

Sex-biased dispersal produces high error rates in mitochondrial distance-based and tree-based species delimitation

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Species delimitation using mitochondrial sequences aims to identify species as morphological expertise and biodiversity both decline. Species delimitation in animals relies completely or in part on 2 criteria: genetic distance and reciprocal monophyly. Using coalescent simulations of populations experiencing continuous and interrupted gene flow we show that these commonly applied criteria incur both high false-positive and high false-negative error rates in species delimitation when dispersal is sex biased, as it is in most mammals. The combination of distance- and topology-based criteria will incur false-positive error rates well above 5% when ancestral effective population sizes are large and when population structure has been in place for 10,000 years or more. This effect persists even with a subtle bias in dispersal of 1 : 4 female : males dispersing in each generation. High false-positive error rates in genetic distances can be overcome by correcting for within-population sequence divergence. We argue that mitochondrial species delimitation requires additional supporting data on ecology, behavior, and morphology, as well as within-population sampling of multiple individuals to ensure that sex-biased dispersal is not the basis for the species limits proposed.

Key words: barcoding, distance methods, error rates, mitochondrial DNA, monophyly, species delimitation

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Species are fundamental biological units in evolution, ecology, and conservation. The ability to rapidly and accurately identify species limits is critical for assessing population persistence, ecosystem function, and conservation targets. Since the 1990s, genetic techniques have enabled species identification without intact organisms (Kyle and Wilson 2007; Russell et al. 2009). The deoxyribonucleic acid (DNA) barcoding approach proposes to apply these same techniques systematically and broadly across all species (Hebert et al. 2003). Barcoding emerged to close the gap between taxonomic expertise and biological diversity and to address 2 key problems: discovering previously unrecognized species (Hebert et al. 2004; Clare et al. 2011), and assigning an unknown sample to known species (Chaves et al. 2012).

Ideally, the barcoding approach works by targeting a single, universally applied gene. In animals, the mitochondrial cytochrome *c* oxidase 1 (*cox1*) gene has become the consensus barcoding locus (Vences et al. 2005). When attempting to identify an unknown specimen using barcoding, the locus is sequenced from that unknown specimen, and the sequence is compared with a database of known reference sequences

through the computation of genetic distances. The intention is that a “barcoding gap” will unambiguously distinguish intra- from interspecific variation, so that distances calculated for a novel sequence could be easily categorized as belonging to one or another population (Hebert et al. 2004). In more sophisticated applications, the evolution of the barcoding locus can be modeled within and between populations so that an explicitly evolutionary framework, instead of just evolutionary distances, aids in species delimitation (Monaghan et al. 2009; Powell et al. 2011). The success of these models crucially depends on keeping the ancestral population size and time since divergence within a certain range. Large ancestral population sizes and/or recent divergences are expected to result in high error rates even when applying sophisticated modeling (Esselstyn et al. 2012).

In principle, the large degree of stochasticity in species divergence makes it difficult to apply simple and universal



rules of thumb for inferring the evolutionary history of speciation (Knowles and Carstens 2007). An empirical evaluation of the barcoding approach comparing signals from a mitochondrial barcoding locus with those from nuclear amplified fragment length polymorphism (AFLP) loci found a large number of false positives where mitochondrial clades were not supported by the nuclear loci (Dasmahapatra et al. 2010b). Phenotypic differences suggested that AFLP divisions were relevant in defining genetically isolated units, and that the mitochondrial barcoding locus tended toward oversplitting. Simulation-based evaluations of the barcoding approach have found high false-negative error rates for recent speciation (< 4 million generations) and/or for speciation reinforced by divergent selection (Hickerson et al. 2006). Meyer and Paulay (2005) also found a large overlap between observed inter- and intraspecific distances in 3 well-studied families of gastropods, resulting in high false-negative error rates of at least 17% using distance thresholds. Importantly, optimum thresholds varied considerably by family, and were not easily generalizable as a function of intraspecific distances. Since intra- and interspecific distances may overlap substantially (Meyer and Paulay 2005), additional criteria are often used to delimit species using barcodes, e.g., requiring species to be reciprocally monophyletic or defining interspecific distances as $10\times$ the average intraspecific distance (the $10\times$ rule).

Even as modeling of single-locus data increases in sophistication (Hendrich et al. 2010; Fujisawa and Barraclough 2013), and despite the burgeoning literature on integrative taxonomy (Padial et al. 2010; Fujita et al. 2012), species delimitation and assignment using DNA barcodes still commonly rely on a combination of distances and monophyly (e.g., Clare et al. 2007, 2011; Kerr et al. 2007, 2009; Borisenko et al. 2008; Baker et al. 2009; Hebert et al. 2010). Reciprocal monophyly in mitochondrial genes is the expected outcome of genetic isolation, given a sufficient number of generations since the cessation of gene flow (Neigel and Avise 1986). In other words, just as distances may incur high error rates, reciprocal monophyly may be an excessively conservative measure of genetic isolation and incur high false-negative error rates (Knowles 2004; Hickerson et al. 2006; Knowles and Carstens 2007).

Beyond the analytical shortcomings of distance- and monophyly-based species delimitation, the choice of barcoding locus is itself consequential. Mitochondrial loci will be a poor reflection of gene flow when dispersal differs between sexes, as it does in many organisms (Williams and Rabenold 2005; Johansson et al. 2008; Paquette et al. 2010). Mammals, the focal organisms of our analyses, are often characterized by sex-biased gene flow in which males disperse widely and females exhibit natal philopatry (Greenwood 1980). Male-biased dispersal has been directly and indirectly observed in many land mammals (Lawson Handley and Perrin 2007), and comparisons of gene flow from both mitochondrial and nuclear markers commonly reveal patterns consistent with male-biased dispersal among bats (Petit et al. 2001; Kerth et al. 2002). However, the combined impact of demographic stochasticity

and male-mediated dispersal on barcoding in mammals remains to be examined, even as distances and monophyly continue to be used in mammalian species delimitation (Bradley and Baker 2001; Baker and Bradley 2006; Clare et al. 2007, 2011; Borisenko et al. 2008; Francis et al. 2010).

Here, we examine the parameter space of distance- and monophyly-based species delimitation using a mitochondrial bar code under demographic models of male-biased gene flow. Because the mitochondrial mutation rate of mammals encompasses variation of 3 orders of magnitude (Nabholz et al. 2008; Galtier et al. 2009), we also investigate the effects of variation in mutation rate on these delimitation criteria. We focus on false-positive errors in which distances or monophyly will diagnose species despite ongoing gene flow, and false-negative errors in which gene flow will be inferred despite its absence. Our analyses suggest that distances and monophyly result in both high false-positive and false-negative error rates when dispersal is male biased, and both are largely insensitive to variation in mutation rates.

MATERIALS AND METHODS

Simulations.—Mammalian life-history parameters were used in our simulations because distance- and tree-based species delimitation has been used in mammals even before the advent of large-scale mitochondrial barcoding projects (e.g., Bradley and Baker 2001). Additionally, the great number of cryptic mammalian species purportedly uncovered using mitochondrial sequences is similar to that of other poorly known vertebrates (Clare et al. 2007, 2011; Mayer et al. 2007; Borisenko et al. 2008; Francis et al. 2010).

To reflect the conditions that structure and isolate populations, we simulated DNA sequences under 2 demographic models: intraspecific population structure with sex-biased dispersal in which females disperse less than males (Fig. 1A), and speciation through complete genetic isolation (Fig. 1B). The intraspecific structure model (model 1) aimed to quantify the probability of falsely rejecting the null hypothesis of no speciation for purely demographic reasons by evaluating the probability that male-biased dispersal combined with a high mutation rate could lead to reciprocal monophyly and high divergence of mitochondrial genealogies despite ongoing gene flow. On the basis of a range of mammal population sizes from small relict populations of 100 individuals to large colony-forming populations of millions (Reed et al. 2003; Schipper et al. 2008; Russell et al. 2011), the ancestral effective population size (N_A) was allowed to vary from 10^2 to 10^7 individuals in all simulations. The ancestral population became structured into 2 equally sized subpopulations (each of size $\frac{1}{2}N_A$) at times $T = 10^2$ – 5×10^6 years ago (Fig. 1). Following the establishment of structured subpopulations, male-biased gene flow continued to connect the subpopulations. We used a range of migration rates based on estimates calculated on subpopulations of North American gray wolf (Wilson and Rannala 2003). We used a migration rate (m) of 1.25×10^{-3} (equivalent to 1% of $\frac{1}{2}N_A$ per generation between populations) for simulated autosomal data and 3 rates

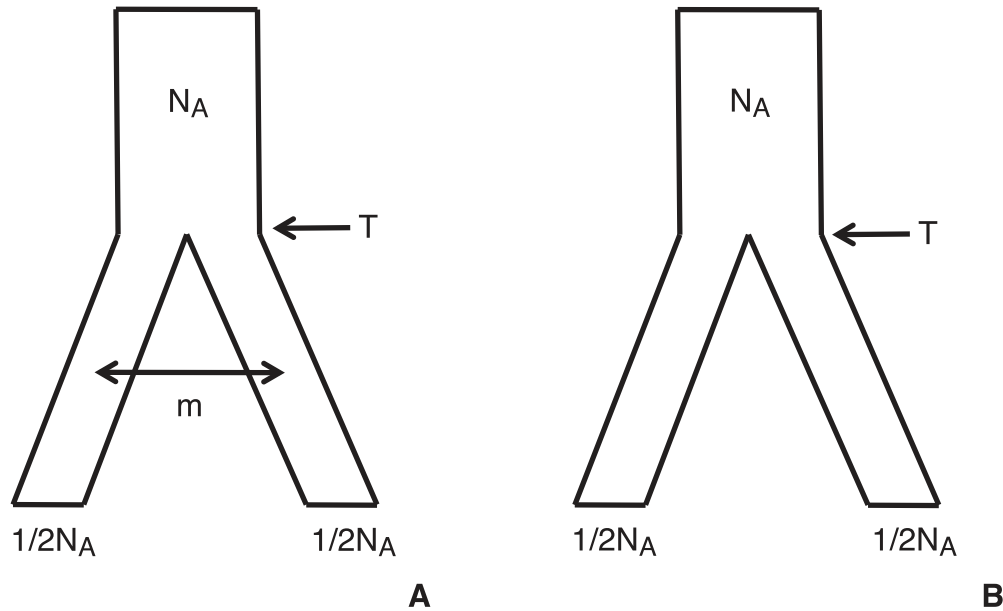


FIG. 1.—Demographic models for simulation analyses. A) Intraspecific population structure model. Ancestral population (N_A) splits at time T into 2 daughter subpopulations, each of size $1/2 N_A$, connected mainly by male-biased gene flow (m). B) Speciation model. Ancestral population (N_A) speciates at time T into 2 daughter species, each of size $1/2 N_A$, without any gene flow in either sex.

for simulated mitochondrial data: 1.25×10^{-6} (0.0025% of $1/2 N_A$ per generation, or 1 female dispersing for every 400 males); 1.25×10^{-5} (0.025% of $1/2 N_A$ per generation, 1 : 40 female : male dispersal ratio), and 1.25×10^{-4} (0.25% of $1/2 N_A$ per generation, 1 : 4 female : male dispersal ratio). A mutation rate $\mu = 10^{-10}$ substitutions site⁻¹ year⁻¹ was used to simulate autosomal data (Lynch et al. 2006). To account for the large variation in mitochondrial mutation rate in mammals, the 1 : 40 female : male dispersal scenario was repeated with low $\mu = 10^{-9}$ substitutions site⁻¹ year⁻¹; background $\mu = 10^{-8}$ substitutions site⁻¹ year⁻¹; and high $\mu = 10^{-7}$ substitutions site⁻¹ year⁻¹ (Nabholz et al. 2008). The background mitochondrial mutation rate of $\mu = 10^{-8}$ substitutions site⁻¹ year⁻¹ was used in all other scenarios. We used the conventional thresholds of 1% and 5% (or $\alpha = 0.01$ and 0.05) to determine false-positive (type I) error rates.

The speciation model (model 2) aimed to quantify the probability of failing to reject the null hypothesis of no speciation despite the absence of gene flow. A false-negative type II error rate of 20% (or $\beta = 0.20$) is considered acceptable in hypothesis testing (Sokal and Rohlf 1995). In recently isolated populations, insufficient time may have passed to permit the accumulation of mutations, resulting in low divergence and reduced pair-wise genetic distance despite the absence of gene flow between daughter populations. Divergence time (T) varied from 100 to 5 million years, at which point the ancestral species diverged into 2 reproductively isolated daughter species, each of size $1/2 N_A$. The speciation model simulated immediate and complete separation of gene pools with zero gene flow after time T . The impact of variation in mitochondrial mutation rate was examined as in the sex-biased dispersal model.

In all scenarios, we assumed a generation time of 2 years, greater than the mean age of female sexual maturity in mammals in the AnAge database (526 days—de Magalhaes and Costa 2009). Genetic data were simulated using the coalescent sampler *ms* (Hudson 2002). The *ms* software implements an infinite-sites model and thus does not conform to finite sequence length and the presence of multiple hits that occur in real molecular data. We used coalescent genealogies from *ms* as input for the molecular sequence simulator Seq-Gen v. 1.3.2 (Rambaut and Grassly 1997), implementing a model based on mammalian mitochondrial evolution, the Hasegawa–Kishino–Yano, with equal base frequencies, a transition/transversion rate ratio of 2, and no among-site heterogeneity (Hasegawa et al. 1985). For each of 60 demographic scenarios (i.e., each combination of N_A and T), we simulated 100 one-kb alignments with 40 individuals, 20 in each subpopulation or daughter species. Both mitochondrial and autosomal data sets were simulated for the population structure and speciation models.

Data analyses.—Two parameters were estimated from each data set: the median Kimura 2-parameter (K2P—Kimura 1981) distance between individuals in different subpopulations or daughter species (with and without accounting for within-subpopulation distances), and the proportion of replicate genealogies showing reciprocal monophyly of daughter species or subpopulations in neighbor-joining (NJ) phylogenetic analyses. These criteria are commonly used to delimit species using mitochondrial sequences (Bradley and Baker 2001; Baker and Bradley 2006; Clare et al. 2007, 2011; Kerr et al. 2007, 2009; Borisenko et al. 2008; Baker et al. 2009; Hebert et al. 2010). PAUP* UNIX v. 4b10 (Swofford 2002) was used to calculate K2P distances between individuals in

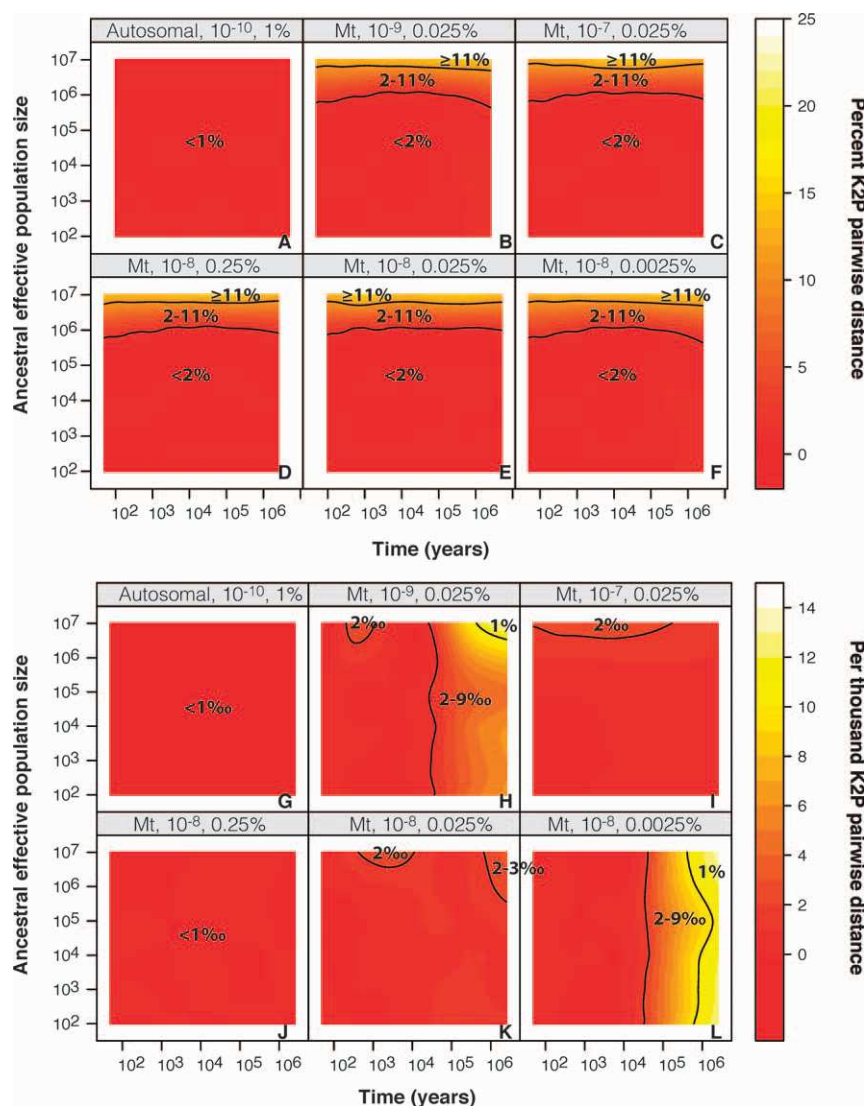


FIG. 2.—Distribution of Kimura 2-parameter distances in a locus as a function of ancestral effective population size and time since restricting female-mediated gene flow between subpopulations. Panels A–F show uncorrected distances between subpopulations, equivalent to most studies with small sample sizes within subpopulation. Panels G–L show results in which intersubpopulation distances were corrected by subtracting the median of within-population distances. Each panel summarizes 3,000 simulations for each locus, mutation rate, and ratio of female-to-male-mediated gene flow. Contours in panels A–F show limits proposed by Bradley and Baker (2001) for genetic distances at intraspecific ($< 2\%$), between-species (2–11%), and generic levels ($\geq 11\%$). Locus types, mutation rates, and migration rates are given on each panel. For example, A is an autosomal locus evolving at 10^{-10} substitutions $\text{site}^{-1} \text{year}^{-1}$ with a migration rate of 1%; and B mitochondrial locus evolving at 10^{-9} substitutions $\text{site}^{-1} \text{year}^{-1}$ with a migration rate of 0.025%. Migration rates are given in percentages of $\frac{1}{2}N_A$ per generation exchanged between subpopulations.

each alignment, and to estimate NJ phylogenies on the basis of those distances. After collapsing all zero-length branches, the resulting trees were queried for monophyly using the *is.monophyletic* command of the Ape v. 2.7-1 (Paradis et al. 2004) R library. Monophyly and distance results from all simulations were summarized using the R statistical language (R Development Core Team 2010). Two-dimensional surfaces of pair-wise distances and reciprocal monophyly were interpolated on the basis of simulated point estimates using the generalized additive model (GAM) smoother implemented in the *panel2ds smoother* command of the *latticeExtra* R library (Sarkar and Andrews 2011).

RESULTS

False-positive rate in distance- and monophyly-based species delimitation.—The population structure model (Fig. 1A) showed that, for the parameter values considered here, distance and reciprocal monophyly at the mitochondrial locus were equally insensitive to variation in mutation rates, and differed in sensitivity to sex-biased dispersal (Figs. 2 and 3). K2P pair-wise distances between subpopulations varied mostly as a function of ancestral population size, with large ancestral populations of $\geq 1,000,000$ individuals yielding distances in the range proposed to encompass between-species variation ($\geq$

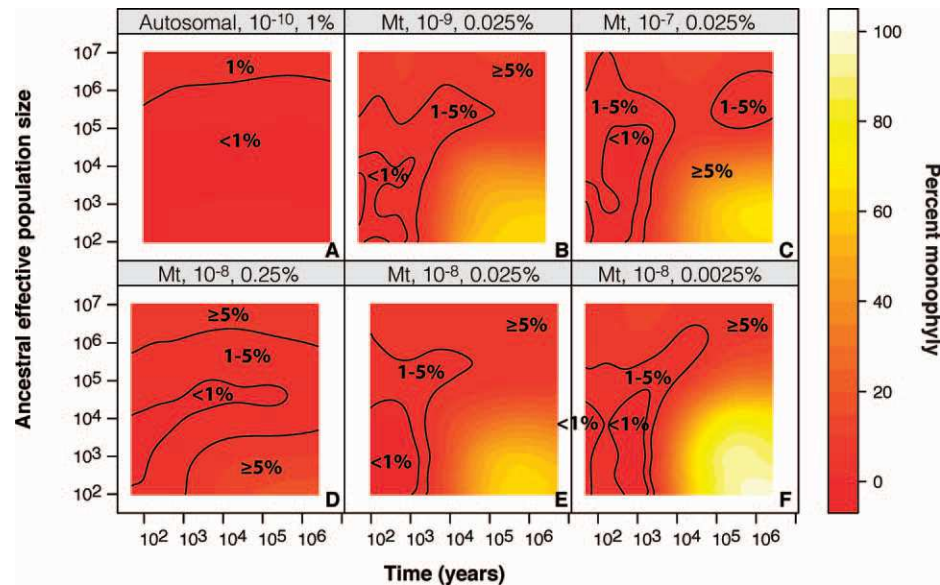


FIG. 3.—Distribution of monophyly in a locus as a function of ancestral effective population size and time since restricting female-mediated gene flow between subpopulations. Each panel summarizes 3,000 simulations for each locus, mutation rate, and ratio of female-to-male-mediated gene flow. Contours show the boundaries in parameter space of critical 1-sided false-positive rates ($\alpha = 1\%$ and 5%). Locus types, mutation rates, and migration rates are given on each panel. For example, A is an autosomal locus evolving at 10^{-10} substitutions site $^{-1}$ year $^{-1}$ with a migration rate of 1% ; and B mitochondrial locus evolving at 10^{-9} substitutions site $^{-1}$ year $^{-1}$ with a migration rate of 0.025% . Migration rates are given in percentages of $\frac{1}{2}N_A$ per generation exchanged between subpopulations.

2% ; Figs. 2B–F [Bradley and Baker 2001; Baker and Bradley 2006]). Results were similar across a range of biases in dispersal, and errors were drastically reduced by systematically accounting for within-subpopulation distance (Figs. 2H–L).

In contrast to the relationship between distance and population size, monophyly responded to time since population subdivision in combination with population size: small (10^2 – 10^4) ancestral populations that became structured 10^4 – 10^6 years ago had the highest probability of reciprocal monophyly, and this effect increased with the scale of sex bias in dispersal (Fig. 3). As expected given the low mutation rate and larger effective population size of autosomal markers (Moore 1995; Rosenberg 2002), distances and reciprocal monophyly were at or below the 1% false-positive (type I error or α) rate in every case (Figs. 2A and 2G, and 3A).

False-negative rate in monophyly- and distance-based systematics.—The speciation model (Fig. 1B) showed that distance and monophyly were equally insensitive to variation in mitochondrial mutation rate, and instead responded to the combination of ancestral population size and divergence time (Figs. 4 and 5). Mitochondrial distance thresholds proposed to delimit species were easily attained with very large effective populations ($\geq 1,000,000$ individuals), or over $1,000,000$ years of isolation, and were unlikely with smaller ancestral effective populations (Figs. 4B–D). Correcting for within-population distances removes the sensitivity of distance to population size and makes between-population distance reflect time since divergence (Figs. 4F–H). Distances estimated with the autosomal locus were consistently $< 1\%$, as expected given its low mutation rate (Figs. 4A). Monophyly tended to underestimate genetic isolation in large populations and

below $10,000$ years of isolation, and this underestimation was greater with the autosomal locus, as expected given its larger effective population size (Fig. 5).

DISCUSSION

The analyses presented here show that widely used distance and monophyly criteria for mitochondrial sequences will falsely infer species-level differentiation in a range of demographic scenarios, including subtle differences in dispersal between sexes (1 female:4 males; Figs. 2 and 3). Simulations of interspecific distances (Meier et al. 2008), sampling sizes (Rosenberg 2007; Ross et al. 2008; Zhang et al. 2010), and divergence time and selection strengths (Hickerson et al. 2006) have previously uncovered pitfalls in species delimitation using mitochondrial sequences. Mitochondrial distance and monophyly used together to determine whether speciation has occurred incurs false-positive error rates above 5% when ancestral populations are large ($\geq 500,000$ individuals, Figs. 2 and 3). These false positives would be inconsequential if male-biased dispersal and female philopatry were rare. Female philopatry, however, is typical among mammals (Greenwood 1980; Petit et al. 2001; Kerth et al. 2002; Lawson Handley and Perrin 2007), and similar patterns have increasingly been reported in other vertebrates (Williams and Rabenold 2005; Johansson et al. 2008; Paquette et al. 2010).

There is one simple way to remedy the high false-positive rates of distance-based species delimitation: correcting for within-subpopulation variation (e.g., Meyer and Paulay 2005). Analyses in which we subtracted the median distance within

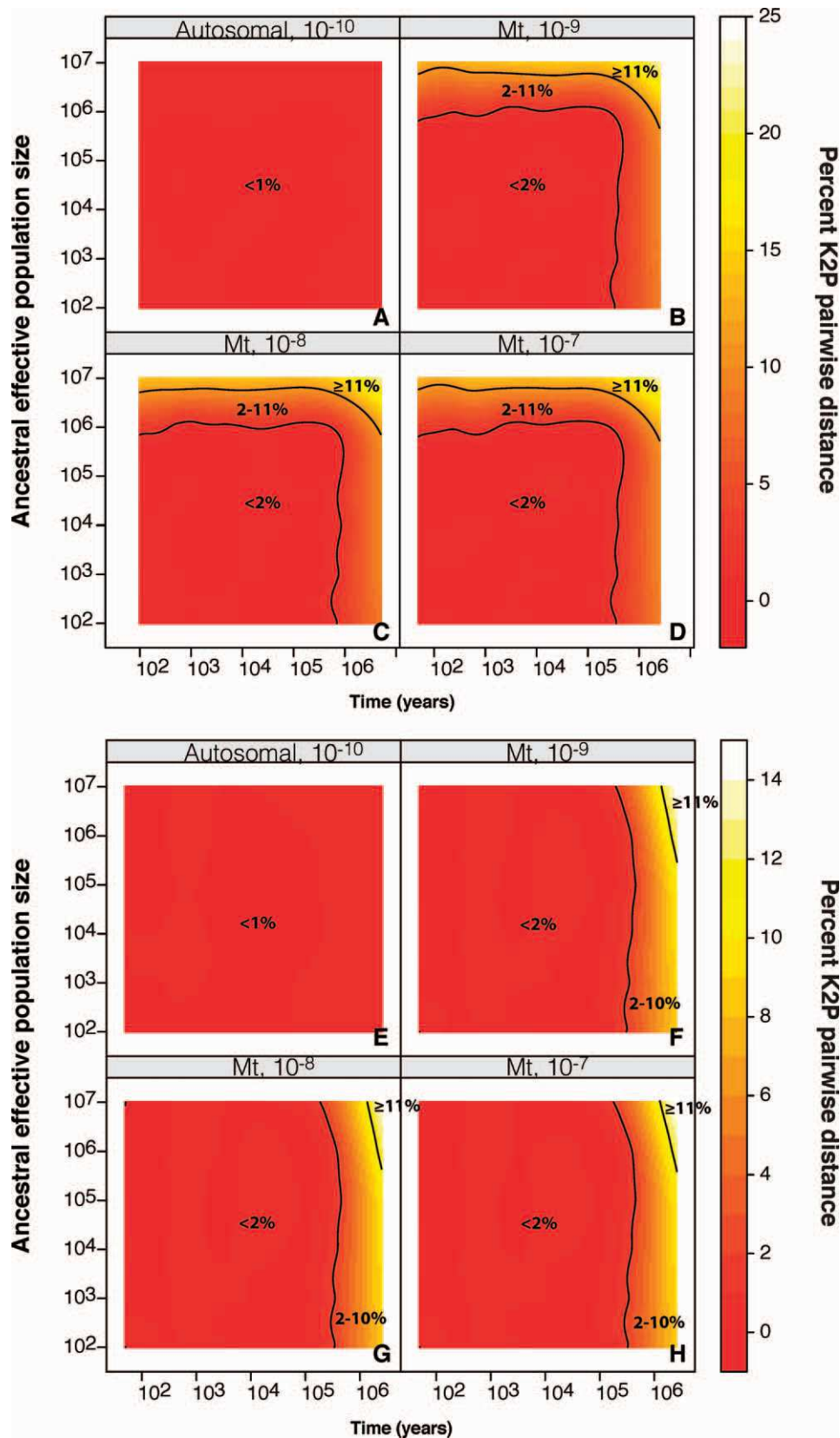


FIG. 4.—Distribution of Kimura 2-parameter distances in a locus as a function of ancestral effective population size and time since completely restricting gene flow for both sexes. Panels A–D show uncorrected distances between subpopulations, equivalent to most studies with small sample sizes within subpopulation. Panels E–H show results in which between-subpopulation distances were corrected by subtracting the median of within-population distances. Each panel summarizes 3,000 simulations for each locus and mutation rate. Contours in panels A–D show limits

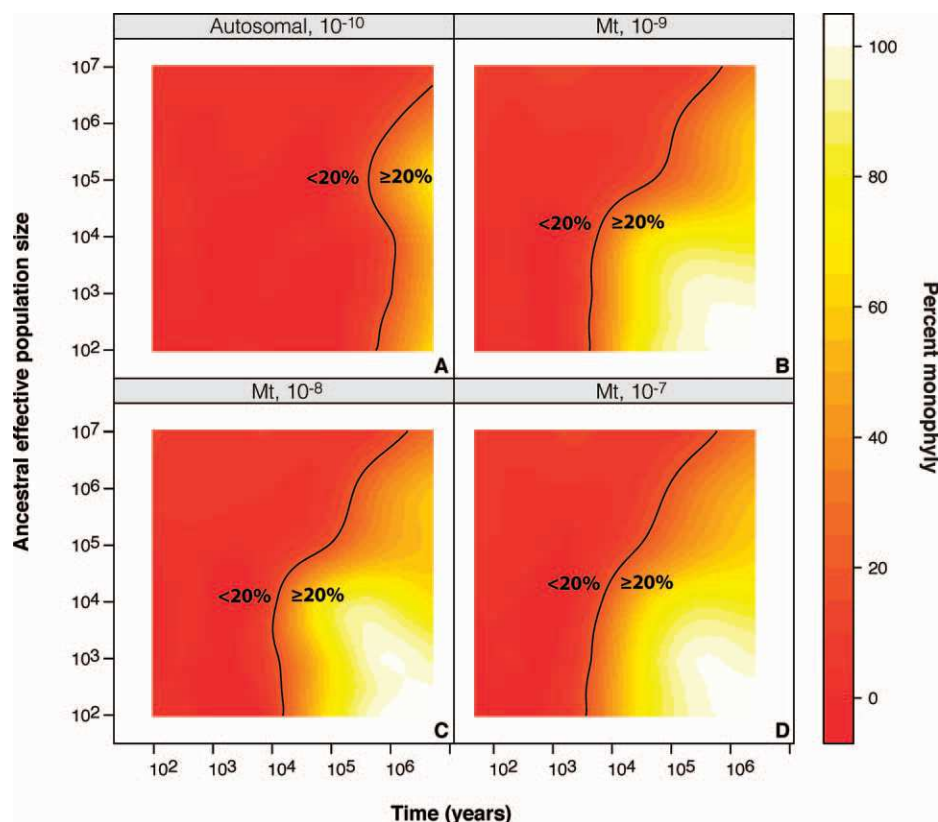


FIG. 5.—Distribution of monophyly in a locus as a function of ancestral effective population size and time since completely restricting gene flow for both sexes. Each panel summarizes 3,000 simulations for each locus, mutation rate, and ratio of female-to-male-mediated gene flow. Contours show the boundaries in parameter space of the target false-negative rate ($\beta = 20\%$ —Sokal and Rohlf 1995). Locus types and mutation rates are given on each panel. For example, A is an autosomal locus evolving at 10^{-10} substitutions site $^{-1}$ year $^{-1}$; and B mitochondrial locus evolving at 10^{-9} substitutions site $^{-1}$ year $^{-1}$.

subpopulations from that between subpopulations rendered distance mostly independent from population size and reduced the between-subpopulation distances by an order of magnitude (Figs. 2H–L). This suggests a straightforward correction in cases in which multiple individuals have been sampled in a subpopulation (e.g., Clare et al. 2011), and cautions against distance- and monophyly-based species descriptions inferred from very small sample sizes (e.g., Dávalos 2004; Velazco and Patterson 2013). It is also important to point out that the correction we applied is ideal: the entire distribution of distances among individuals within a subpopulation was known and these individuals were randomly sampled from the subpopulation and hence unrelated. In situations in which the number of sampled individuals in each subpopulation is small, or in which sampled individuals may be related, correcting for within-subpopulation distance may not reduce

the false-positive error rate as substantially as in these simulations.

The simulations presented here were not intended to test any specific speciation scenario for a particular taxon. We emphasize that mapping parameters resulting from real data onto these simulations is only legitimate if the natural history and genetic characteristics assumed here match those of the populations in question. Because the processes giving rise to the genetic variation presented in the simulations are idealized and completely controlled, our results may be used to draw conclusions about the resulting generalized parameter space given by sex-biased dispersal in combination with varying population sizes and time since structure or genetic isolation. They were also generalized to account for variation in mutation rates documented extensively in mammals, and to a certain extent in birds (Nabholz et al. 2009; Bromham 2011).

When there is uncertainty regarding the extent of sex bias in dispersal, the sensitivity of mitochondrial (mt)DNA-based species delimitation to this natural history parameter needs to be evaluated in light of additional genetic or other data (e.g., ecological, behavioral, or morphological variation; see Hoelzer [1997]; Dávalos and Porzecanski [2009]; Damm et al. [2010]). We tested known evolutionary histories using simulated mitochondrial and nuclear genetic data, and did not include

←
proposed by Bradley and Baker (2001) for genetic distances at intraspecific ($< 2\%$), between-species ($2\text{--}11\%$), and generic levels ($\geq 11\%$). Locus types and mutation rates are given on each panel. For example, A is an autosomal locus evolving at 10^{-10} substitutions site $^{-1}$ year $^{-1}$; and B mitochondrial locus evolving at 10^{-9} substitutions site $^{-1}$ year $^{-1}$.

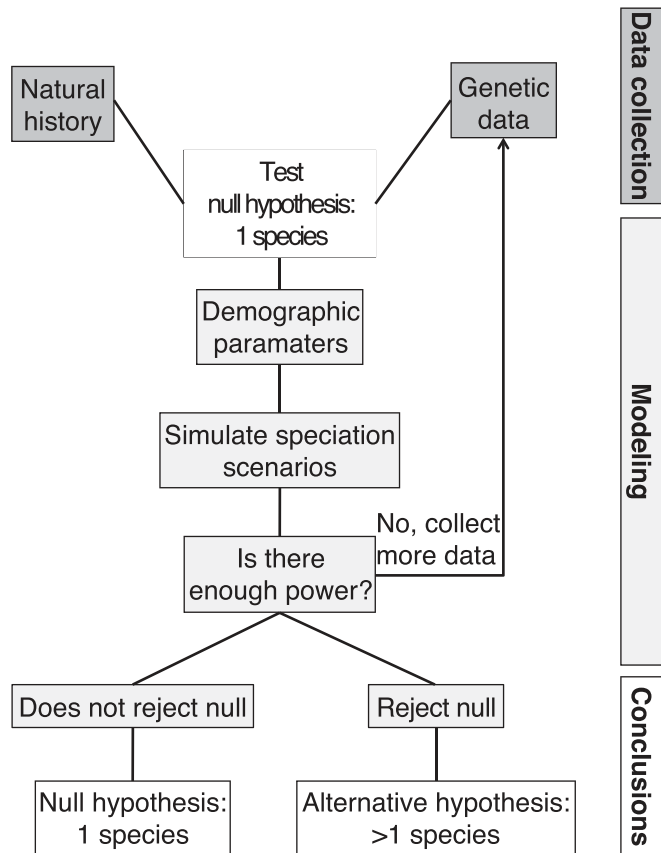


FIG. 6.—Flowchart illustrating a general model for delimiting species with genetic data by using history to parameterize null no-speciation and speciation scenarios.

any phenotypic data (e.g., ecology, behavior, morphology). In the absence of selection, male-mediated gene flow would tend to reduce phenotypic differences in a structured population, reflecting the lack of genomic isolation (Nuismer et al. 1999).

Given even subtle sex bias in dispersal, monophyly of mtDNA loci by itself is a poor indicator of genetic isolation, with high false positives that rise most acutely with small ancestral population size. Small ancestral populations may be common through founding events between habitat islands, and more generally among tropical species (Lee 2002; Leaché and Fujita 2010). The small population size of ancestral populations may even lie behind the high false-positive rates in recent applications of barcoding in tropical species (Dasmahapatra et al. 2010a). Again, if natural history (e.g., biogeography, current population sizes) suggests that ancestral populations would have been small, the sensitivity of tree-based single-locus species delimitation needs to be evaluated.

Conversely, when population sizes were large ($\geq 100,000$ individuals), the combination of mitochondrial distances and monophyly systematically incurred false-negative error rates above 20% with $\leq 100,000$ years of isolation. Arguably, genetic isolation resulting in speciation in 10,000 years or less is probably rare, and might only occur under strong selection (Machado and Hey 2003; Orr et al. 2004). Nonetheless, it is important to note this lower limit because our results indicate

that hypotheses linking speciation to the allopatric isolation of Pleistocene refugia over the last 50,000 years will lack support from mitochondrial distances and monophyly in the vast majority of cases (Figs. 4 and 5). Several empirical studies have shown this result before (e.g., Carnaval and Bates 2007; Martins et al. 2009; Russell et al. 2011), and our results imply that even order-of-magnitude increases in mitochondrial mutation rates are unable to overcome the lower limit on reciprocal monophyly in mitochondrial markers arising from large effective population sizes and recent divergences (Fig. 5). Only small ancestral effective population sizes were consistent with mitochondrial markers evolving reciprocal monophyly in less than 100,000 years (Fig. 5).

On the basis of our results, we advocate a hypothesis-testing approach to species delimitation using molecular sequences. Species delimitation must start with a hypothesis about the extent of historical and current genetic isolation, with no genetic isolation as the null hypothesis (Fig. 6). The demographic parameters of the population together with the proposed evolutionary trajectory of putative populations provide alternative scenarios modeled using the coalescent. The statistical power of the tests should be modeled to determine whether the extent of sampling allows for testing the speciation hypothesis (Knowles and Carstens 2007; Rosenberg 2007; Ross et al. 2008; Zhang et al. 2010). Explicit tests of speciation scenarios will require estimating more parameters than just distances between and distance-based phylogeny of the putative populations. Instead, a suite of statistics describing both within- and between-population genetic diversity (e.g., nucleotide diversity [π], number of segregating sites [S], number of singleton mutations [η], distances, reciprocal monophyly, and F_{st} or fixation index) must be collected (Beaumont et al. 2002). Many powerful new pipelines to automate simulations and collect summary statistics in demographic scenarios have recently become available (Hickerson et al. 2007; Cornuet et al. 2008; Lopes et al. 2009; Wegmann et al. 2009), and can be readily deployed to test proposed species limits. If there is sufficient power given the genetic data, and the null is rejected, the hypothesis of genetic isolation is supported.

The simulations presented here did not exhaust the possible variation in demographic and evolutionary parameters expected under different mammalian speciation scenarios. Natural or sexual selection (Machado and Hey 2003; Orr et al. 2004) and postisolation changes in population size were not modeled (Russell et al. 2011). Nevertheless, by describing the parameter space of high error rates under simple demographic scenarios and a range of mutation rates, our simulations strongly caution against using distances and phylogenies based on single mitochondrial loci to indicate the presence or absence of gene flow.

Sequence-based species delimitation has rightly been moving away from descriptions of a few genetic parameters such as distances, to an integrative taxonomic approach that tests hypotheses grounded on natural history, including morphology, ecology, and behavior (Padiá and De la Riva

2010). The analyses presented here strongly argue for an integrative approach, particularly when male-biased dispersal is present, and when ancestral population sizes and proposed divergence times are either small or large (Figs. 2–5). Since in most cases researchers will not know a priori where their system lies along the distribution of population sizes and time since divergence, the hypothesis-testing approach should be the default in species delimitation. To minimize error rates, species delimitation requires hypothesis-driven approaches accounting for multiple data types (including morphology, ecology, and behavior), a range of divergence times, and criteria beyond sequence divergence and monophyly. Finally, our analyses raise doubts regarding the Last Glacial Maximum as a common geological mechanism for observed genetic isolation using vertebrate mitochondrial data: even with high mutation rates it is highly unlikely for this event to drive reciprocal monophyly in populations estimated to have $\geq 10,000$ individuals effectively contributing to reproduction.

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