

## COMBINING ISOTOPIC AND GENETIC MARKERS TO IDENTIFY BREEDING ORIGINS OF MIGRANT BIRDS

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**Abstract.** A quantitative method for linking reproductive and nonreproductive phases of migratory life cycles is fundamental to understanding the biology of migratory organisms. Here we combine genetic (mtDNA) and biochemical (stable isotope) information to examine seasonal movements in the Swainson's Thrush (*Catharus ustulatus*), a Neotropical migrant. We show that when these intrinsic markers are used in concert, they can predict the site-specific origin of thrushes with 76–80% accuracy. Genetic and isotope data needed for these classifications can be obtained from migratory organisms at any phase of the life cycle. We demonstrate how this classification analysis can be used to infer breeding origins of samples gathered during the nonreproductive phases of the life cycle. Based on these results, we argue that further integration of methodologies will refine the scale at which linkages between reproductive and nonreproductive phases of the life cycle can be quantified.

**Key words:** *Catharus ustulatus*; deuterium; hydrogen; mitochondrial DNA; stable isotope; sulfur; Swainson's Thrush.

### INTRODUCTION

Animals that migrate long distances typically spend the majority of their lives away from their breeding sites. An open question in the ecology of migrant species is how events that occur during nonreproductive phases, which generally occur away from the breeding region, may affect productivity and survival on breeding grounds and vice versa (Sillett and Holmes 2002, Norris et al. 2003). To make progress in this area, we need to understand movements of individuals and populations in detail. For the largest vertebrates, satellite transmitters have provided new insights into these questions (Clausen et al. 2003), but high costs limit the number of individuals that can be sampled and the mass and size of satellite transmitters exclude their use for most migrants.

A successful migratory life history depends on an organism's ability to find suitable habitat in all phases of the migratory cycle. Therefore, conditions in the breeding, wintering, and migratory ranges of these organisms are equally important to survival and repro-

duction. To understand the links among phases of a migrant's life cycle, ecologists have used intrinsic markers, such as adventitious coloration (Alisauskas et al. 1998) and parasites (Alekseev et al. 2001), to infer geographic origins of migrants. The primary metric used has been subspecific variation in coloration and meristic characters (e.g., Bond 1963). Although a great deal of what we know about migratory patterns has come from such studies, these studies rarely provide sufficient geographic resolution to allow assignment of nonbreeding animals to specific breeding sites. This limited resolution is of particular concern when conservation actions, such as those related to declining populations, depend on the ability to assign nonreproductive organisms to breeding sites or natal origins.

In the past decade there has been rapid increase in the use of stable isotope and genetic approaches to understanding migratory biology. Although the feasibility of applying these techniques to questions in migratory biology has been demonstrated (Chamberlain et al. 1997, Hobson and Wassenaar 1997, Kelly et al. 2001, Rubenstein et al. 2002), the level of resolution that can be achieved through these methods continues to evolve. Recently, Lovette et al. (2004) showed that the ability to use mtDNA to resolve migratory con-

Manuscript received 15 November 2004; revised 12 January 2005; accepted 26 January 2005. Corresponding Editor: T. R. Simons.

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nections within known subspecies was only possible at a broad geographic scale. A similar concern over lack of population-level specificity has also begun to emerge in isotopic studies (Wunder et al., 2005). Several studies have reported that, using regression techniques based on H isotope ratios, assignment of individuals to any subset of the species' breeding range was unproductive (Kelly et al. 2001, Meehan et al. 2001). However, this isotopic marker has also been useful in uncovering the existence of previously unknown breeding populations (Hobson et al. 2001, 2004). Dockx et al. (2004) have demonstrated the potential benefits of combining stable isotope data with other geographic markers, such as thin-layer chromatography. Although many investigations have explored the limits of resolution of both genetic and isotope methodologies, few studies (e.g., Clegg et al. 2003) have integrated these methods to study patterns of connectivity between breeding and wintering ranges.

Here we use both stable isotope and genetic data to assign migratory birds to their populations of origin. We chose these markers because animals carry them throughout their annual cycle. In particular, we measured the stable H ( $\delta D$ ) and S ( $\delta^{34}S$ ) isotope ratios of feathers. We focused on these isotope ratios because several studies have demonstrated that they provide information on the latitude of molt location ( $\delta D$ ; e.g., Kelly et al. 2001, Norris et al. 2004) and proximity to the coast ( $\delta^{34}S$ ; Lott et al. 2003). Hydrogen isotope ratios in birds' feathers reflect those of local precipitation (Chamberlain et al. 1997, Hobson and Wassenaar 1997). Because there is a strong north to south gradient in the H isotope ratios of precipitation, this measure has been useful in assessing the origins of migratory species (Wassenaar and Hobson 2000). Likewise, marine sulfur is enriched in heavy isotopes relative to terrestrial sulfur (Krouse 1989). This sulfur signature has been useful in detecting a marine influence in feathers of migratory birds that were collected in coastal environments (Lott et al. 2003).

Swainson's Thrush (*Catharus ustulatus*) is a New World Nearctic–Neotropical migrant whose migratory biology has been intensively studied. The breeding range of Swainson's Thrush includes mountain and coastal western North America and across the boreal forest regions of Alaska, USA, and Canada. Individuals then migrate to a winter range that extends from Mexico through Latin America and into South America. Researchers have used mtDNA (Ruegg and Smith 2002) and stable isotope ratios (Wassenaar and Hobson 2001a) to distinguish geographic origins of Swainson's Thrushes. However, the level of geographic resolution at which breeding origins could be distinguished with each of these methods was relatively coarse. Here we combine approaches using both isotopic and genetic (mtDNA) data to explore ways to improve on previous approaches and assign individuals to discrete sampling sites.

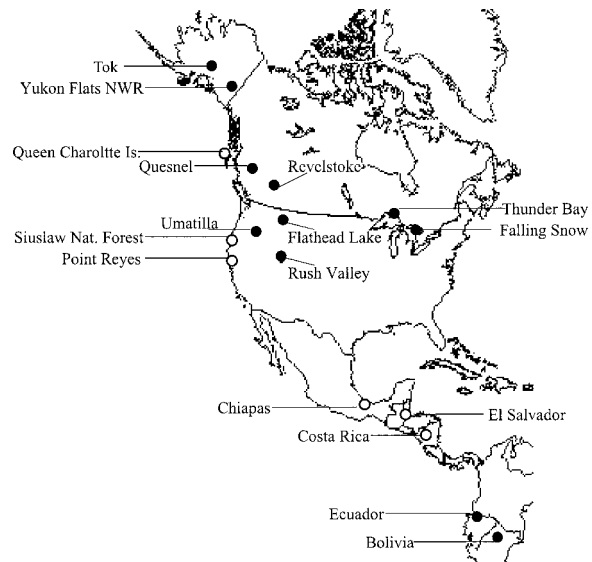


FIG. 1. Sites from which samples of breeding and wintering Swainson's Thrushes were obtained. Open circles indicate sites where birds with coastal haplotypes were found; closed circles indicate sites where inland haplotypes were found.

#### METHODS

Birds were captured in mist nets and their two outer rectrices were collected before they were banded and released. Feather samples were collected from individuals at 12 breeding sites ( $n = 104$  birds) and five wintering sites ( $n = 43$  birds; Fig. 1). Precautions were taken to reduce the probability that nonbreeding individuals were included in breeding location samples. Only adults from sites where the species is known to reproduce were included. Individuals were examined for the presence of a cloacal protuberance (males) or brood patch (females) to assess breeding activity (Pyle 1997). Sampling feathers only from birds in breeding condition also ensured that samples were collected before the post-breeding molt (Evans and Wang 1999). The pre-molt feathers carry the isotopic signature generated at the end of the previous breeding season, thereby providing information on molting location for the previous year.

Analysis of both H and S isotope ratios was done in continuous-flow mode. Feather sample preparation followed the methods of Kelly et al. (2001). Feathers were washed in detergent and thoroughly rinsed to remove oil, dirt, and residual detergent. Before isotopic analysis, feathers were oven-dried at 100°C to remove water.

For H isotope analysis, 0.1–0.2 mg of the distal end of a feather was removed and wrapped in a silver capsule. All isotope analyses were conducted at the University of New Mexico's Stable Isotope Laboratory, Albuquerque, New Mexico, USA. Feathers were loaded into an autosampler from which they dropped into a

high-temperature reduction furnace (Finnigan TC/EA; Finnigan, Bremen, Germany) interfaced through an open split (Finnigan MAT ConFlo II) with a mass spectrometer (Finnigan MAT Delta plus XL). The reduction furnace was used to pyrolyze feather samples at 1450°C. We present  $\delta D$  values of bulk feather tissues. They are not corrected for the exchangeable fraction of H contained in the feathers (Wassenaar and Hobson 2001*b*, 2003). Although we recognize that reporting  $\delta D$  values that are corrected for exchangeable H is preferable to uncorrected values, our samples were analyzed before a method of comparative equilibration was widely known or available. All samples were air-equilibrated for two weeks prior to sampling, so that differential exchange is not a problem. We express the ratio of stable hydrogen isotopes ( $H_2/H_1$ ) in a sample as the parts per thousand (‰) deviation from standard mean ocean water (vSMOW = 0‰). We report deviation from these standards in delta notation ( $\delta D$ ,  $\delta^{34}S$ ). The precision of the analyses for hydrogen was  $\pm 2.0\%$ .

For S isotope analysis, we removed 0.3–0.4 mg of feather from the distal end of the feathers and wrapped it in a tin capsule. These samples were combusted at 1020°C in a Carlo-Erba (Milan, Italy) elemental analyzer following the methods of Fry et al. (2002). Stable sulfur isotope ratios ( $S_{34}/S_{32}$ ) are expressed as parts per thousand deviations from the Canyon Diablo standard (vCDT); the analytical precision for sulfur was  $\pm 0.2\%$ . Analysis of S isotope ratios via continuous-flow methodology is an evolving field. For plant and animal tissues, differences of 1–3‰ between isotope ratios done in continuous-flow mode and those from more traditional offline methods have been demonstrated Fry et al. (2002). These differences are thought to result from incomplete combustion of S in continuous-flow mode. We followed the procedures of Fry et al. (2002) to correct for incomplete combustion. Specifically, we used quartz ( $SiO_2$ ) chips in an equilibration furnace (i.e., the furnace typically used for reduction when analyzing C and N isotope ratios). Fry et al. (2002) found that this procedure eliminated the need to correct S isotope ratios measured through continuous flow. A further difficulty in the analysis of S isotope ratios is the lack of organic standards. Although this limitation reduces our confidence in the absolute value of S isotope ratios that we report, it does not impact the relative differences among samples.

In order to determine the genetic identity of all 147 samples from breeding and wintering sites, we extracted DNA from feather tips following the technique of Milá et al. (2000) and screened using restriction site methods described in detail in Ruegg and Smith (2002). All of the genetic samples screened in the current study were also screened and reported in Ruegg and Smith (2002). In short, phylogenetic analysis of the control region sequences identified two reciprocally monophyletic haplotype groups within the Swainson's Thrush: the coastal haplotype group restricted to the Pacific

coast of North America and the inland haplotype group found throughout the remainder of the breeding range (Ruegg and Smith 2002). To survey the distribution of these clades in our breeding and wintering samples, we amplified the 800 base pair fragment of the control region using the primers L437 and H1248 (Tarr 1995). We then used the restriction enzyme Sfc I to assay a variable site in which cleavage is diagnostic of the coastal clade. Five  $\mu L$  of the digest reaction was electrophoresed on a 6% polyacrylimide gel and restriction fragments were stained with ethidium bromide and visualized under ultraviolet light.

Among Swainson's Thrushes in the East, after-hatch-year (AHY) birds move south of their breeding sites to molt (Cherry 1985, Winker et al. 1992). Wassenaar and Hobson (2001*a*) showed that this molt migration was detectable as enrichment (21–28‰) in the  $\delta D$  values of after-second-year birds (ASY) captured during migration. During the breeding season, when our samples were collected, most first-year adults (i.e., AHY birds) cannot be reliably distinguished from older adults (i.e., ASY birds; Pyle 1997). Consequently, our samples of AHY birds probably contained both first-year and older adults. It is possible, therefore, that the distribution of  $\delta D$  values in our sample would be bimodal. One mode would reflect the first-year adults that were carrying feathers grown at their natal origin in the previous year. The second mode would reflect the ASY birds that molted south of their breeding sites in the previous year. Because we did not have enough samples to evaluate the modality of these distributions at each site, we calculated a mean  $\delta D$  value for each site and then subtracted this mean from each individual measure. We then examined these deviations from the site mean to determine whether, in fact, the distribution was bimodal. We found no indication of bimodality in the distribution of  $\delta D$  values; there was a single prominent mode centered on the mean. Although this pattern does not necessarily contradict previous reports of molt migration in eastern Swainson's Thrushes, it does not provide any justification for adjusting  $\delta D$  values for post-breeding movements.

For the breeding samples, we expected (1)  $\delta D$  values of feathers to vary with latitude of the collection sites, and (2)  $\delta^{34}S$  values to differ between coastal and inland sites. We examined plots of latitude of sample sites and  $\delta D$  values of feathers. To describe the observed patterns, we report slope and intercept coefficients from simple least-squares regressions. We compared the  $\delta^{34}S$  values of coastal and inland haplotype birds with a *t* test. Finally, we plotted  $\delta D$  vs.  $\delta^{34}S$  for all populations to examine the relative distinctness of populations with respect to these stable isotope ratios.

To test the ability of these genetic and biochemical markers to correctly assign individuals to specific breeding populations, we employed a two-step process. First, we used the results from the genetic screening to divide birds into those with coastal ( $n = 26$  birds)

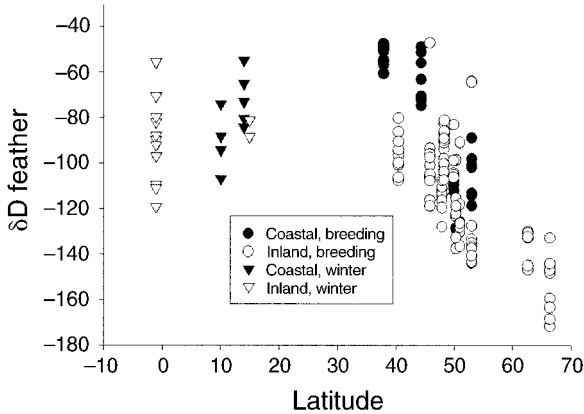


FIG. 2. Relationships between  $\delta D$  values and latitude for Swainson's Thrushes with inland and coastal haplotypes. For breeding season samples, the relationship between latitude and  $\delta D$  values was  $\delta D = -3.1 \times \text{latitude} + 47.9$  ( $R^2 = 0.60$ ,  $P < 0.001$ ). For winter season samples, the relationship between latitude and  $\delta D$  values was  $\delta D = 1.3 \times \text{latitude} - 91.6$  ( $R^2 = 0.29$ ,  $P < 0.001$ ).

and inland ( $n = 78$ ) haplotypes. Then we then used isotopes to classify birds using a quadratic discriminant function analysis in which the sampling sites were the dependent (group) variable and  $\delta D$ ,  $\delta^{34}S$  values were the independent variables. Classification criteria were based on individual within-group covariance matrices and prior probabilities for each sampling site were proportional. We applied separate discriminant functions to birds with coastal and inland haplotypes. To get a better measure of accuracy of the discriminant analyses, we (1) randomly selected 34% of our breeding ground samples to be excluded from initial analysis; (2) used the remaining 66% of the data (i.e., training data) to construct discriminant functions; (3) used these functions to classify the remaining 34% of the samples (i.e., test data); and (4) used the functions derived from the breeding data to estimate relative probabilities of wintering birds having originated from breeding sites. Within all of these analyses, we only classified observations for which the probability of membership in one of the sites exceeded 0.5.

### RESULTS

As predicted, there was a distinctive latitudinal pattern in  $\delta D$  values from north to south among breeding populations (Fig. 2). There was also a tendency for coastal populations to be enriched in  $\delta D$  values, as has been found in other studies (e.g., Chamberlain et al. 1997); this reflects the enriched  $\delta D$  values of coastal precipitation (Bowen and Revenaugh 2003, Meehan et al. 2004). A latitudinal pattern was also apparent in the winter H isotope data, suggesting that birds that breed at lower latitudes migrate shorter distances and to more northern winter grounds than do birds that breed at high latitudes. In addition, birds with coastal haplotypes did not differ in  $\delta^{34}S$  values ( $6.5\text{‰} \pm 6.9\text{‰}$ , mean  $\pm$  SD;

$n = 26$ ) from those with inland haplotypes ( $4.9\text{‰} \pm 3.5\text{‰}$ ;  $n = 78$ ; Fig. 3,  $t = 1.1$ ,  $P = 0.3$ ). Although there was no population-level difference, it should be noted that four birds from Point Reyes, California, USA were substantial outliers with respect to  $\delta^{34}S$  values (Fig. 3). It is unclear why the variation in  $\delta^{34}S$  values at this site was so much greater than at other sampling sites. If these four birds are removed, then there is a population-level difference in  $\delta^{34}S$  between feathers from birds with coastal vs. inland haplotypes. Visual inspection of the bivariate plot of S and H isotopes for 12 sampling sites indicates that sites are arrayed with limited overlap in  $\delta D$  and  $\delta^{34}S$  values of feathers of birds from different sites (Fig. 4).

Coastal and inland discriminant function analyses correctly classified 93% and 83% of the observations from the training data (Table 1). The only bird misclassified among those of coastal haplotypes was assigned to Point Reyes, California when its true origin was Queen Charlotte Island, British Columbia, Canada. In total, eight birds with inland haplotypes were misclassified (Table 2). When applied to the test data, these functions correctly classified 80% of coastal birds and 76% of inland birds to their correct population of origin (Table 1). Only the two Alaska populations had classification rates that reached or dipped below 50% (Ta-

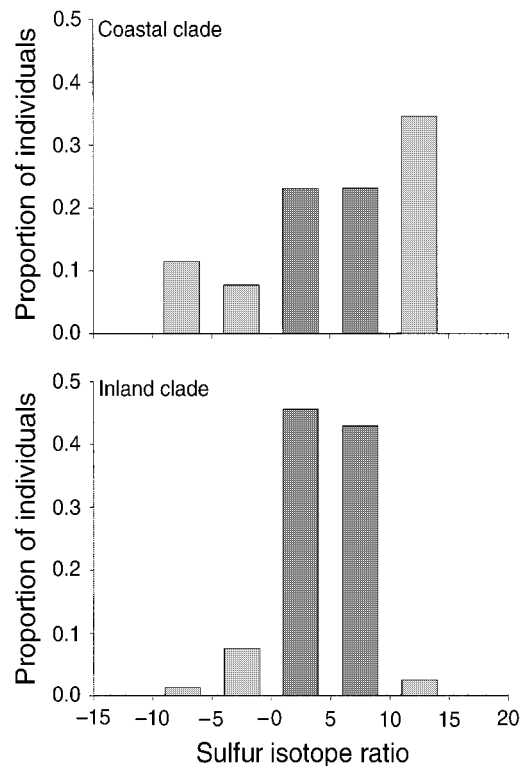


FIG. 3. Distribution of  $\delta^{34}S$  values for Swainson's Thrushes with coastal and inland haplotypes. Note that the coastal birds tend to have more individuals with enriched values, but also have a few with very depleted values.

TABLE 1. Numbers of Swainson's Thrushes assigned to coastal and inland breeding regions based on H and S isotope ratios used in discriminant function analysis.

Clade	Training data			Test data		
	Correct (%)	N	Not classified	Correct (%)	N	Not classified
Coastal	94	16	0	80	10	0
Inland	83	47	6	76	21	4

ble 2). When applied to winter populations, these discriminant functions predicted that birds from winter sampling sites were probably from a relatively restricted portion of the breeding range (Fig. 5). No winter birds were assigned to the two sampling sites in Alaska, USA, or to Fallingsnow, Ontario, Canada (Table 3). The remaining sites were assigned 1–6 winter birds. Nearly all winter samples with inland haplotypes had a high probability of being from breeding sites in the interior mountain West. Only one winter sample with a coastal haplotype was assigned to Point Reyes, California; the remaining birds were nearly equally split between Queen Charlotte Island, British Columbia and Siuslaw, Oregon, USA.

DISCUSSION

To our knowledge, this analysis is the first demonstration that combining genetic and isotopic data allows the correct classification of three out of every four individual Nearctic–Neotropical migratory breeding birds of unknown origin to site-specific breeding locations. The probability of random assignment of birds to their correct origin, given 12 possibilities, was <9%. Although the degree to which these methods will transfer to other systems remains to be tested, there is no

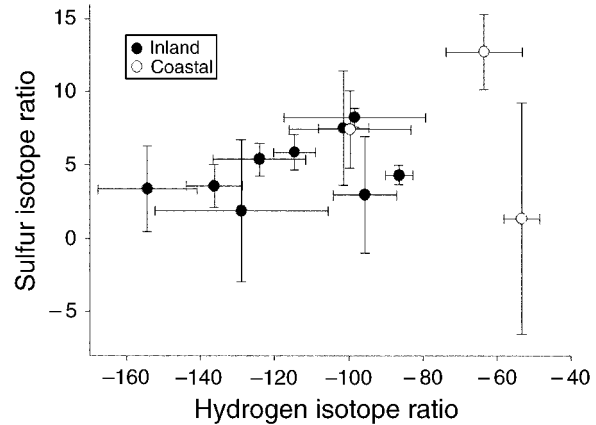


FIG. 4. Plot of δ³S values vs. δ²D values for Swainson's Thrushes with coastal and inland haplotypes. Error bars reflect standard deviations (±SD) from the means of the 12 sampling sites.

apparent idiosyncratic genetic or biochemical pattern unique to Swainson's Thrush that would limit similar applications.

Because S isotope ratios did not show the expected coastal–inland gradient, we thought that this information might not be helpful in assigning birds to sampling sites. Indeed, removing S isotope data from our discriminant analyses of coastal birds did not diminish our assignment rates substantially (81% correct classification for training data and 91% for test data). However, for inland sites, removal of S isotope data from the analyses substantially reduced our ability to correctly assign birds to sample sites (62% correct for training data and 55% for test data). The differential effect of removing S isotope data on the performance

TABLE 2. Sample origins and the sites to which samples were misclassified using discriminant function analyses.

Sample origin	Training data†			Test data‡			Misclassified sites
	N	Not classed§	Correct (%)	N	Not classed	Correct (%)	
Yukon Flats, AK	5	1	100	3	1	50	(Tok, Alaska, USA)
Tok, AK	3	1	50	3	2	0	Revelstoke, BC, Canada (Revelstoke, BC)
Queen Charlotte, BC	5	0	80	4	0	100	Point Reyes, California, USA
Quesnel, BC	4	1	67	6	0	83	Revelstoke, BC (Umatilla, Oregon, USA)
Revelstoke, BC	6	0	83	3	0	67	Flathead, MT (Umatilla, OR)
Siuslaw, OR	4	0	100	3	0	67	(Point Reyes, Ca)
Point Reyes, CA	7	0	100	3	0	67	(Siuslaw, OR)
Umatilla, OR	7	0	100	3	0	100	
Rush Valley, UT	7	1	83	2	0	100	Fallingsnow, ON
Flathead, MT	8	1	86	1	0	100	Revelstoke, BC
Fallingsnow, ON	10	1	66	0			Rush Valley, UT¶; Umatilla, OR
Thunder Bay, ON	3	0	100	4	1	67	(Rush Valley, UT)

Note: Location abbreviations are: AK, Alaska; BC, British Columbia; OR, Oregon; CA, California; UT, Utah; MT, Montana; ON, Ontario.

† Training data represent 66% of the total original data that were randomly selected to generate discriminant functions.

‡ Test data were those 33% of the data not used to train the discriminant functions.

§ Observations for which probability of membership did not exceed 0.5 for any site were not classified.

|| Sites to which birds were incorrectly classified by discriminant functions. Site in parentheses are associated with discriminant analysis of test data, whereas those not in parentheses are associated with training data.

¶ Two observations were misclassified to this site.

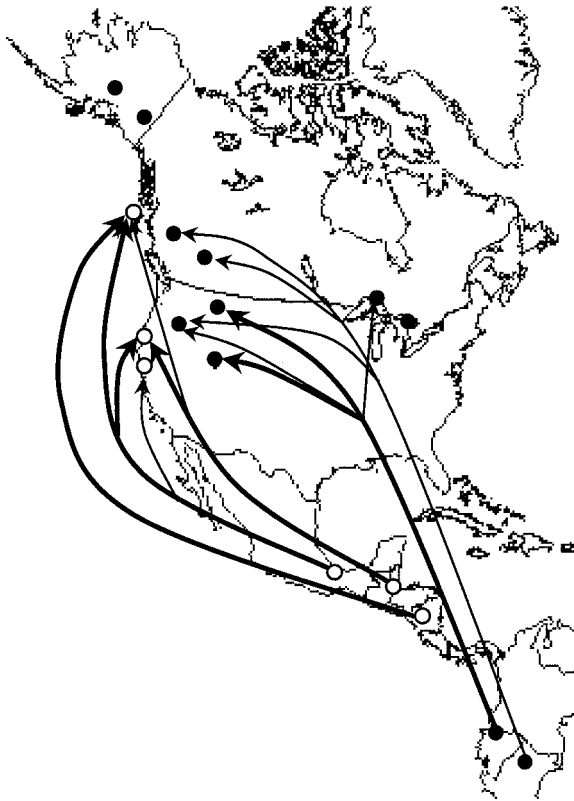


FIG. 5. Map of the predicted breeding sites of Swainson's Thrushes sampled at five wintering sites. The weight of the arrows reflects the number of individual birds predicted to share that breeding origin. Heavy arrows indicate that 4–6 individuals share the origin; light arrows indicate that 1–3 individuals share the origin.

of discriminant analyses for coastal and inland sampling sites is due to the clear separation of the coastal sites on the H isotope axis and the lack of separation of inland sites on this axis (Fig. 4). Likewise, with only the mtDNA information, the probability of correct classification for birds of coastal or inland haplotypes was only 33% and 11%, respectively. When mtDNA data were excluded from the analysis and only isotope data

were used to build the discriminant functions, only 61% of observations were correctly classified to sampling site.

It appears that combining genetic and isotopic data provides better resolution than either of these techniques has produced in isolation. For example, several recent studies have used isotopes of multiple elements to classify birds to breeding origins. Using carbon and nitrogen isotope ratios, Wassenaar and Hobson (2000) correctly classified 80% of Red-winged Blackbirds to sites. However, this analysis was not separated into training and testing phases, so the ability to classify unknowns was probably <80%. Wunder et al. (2005) correctly classified 55–64% of Mountain Plover chicks among 11 breeding sites using hydrogen, carbon, and nitrogen stable isotope ratios. Also of note was that Wunder et al. (2005) controlled for other sources of variation such as: year of sample collection, age of sampled birds, and uncertainty regarding site of origin. Comparisons of these results suggest that the information contained in genetic and isotope markers is largely additive, at least for Swainson's Thrushes. Given these results, it seems that integration of mtDNA and isotope ratios of multiple elements were important to the high rates of correct discrimination among sites. This example argues for further exploration of methods to integrate isotope ratios of multiple elements with other genetic and biochemical markers in studies of migration ecology.

A number of authors have suggested that integration of such data sets ought to yield better estimates of migratory connectivity (e.g., Webster et al. 2002, Clegg et al. 2003, Smith et al. 2005). In general, however, this potential had not been fully realized. Although our results suggest that this structure exists, our analyses also reach a limit in resolution. We think that this limit is evident in the misclassification of observations. We find that many of these misclassifications were neighboring or relatively nearby sites, suggesting that some of our sites were too close together to be effectively distinguished with our methods (e.g., Revelstoke, British Columbia, and Flathead, Montana, USA). We ac-

TABLE 3. Number and percentage of birds sampled during winter that were assigned to breeding sites using discriminant function analysis.

Winter site	Breeding site	No. assigned	Assigned (%)
Ecuador	Umatilla, OR	1	8.3
	Rush Valley, UT	4	33.3
	Flathead, MT	5	41.6
	Thundercape, ON	2	16.6
Bolivia	Quesnel, BC	1	33.3
	Revelstoke, BC	1	33.3
	Rush Valley, UT	1	33.3
El Salvador	Queen Charlotte Is., BC	1	20.0
	Siuslaw, OR	4	80.0
Costa Rica	Queen Charlotte, Is., BC	4	100.0
Chiapas, Mexico	Queen Charlotte, Is., BC	6	46.2
	Siuslaw, OR	6	46.2
	Point Reyes, CA	1	7.6

knowledge these limits, but these results also imply that it is possible to classify unknown birds to sites or regions at a reasonably high rate.

Significantly, the markers that we used in our analysis can be collected at any point during the life cycle of a migrant organism. Once patterns that distinguish breeding sites are described, then it becomes possible to map the nonbreeding individuals to these sites with some level of confidence. We demonstrate this approach by using breeding-season discriminant functions to assign winter birds to 12 breeding sites that we sampled. Obviously, all birds that we sampled during winter molted some distance from, rather than at, the nets in which breeding birds were captured. Therefore, in the narrowest sense, we know that the winter migrants did not originate from exactly where any of the breeding samples were collected. Moreover, the small number of samples of birds from most winter locations limits our ability to make generalizations about the proportion of birds wintering at these locations that might migrate to specific sites in the United States and Canada. These types of generalizations would require systematic sampling of sex and age classes across the variety of vegetation types in which a species winters. Although our data do not achieve this goal, the geographic variation in the markers that we examined allows us to suggest that it is likely that most, but not all, of the wintering individuals that we sampled from Ecuador and Bolivia bred in the intermountain West and that the birds we sampled in Costa Rica, El Salvador, and Chiapas, Mexico bred primarily on the coast of the northern United States and southern Canada.

If the patterns found here are also found in other migrant species, then future studies will be able to link winter ecology of songbirds with a probabilistic knowledge of where those individual migrants will breed. Improvements in both methods of extracting information contained in the genome and other macromolecules, along with advances in methodologies for analyzing and understanding this information, are certain to improve our ability to resolve such patterns (e.g., Bensch et al. 2002, Webster et al. 2002, Smith et al. 2005).

#### ACKNOWLEDGMENTS

We thank V. Atudorei and Z. Sharp for access to, and assistance with, the University of New Mexico's Stable Isotope Laboratory, and M.B. Wunder for productive discussions regarding statistical approaches to classification. D. Rubenstein provided a critical review. We thank D. DeSante and the Institute for Bird Populations, the Monitoring Avian Productivity and Survivorship Program (MAPS) banders, the Point Reyes Bird Observatory, and the many independent banders across the hemisphere that donated tissue samples. This work was supported in part by grants from NIH [National Institutes of Health] Office of Research on Minority Health (grant #5P20RR11805), The Turner Foundation, the Environmental Protection Agency (R827109-01-0), and National Science Foundation (DEB-9726425 and IRCEB-9977072) to T. Smith.

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