	6	6	7	7	8(1)	7(2)	41	0.07
	Pinta	3	4	6	3	6	4	26	0.00

Genovesa	2	2	3	3	4	2	16	0.00
Española	8(8)	2(1)	1	2(1)	3	1	17	0.59
Total no. alleles	16	11	14	11	11	10		

Table 2 Measures of genetic variation in six Galápagos mockingbird (*Mimus* spp.) populations based on six microsatellite loci (n = sample size, A = total number of alleles, R_S = average allelic richness, H_E = average expected heterozygosity, H_O = average observed heterozygosity). Data from both the full sample and the subsample are presented.

	Full Sample					Subsample				
Island	n	A	R_S	H_E	H_O	n	A	R_S	H_E	H_O
Santa Cruz	43	46	7.12	0.720	0.725	25	44	6.92	0.736	0.740
Isabela	40	49	7.32	0.732	0.746	34	46	6.60	0.725	0.730
Fernandina	25	44	7.33	0.769	0.747	18	41	6.83	0.778	0.741
Pinta	28	27	4.43	0.550	0.536	19	26	4.29	0.553	0.509
Genovesa	34	16	2.62	0.429	0.422	19	16	2.65	0.445	0.430
Española	45	17	2.71	0.298	0.278	33	17	2.69	0.311	0.298

Table 3 Pairwise microsatellite differentiation among populations of Galápagos mockingbirds (*Mimus* spp.). F_{ST} values (Weir and Cockerham 1984) for the larger sample are reported above the diagonal (*n*=215) and values for the subsample (*n*=148) are below. All pairs of populations are significantly differentiated from each other for both datasets.

	Santa Cruz	Isabela	Fernandina	Pinta	Genovesa	Española
Santa Cruz	~	0.143	0.119	0.246	0.252	0.437
Isabela	0.131	~	0.030	0.213	0.258	0.482
Fernandina	0.101	0.033	~	0.216	0.298	0.500
Pinta	0.249	0.213	0.213	~	0.361	0.560
Genovesa	0.222	0.235	0.259	0.348	~	0.590
Española	0.438	0.469	0.489	0.555	0.589	~