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Determining geographic patterns of migration and dispersal using stable isotopes in keratins

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Stable isotopes in metabolically inert tissues of migratory animals can be used to infer migratory and dispersal histories. The general approach for estimating geographic origins of migratory animals based on stable isotope values of their keratinous tissues is to develop or calibrate an assignment model based on tissues of known geographic origin. This paper reviews the general forms and evaluates the application of the 3 assignment approaches. Two of these approaches are considered as nominal assignment frameworks because they require prior declaration of named locations as the set of candidate origins. Individual samples can be sorted into the most likely location using a classification tree or a likelihood-based assignment test. The 3rd and more recent approach is considered a continuous assignment framework because it does not require a predetermined list of candidate locations. This approach depends on an underlying mechanistic geographic model of variation in isotope values. Such models can be developed directly from spatially intensive sampling of keratins or by calibrating a spatial model for isotopes in physical (water or soil) or biological (dietary species) resources. Productive approaches to increase spatial resolution of assignment models will use experiments designed to identify specific geographic-based, variance-generating mechanisms, especially if the contributing factors can be quantified for animals that are released back to the wild.

Key words: assignment models, geographic migration, hair, isoscape

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Over the past few decades, patterns of stable isotopes in animal keratins including hair, claw, skin, nail, horn, baleen, and feathers have been increasingly useful for studying animal migration (Ben-David and Flaherty 2012; Hobson and Wassenaar 2008). Landmark studies (Best and Schell 1996; Chamberlain et al. 1997; Hobson and Wassenaar 1997) demonstrated the potential for using stable isotopes to track migration by documenting systematic geographic patterning in stable isotopes in tissues from wild animal populations. Since then, stable carbon (^{13}C) and nitrogen (^{15}N) isotopes have been widely used to elicit spatiotemporal structure in patterns of mammalian migration in both marine (Best and Schell 1996; Lee et al. 2005; Witteveen et al. 2009) and terrestrial (Cerling et al. 2006) systems. Stable hydrogen (^{2}H) isotopes have been used to study patterns of migration and connectivity for a broad range of species of birds (Inger and Bearhop 2008) and bats (Britzke et al. 2009; Cryan et al. 2004, 2012; Fraser et al. 2010), and are increasingly finding utility in human forensics applications (Ehleringer et al. 2008, 2009; Fraser et al. 2006). In general, these isotopes have been used because of relatively predictable isotopic patterns over geographic and other ecologically meaningful gradients. Because animal keratin is metabolically inert once formed, the chemical composition of keratin reflects the environmental conditions under which it was developed, even if the animal subsequently moves to a novel environment. If an animal molts in 1 location and then migrates across a relatively steep gradient in any of these isotope landscapes, the isotopic signature held in keratin becomes a migratory tag that can be used to estimate where that animal was when it grew the keratin.

At natural abundance levels, stable carbon, hydrogen, and oxygen (^{18}O) isotopes in plants and water vary geographically along temperature and humidity gradients (West et al. 2006), and radiostable isotope values of strontium ($^{87}Sr/^{86}Sr$) vary with age and type of bedrock (Beard and Johnson 2000). One important assumption in linking isotopes in animal tissue to geographic locations is that these geographic patterns from 1st principles are predictably maintained through food webs such that they remain identifiable in animal keratins sampled from across the same geographic gradients. And indeed, the process

of tissue synthesis by animals more or less predictably modifies isotope abundances found in their diets (Ben-David and Flaherty 2012; Martínez del Rio and Carleton 2012), such that geographic patterns in keratin are related to those in water and dietary resources (Bowen et al. 2005; Chamberlain et al. 1997; Chesson et al. 2008; Ehleringer et al. 2008; Hobson and Wassenaar 1997), and with the age of the bedrock where animals are foraging (Sellick et al. 2009). However, these patterns are not perfectly predictable at any level because of vagaries in available energy over time, because not all animals at a given location are eating the exact same well-mixed diet, and because individual animals experience different stresses and possess different nutritional conditions during tissue synthesis (e.g., Betini et al. 2009; McKechnie et al. 2004). These variances from the average expected patterns are used to predict the most likely geographic links between tissue and location.

The reliability of isotope-based estimated geographic links, therefore, depends on our ability to quantify variance-generating processes that operate on resources available at the same locations (Wunder and Norris 2008a). The structure of these variances is used directly to make quantitative statements about the relative probabilities that any predefined candidate location was the keratin origin relative to other predefined candidate locations (Wunder 2010; Wunder et al. 2005; Wunder and Norris 2008b). Herein, I provide a brief introduction to current approaches for relating isotope values in keratins to geographic locations.

What are Calibrations and Assignment Models?

Quantitatively, the 1st and most critical step to identify geographic origins from isotope values in animal tissue is to calibrate an assignment model to link isotopes and geography using tissues of known origin (Wunder and Norris 2008a). Calibration simply refers to adjusting a model to relate geography to expected isotope values for keratins. This model can take 1 of several different forms, but all assume that isotopic discrimination between diet and tissue is predictable over time and space for all animals in the study population. There are 2 broad assignment model approaches to link isotopes and geography.

The 1st approach is usually sample-based and does not rely on any specific mechanism to explain geographic patterns in the isotope variance. I refer to this approach as nominal because it requires a priori definitions of potential areas of origin such that the overall potential geographic range of origin is divided into smaller named blocks of geography that are meaningful from a biological or management perspective. In this approach, the predictor variables (the isotope values) are continuous measurement variables and the response variable is categorical (geographic origin), usually having only a few to several possible levels (locations). Nominal assignment approaches use a classification tree or discriminant function–like model for generating the assignments. Because the target regions are sharply defined, the design and selection of geographic units as targets will influence the efficacy of the approach.

The 2nd general approach is model-based and relies on functionally smoothed or interpolated patterns that generate predicted results across the full range of spatial scales for possible geographic locations. These interpolation functions are usually described for inorganic materials, but there is nothing preventing interpolations based on a carefully considered spatial sampling frame that yields isotope values directly for the tissue of interest. For example, Hobson et al. (1999) developed a spatially interpolated model for monarch butterflies (*Danaus plexippus*) from a spatially exhaustive sample set collected in cooperation with a network of volunteers who each reared caterpillars locally. However, in the majority of cases such exhaustive spatial sampling is unfeasible and so this 2nd approach usually requires a smaller subset of training data to calibrate the inorganic-based model. This calibration both adjusts the spatially explicit function so that it predicts values expected for the organic tissue that will be sampled, and also quantifies the expected magnitude of variation from the training data sites. This approach does not specifically impose any predetermined geographic constraints, so I refer to it as a continuous-surface assignment approach; questions can be asked at a variety of spatial scales that are not specifically predetermined by the sampling design for collecting the calibration data (Wunder 2010). In this approach, the predictor variables (the isotope values) are continuous measurement variables and the response variable also is continuous (geographic coordinate anywhere within the range of possible origins). Continuous-surface assignment approaches used thus far have relied on regression-based calibrations and Gaussian probability densities (Hobson et al. 2009b).

Do We Care About an Assignment for the Mean or the Mean of the Assignments?

Questions about the origin of migratory populations are best addressed by 1st assigning geographic origins to individual animals and then compiling the assignments, rather than by finding a location that is associated with the (single) mean isotope value for the sample (Wunder and Norris 2008a). This is because the isotope values are not interesting by themselves, but the transformations of those values to geographic locations are. And because perfect 1:1 transformations of isotope to geography are rare, the mean of the assigned geographies will not equal the geographic assignment for the mean of the isotope values (Fig. 1).

Isotope values for different individuals from the same population foraging at the same location are never identical. In fact, even repeat samples of the same tissue from the same individual are not expected to be identical (Wassenaar and Hobson 2006). Because of this, the transformation from isotope values to geographic location is not done with complete certainty. By assigning individuals to geographic locations 1st, we propagate the uncertainty associated with the transformation, and provide a less biased and more honest answer about

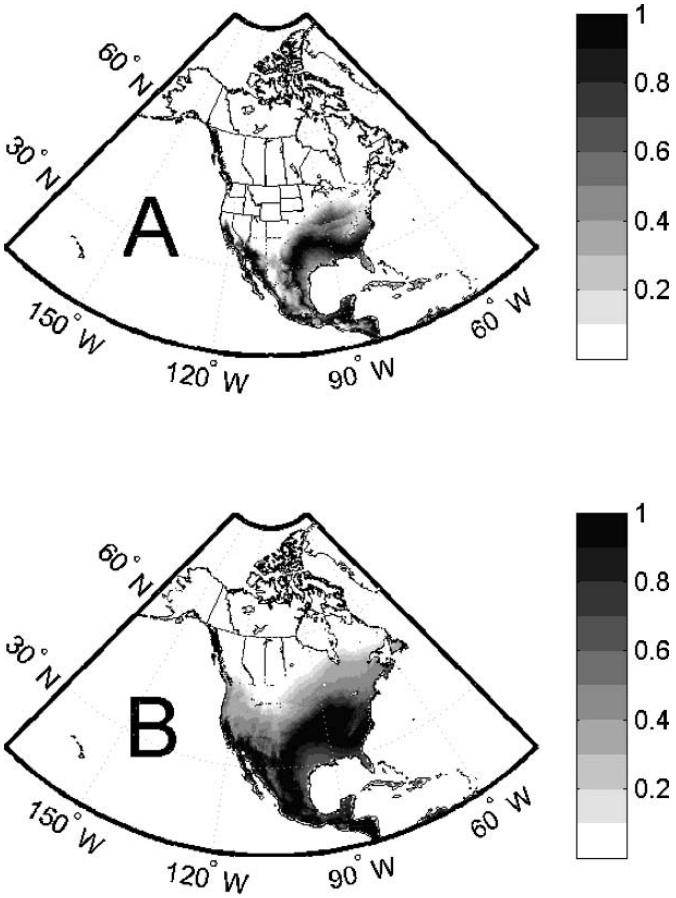

Fig. 1.—Simple demonstration depicting spatial differences in inference from a single continuous-surface assignment for the mean isotope value from a sample and for the mean of individual-level assignments. Simulated results are based on a precipitation-based model for hydrogen that was calibrated for keratin using $\delta^2H_{keratin} = 1.4 \times \delta^2H_{precipitation} + 14$ as the calibration function (Paxton et al. 2007). A) The result from fitting a single surface to the average value of keratin hydrogen for a sample of 60 individuals. B) The result from 1st fitting a unique surface for each of the 60 samples and then averaging those surfaces. Generating a single assignment surface for the mean as in panel A gives the misleading impression that the range of origin is narrow relative to that for the average of the individual assignment surfaces.

the geographic structure of the sample population (Wunder 2010). More importantly, the goal of most migration studies is to determine if there is any geographic structure in the sampled data, and not to assume the structure follows a normal distribution with a mean geographic location associated with the mean isotope value for the population that was sampled.

Nominal Assignment Approaches: An Overview

Nominal assignment approaches start by defining a set of candidate geographic locations of origin, characterized by some weighted distribution of possible isotope values. Once tissues of known origin have isotopically characterized a set of candidate locations, individuals of unknown origin are assigned to the location most consistent with the isotope values from their tissue sample. Nominal assignment methods use classification trees (Hebert and Wassenaar 2005; Witteveen et al. 2009), discriminant function–like models (Caccamise et al. 2000; Farmer et al. 2004; Kelly et al. 2005; Rocque et al. 2006; Sellick et al. 2009; Szymanski et al. 2007; Wassenaar and Hobson 2000b), and Bayesian extensions thereof (Norris et al. 2006; Royle and Rubenstein 2004; Wunder et al. 2005). Ideally the characterizations are based on values observed in tissues of known origin, and all possible regions of origin are characterized. The efficacy of nominal assignment approaches depends strongly on this definition of potential geographic origins for the animal, and also on the choice of which and how many isotopes to use.

For studies using the nominal assignment approach, the geographic divisions are determined by the sampling design, where there is a trade-off between sample size within a given geographic area and the number of geographic areas considered. Decisions about when to pool and when to split samples over locations can have broad impacts on the resultant assignments. Pooling over locations tends to define larger regions that ignore potentially informative structure in the variance, and splitting runs the risk of identifying artificial divisions based on spurious findings in the variance structure of the samples collected. Few studies have formally considered the impacts on assignment efficacy that are caused by decisions of pooling versus splitting.

Wunder et al. (2005) reported performance differences in a nominal assignment approach for 2 spatial resolutions (1 consisting of 6 locations, and another that collapsed those 6 locations into 3) and for various combinations of 3 different isotopes (δ^2H alone, the joint distribution of δ^2H and $\delta^{13}C$, and the joint distribution of δ^2H, $\delta^{13}C$, and $\delta^{15}N$) in mountain plover (*Charadrius montanus*) feathers. This study was conducted exclusively with feathers of known origin from central North America, so the correct geographic origin was known for all cases. Results for 2 different evaluation methods were presented (leave-1-out cross validation and independent data sets for model generation and evaluation). In all cases, correct assignment rates were improved by using all 3 isotopes as compared with using only hydrogen, and by pooling over geographically proximal locations. The highest rates of correct assignment (93–95%) were achieved using 3 isotopes to assign birds to 1 of 3 adjacent locations, each spanning approximately 3° of latitude, whereas the lowest rate (25%) was using 1 isotope to assign birds to 1 of 6 different locations spanning the same range of latitude. The greatest disparities in performance (88% correctly assigned as compared with 25%) were derived from decisions about the spatial structuring of the locations (pooling as opposed to splitting), not the number of isotopes used. Sellick et al. (2009) report similar findings for a study using 2 isotopes and 18 breeding locations.

Nominal Assignment Approaches: Classification Trees

A classification tree is a derived hierarchy of decision rules for assigning novel data to 1 of 2 or more classes that relies on

recursive clustering algorithms. In theory, it works like a dichotomous key does for species identification. It begins with the most general bifurcation rule and proceeds to more specific rules as supported by the data. Each decision provides a fork in the flow toward assigning a tissue sample as having originated in 1 of the predefined locations. Classification trees require no assumptions about data distributions and can incorporate 1 or more discrete and continuous covariates as determinants along the branches of the tree. For example, Hebert and Wassenaar (2005) used univariate thresholds for decision branching to assign mallards (*Anas platyrhynchos*) and northern pintails (*A. acuta*) to 1 of 4 predefined geographic regions based on δ^2H, δ^{34}S, δ^{13}C, and δ^{15}N in feathers; and Witteveen et al. (2009) used similar univariate decision branching rules based on δ^{13}C and δ^{15}N in skin for sorting North Pacific humpback whales (*Megaptera novaeangliae*) into 6 different breeding regions.

Branching rules in classification trees are based on averages and are fixed once determined for the particular data set under analysis (Fig. 2). Once determined, latent variability in the threshold values is ignored. Therefore, direct classification tree applications do not quantify the uncertainty of any individual assignment. Most algorithms to fit classification trees optimize the trade-off between number of branching splits and predictive accuracy to increase performance, but these optimizations are necessarily limited by the data on hand. Because classification trees do not inherently propagate uncertainty in mean isotope profiles for locations, they are most useful for exploratory work and pilot studies. This is especially true as more isotopes and trace elements are measured because classification trees are flexible tools for determining general patterns and divisions in any combination of continuous or discrete predictor variables. Future applications of classification trees would benefit from resampling algorithms that quantify the sensitivity of classifications to variance in the data used to generate the tree.

Nominal Assignment Approaches: Likelihood and Bayesian Methods

The most common and broadly applied likelihood-based nominal assignment approaches include discriminant function analysis and related methods for inverting analysis of variance–type models (e.g., Caccamise et al. 2000; Farmer et al. 2004; Kelly et al. 2005; Norris et al. 2006; Rocque et al. 2006; Sellick et al. 2009; Szymanski et al. 2007; Wakelin et al. 2011; Wassenaar and Hobson 2000b). These methods assign a sample of unknown origin to 1 of 2 or more locations based on characterizations of isotope distributions for those locations. Once the set of candidate locations has been defined, each location is characterized by a probability density that has been parameterized from the sampling distribution of isotope values in tissues that were synthesized at the location. These conditional probabilities are then inverted using Bayes' rule, giving a posterior probability distribution over all candidate locations for a given isotope value. The lines of density

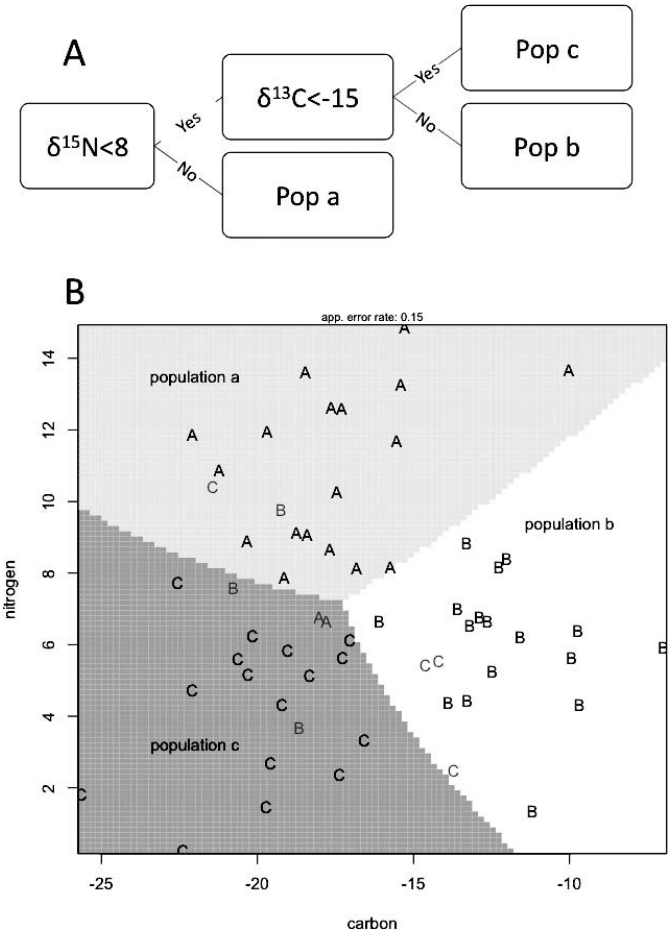

Fig. 2.—A) Simple demonstration depicting decision splits in a nominal classification tree and B) 2-dimensional boundaries in a nominal likelihood assignment model. In the hypothetical assignment problem depicted here, there are 3 named locations to which individuals can be assigned (a, b, and c); carbon and nitrogen isotope measurements are used to differentiate among them. Location b is distinct from a and c based solely on average carbon values. Locations a and c are indistinguishable in terms of average carbon values, but differ in terms of average nitrogen values. Most classification problems parameterized from data are more complex than that shown here, but all use the same variety of branching rules and dividing lines that are based on fixed decision values estimated as conditional averages.

overlap are identified (Fig. 2) and the animal is then assigned to the single location with the highest posterior probability value.

Bayes' rule for this model is written as:

$$P(\mathrm{l}|\mathrm{i}) = \frac{P(\mathrm{i}|\mathrm{l})P(\mathrm{l})}{\int_{\mathrm{L}} P(\mathrm{i}|\mathrm{l})P(\mathrm{l})d\mathrm{l}}, \quad (1)$$

where l is a location, i is an isotope value, and L is the set of candidate locations that were determined beforehand. The numerator consists of the probability based on the sampling distribution (i.e., P(ill) the probability density for isotopes from location l), and the prior probability for location l, P(l), which describes the probability that location l was the origin

in the absence of any information about isotope values. Discriminant analysis is a special case of Bayesian analysis where the sampling distribution is normally distributed for each geographic location, and the prior probability distribution is uniform over the set of locations. As such, the Bayes' rule inversion is done automatically by most software implementations of discriminant analysis, but it should be noted that it also could be done easily enough by hand using the equation:

$$P(\mathrm{l}|\mathrm{i}) = \frac{P(\mathrm{i}|\mathrm{l})}{\sum_{\mathrm{l}=1}^{\mathrm{L}} P(\mathrm{i}|\mathrm{l})}, \quad (2)$$

which states that the probability that location l (given isotope value i) is the origin is equal to the probability density for isotope value i assuming location l, divided by the sum of such probability densities for all locations in L. This forces the assignment probabilities for all locations to sum to 1, enabling us to evaluate the strength of support for assigning an animal to 1 location over another. This is a major advantage of likelihood-based methods over classification-tree approaches.

For example, if the isotope data provided no information to help decide which of 4 potential locations was the origin for an animal, then each of the 4 locations can be considered equally likely, or as having a posterior probability of 0.25 as the origin. This is effectively a null model against which we can evaluate our isotope-based results. Suppose a particular isotope value yields a posterior probability distribution of (0.1, 0.15, 0.15, 0.6) over those 4 sites. Odds ratios can be used to help clarify the difference between the 2nd posterior distribution that was based on isotope data, and the 1st distribution based on random chance. In the isotope-based distribution, the highest probability of assigning an animal to any single location is 0.6, which means the probability of not assigning it to that location is 0.4. So the odds of assigning an animal to a single location based on this posterior distribution are 0.6/0.4 or 1.5. Likewise, the highest posterior probability of assigning an animal to any location under the null model is 0.25, making the probability of not assigning it to that location equal to 0.75, giving odds of 0.25/0.75 or 0.33. Thus, the odds ratio of the isotope-based assignment model relative to the null assignment model is 1.5/0.33, or 4.5. This means the structure of isotope data among the locations for that animal is about 4.5 times more informative than the uniform, or random, case.

We can use these comparisons to generate decision thresholds for determining the potential reliability of the assignments. For example, Wakelin et al. (2011) reported that 6 of 7 wintering blue swallows (*Hirundo atrocaerulea*) could be unambiguously assigned to 1 of 3 distinct breeding locations based on posterior probabilities that ranged from 0.86 to 0.99, all dramatically higher than the 0.33 that would reflect random assignment. These differences reflect odds ratios of 12:1 to 198:1, respectively, in favor of the isotope assignment model relative to random chance. The highest posterior probability for the 1 bird that was not unambiguously assigned was 0.55, which translates to a relatively meager 1.7-fold improvement over random chance in a 3-location system. Similarly, Rocque et al. (2006) considered assignments to 1 of 2 different locations as reliable only if they were based on posterior probabilities > 0.80, which translates to a 4-fold improvement in odds over random assignment. This decision criterion resulted in unambiguous assignment of only 41% of 10 American golden-plovers (*Pluvialis dominica*) and 17 Pacific golden-plovers (*P. fulva*) of known origin to the correct breeding or wintering location, based on isotope values in feathers. Although the specific reasons for differential performance of isotope assignment models in these 2 studies remain unknown, contributing factors include decisions about defining target regions of origin and the respective levels of population-level variance in the isotope data for those regions. Regardless, the most important and consistent result from both studies is that the assignment probabilities were quantified and so could be compared against a null model of random assignment.

A simple Bayesian extension of discriminant analysis is accomplished by structuring the prior probability distribution. When informed by reasonable ancillary information, structured priors can reduce undue influences of otherwise spurious isotope-based results. For example, Royle and Rubenstein (2004) assumed normal sampling distributions for isotopes in feathers for each of 3 regions and used relative abundance for these regions to describe the prior probability distribution. In other words, they assumed the probability of an individual originating from any of the regions was proportional to the relative abundance of animals in those regions regardless of what the isotope data suggest. Wunder et al. (2005) used the number of known-origin individuals sampled from each location as a proxy for relative abundance to shape the prior probability. The Bayesian assignment model in this case evaluates the relative strength of partitioning attributed to information on estimates of relative abundance in the predetermined regions against that based on differences in isotope values. Bayesian extensions also can admit other sources of structure that may not necessarily be related directly to geography. For example, Wunder and Norris (2008b) analyzed a hierarchical variance model that considered variation in δ^2H values that arose from 3 sources, including differences among individuals growing feathers at the same site, uncertainty in a spatial interpolation model, and uncertainty from laboratory-based analytics, to explore sensitivity of conclusions to the assumptions that isotope maps are perfectly predicted and that δ^2H in feathers is perfectly measured. This hierarchical modeling approach can easily be extended to include other factors such as variable isotopic discrimination among individuals based on age, sex, or health, or variable availability of dietary resources among locations.

Although all the studies I described assumed normal distributions for the likelihood function describing isotopes at each location, this is not necessary. If fully Bayesian models are used, the assumption of normality no longer exists, and the models can, like classification trees, simultaneously accommodate both discrete and continuously distributed covariates.

There are no off-the-shelf software applications for fitting such models, however.

Continuous-Surface Assignment Approaches: An Overview

Continuous-probability surfaces model the probability for all points in geographic space as the true origin of an individual animal, given the measured isotope values for a tissue sample from that animal. These models are created by spatially interpolating an exhaustive sample of tissue of known origin (Hobson et al. 1999) or by calibrating an existing spatial model based on isotope values from some other material, such as δ^2H in rainwater (Bowen et al. 2005). For example, if human hair isotopes are to be compared against a water-based isotope landscape (isoscape), that isoscape must 1st be calibrated to reflect isotopic discrimination between hair and water (Ehleringer et al. 2008). Alternatively, if the baseline model was derived directly from the δ-values in hair collected from across the spatial range of interest, then no further calibration would be required.

Wunder (2007) presented the 1st continuous-surface assignment model as applied to the problem of determining the migratory origins for individual mountain plovers. The generalized basic model is presented in detail elsewhere (Wunder 2010). Briefly, the basic algorithm 1st calibrated a δ^2H isoscape for water from Bowen et al. (2005) with feathers of known origin using a simple linear regression. Next, a hierarchical variance model was developed from 3 nested variance-generating processes. First, analytical error (laboratory measurement error) was incorporated as described in Wunder and Norris (2008b). Second, expected variances within and among feathers from the same individual were estimated from values published by Wassenaar and Hobson (2006). Third, the distribution of among-individual, within-location variances was estimated from 112 values from the published literature (Wunder 2007). These 3 nested variance-generating processes were modeled using gamma distributions, and Monte Carlo integration was used to characterize the posterior probability density for variance around the expected values from the linear regression calibration of the water-based isoscape.

The resultant model featured an average isotope value expected for feathers that changed as a function of geography (a calibrated isoscape), and a spatially constant variance structure that accounted for deviations expected from analytical error measuring δ^2H, from differences among and within feathers that arise from physiological and dietary vagaries within individual birds, and finally from differences that arise among individuals using different dietary resources and experiencing different physiological stresses while growing feathers at the same location. This model produced probability density values for the full spatial extent of the entire breeding range of mountain plovers, given the δ^2H for a feather that was grown during the breeding season. This approach makes all sources of variance very transparent, and identifies obvious information gaps and needs for strategic experimentation. Because of this, it offers great potential for providing the back-and-forth dialogue between experimental and applied researchers that is necessary to advance isotope-based methods for determining geographic origin.

The continuous-surface assignment approach is relatively new and has not been as widely applied (but see Hobson et al. 2009a, 2009b). Just as with the nominal assignment models, odds ratios can be used with continuous assignment surfaces to evaluate the strength of evidence favoring any location relative to any other (Hobson et al. 2009b). One difference is that regions can be assigned a posteriori with the continuous assignment model because the probability density is not defined by decisions of how to partition the potential geographic range, as is the case for nominal assignment methods. Alternatively, because continuous assignment models result in a single probability density, one can use a quantile-based approach to define spatially explicit confidence bands for assignments. As software algorithms for generating continuous assignment models are developed, so too will additional applications and interpretations of the resultant probability densities be developed. Currently, however, there are no off-the-shelf computer applications for fitting continuous assignment surfaces.

Additional Design and Application Considerations

The most informative applications of isotope-based assignments will feature well-designed calibrations for the means portion of the model. For nominal assignment methods, all potential locations of origin should be characterized from tissues of unambiguous origin; the different locations should be selected such that they potentially represent maximum differences in isotopic structure (i.e., are most easily discriminated), although rarely will this be known before sampling.

For continuous-surface assignments based on gradients, the full isotopic range of the gradient should be calibrated using tissues of unambiguous origin. At a minimum, the extreme ends of the gradient need to be sampled. For example, if the range of predicted isotope values across a specific geographic target range spans 100‰, a minimal approach would be based on a 2-point linear calibration by collecting freshly grown tissue from the locations associated with the end points of that 100‰ range. An improved design would include more than 2 points and would consider both linear and nonlinear calibrations over the gradient.

Equally important in all empirical calibrations of the mean relationship between stable isotopes and geography is the specification of all known sources of deviation from the mean. Deviations caused by laboratory measurement procedures can be unpredictable when measuring bulk hydrogen in keratin because of uncontrolled isotopic exchange between hydrogen in ambient moisture and noncarbon bound hydrogen in the keratin. It is therefore important for laboratories to provide isotope values that relate only to the stable, nonexchangeable

fraction of hydrogen in the keratin by adopting additional analytical protocols (see Wassenaar and Hobson 2000a, 2003). Apart from laboratory practices specific to hydrogen, the best-documented sources of variance include differences among repeated samples of the same tissue within individuals (Wassenaar and Hobson 2006), among age classes (Britzke et al. 2009; Meehan et al. 2003), between species (Rocque et al. 2006), among years (Farmer et al. 2002), and among individuals within age class and species (Betini et al. 2009; Langin et al. 2007; Wunder et al. 2005). Effective study designs will consider these factors by blocking over them in the sampling design or by modeling them directly as part of the variance structure.

With nominal assignment models, the researcher determines the spatial resolution of the assignment model by default when the target regions are identified. Spatial resolutions of continuous assignment models are determined by the variance structure of the background isoscape. Although it is tempting to try increasing the spatial resolution of assignment models by increasing the number of locations or the number of markers (isotopes, trace elements, genetics, etc.), if this ad hoc approach does not generate consistent results among different studies, interpretation of results becomes more difficult. Experiments designed to identify variance-generating mechanisms will be more useful for refining assignment models (Martínez del Rio et al. 2009), especially if those variance-generating factors can be nondestructively quantified for animals sampled from the wild.

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