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Author: Lovette, Irby J.

Source: The Auk, 121(1) : 1-6

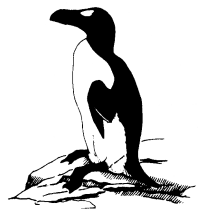
Published By: American Ornithological Society

URL: https://doi.org/10.1642/0004-8038(2004)121[0001:MDAMSF]2.0.CO;2

The Auk

A Quarterly
Journal of Ornithology

Vol. 121 No. 1 January 2004



The Auk 121(1):1–6, 2004

PERSPECTIVES IN ORNITHOLOGY

MITOCHONDRIAL DATING AND MIXED SUPPORT FOR THE "2% RULE" IN BIRDS

Irby J. Lovette[1]

Evolutionary Biology Program, Cornell Laboratory of Ornithology, and Department of Ecology and Evolutionary Biology, Cornell University, 159 Sapsucker Woods Road, Ithaca, New York 14850, USA

The use of a molecular clock to date mitochondrial lineages is based on the assumption that mutations in the mitochondrial genome (mtDNA) accumulate over time at rates that can be calibrated against absolute dates derived from fossils or geological evidence. Mitochondrial DNA (mtDNA) has a higher mutation rate than most nuclear DNA, and mtDNA has long served as the most informative marker for inferring relationships among avian species and populations. By allowing the absolute ages of those lineages to be estimated, mitochondrial clocks have had a revolutionary influence on our understanding of the pace of bird diversification, patterns of historical avian biogeography, and rates of morphological and behavioral change.

A partial list of insights based on mitochondrial dating includes refutation of the hypothesis that late Pleistocene glacial cycles caused widespread speciation in north temperate avifaunas (e.g. Bermingham et al. 1992, Zink and Slowinski 1995, Klicka and Zink 1997); rejection of the Pleistocene refugia theory for the high species diversity of Amazonia (e.g. Bates et al. 1999); documentation of disparate rates of diversification in avian communities or clades (e.g. Lovette and Bermingham 1999, Sato et al. 1999, Voelker 1999, Ricklefs and Bermingham 2001); recognition that rates of morphological, behavioral, and ecological change may vary dramatically among avian lineages (e.g. Omland and Lanyon 2000, Price et al. 2000, Lovette et al. 2001, Burns et al. 2002); and reconstruction of historical changes in population size and other demographic parameters related to environmental change (e.g. Milot et al. 2000, Ruegg and Smith 2002).

Many of those conclusions depend critically on dates derived from measurements of mitochondrial divergence, which in turn depend on calibrations of the rate of change in mitochondrial DNA. There is currently widespread use of an avian mitochondrial clock that is thought to "tick" at a mean rate of ~2% sequence divergence per million years, a rate very similar to that reported for various mammalian groups. This avian mitochondrial clock is now employed so broadly that papers in the ornithological and phylogenetic literature frequently refer to the "standard avian calibration" or "widely accepted" rate of ~2% Ma^{-1}.

Given the prevalence of this "2% rule," it is worth asking, What is the origin and support for this standard avian mitochondrial clock calibration? Avian mitochondrial clock calibrations have received surprisingly little critical attention, especially compared to the ongoing debate about avian nuclear-locus clocks that are used

[1]Email: ijl2@cornell.edu

to date much earlier nodes (e.g. among families and orders) in the avian tree (e.g. van Tuinen and Hedges 2001, Feduccia 2003). Here I review the small number of avian mitochondrial clock calibrations, discuss their power and potential limitations, and identify some measures that can be taken to improve their utility.

Mitochondrial clocks were first used to estimate the time of divergence among humans, chimpanzees, and gorillas (Brown 1979); and they have since been applied to hundreds of organisms. Most of their potential biases are not specific to birds and have been reviewed extensively elsewhere (see Arbogast et al. 2002 for a comprehensive recent treatment of those issues). Potential sources of error include underlying rate differences between evolutionary lineages and among genetic loci, difficulty of accounting for saturation (successive changes at single nucleotide sites) in calculating genetic differences, and potential for overestimating the time since two lineages split if their shared ancestral population itself contained substantial mitochondrial diversity. Even with a perfect rate calibration, this combination of issues suggests that mitochondrial clock dates are usually associated with high (if difficult to estimate) error terms, and these errors will increase when the evolutionary nodes being dated are temporally distant from the nodes used in the rate calibration (Arbogast et al. 2002).

The mitochondrial clock issues specific to birds involve the calibrations of the rate of avian mitochondrial DNA evolution against absolute dates derived from fossil or geological data. Unfortunately, opportunities to perform those rate calibrations are rare, especially given the relatively poor fossil record of most avian lineages. There have correspondingly been mitochondrial clock calibrations for only a handful of avian groups. Although most of the available avian mtDNA rate calibrations cluster around 2% Ma^{-1}, a review of those calibrations demonstrates that there is substantial variance around that value both within and across studies. Furthermore, the total number of calibrations is small, and all such studies are subject to many potential sources of error. Users of these calibrations should therefore be aware that for any particular clade of birds, the 2% rule could be a substantial over- or underestimate of the actual rate of mitochondrial change.

The 2% rule applies only to protein-coding mitochondrial DNA markers, and the calibrations summarized below all address rates of change in protein-coding mtDNA (almost exclusively the cytochrome-*b* gene). A few similar calibrations have been generated for the non-protein-coding mitochondrial control region (Quinn 1992, Baker and Marshall 1997, Drovetski 2003), which contains several subregions that often evolve at a substantially faster rate than protein-coding markers (but not always; Zink and Blackwell 1998, Ruokonen and Kvist 2002, Zink and Weckstein 2003). To date, mitochondrial rate calibrations have been generated (in chronological order) for geese, Hawaiian honeycreepers, cranes, partridges, procellariiform seabirds, and ratites.

Avian mtDNA Rate Calibrations

Geese.—Shields and Wilson (1987) generated one of the first mitochondrial clock calibrations for birds based on comparisons of divergence between the genera *Anser* and *Branta*. Their study was conducted before DNA sequencing was commonplace and was based on divergences estimated from mitochondrial restriction fragment length polymorphisms (RFLPs). From fossil evidence, they assumed that *Branta* and *Anser* last shared a common ancestor 5 mya. The RFLP-based mitochondrial divergences between those genera varied between 7.8% and 9.9%, giving a rate calibration of 1.56% to 1.98% Ma^{-1}, or the more commonly cited "~2% Ma^{-1}" (Shields and Wilson 1987). Paxinos et al. (2002) recently revisited this calibration using mtDNA sequence data, a 4.5 mya calibration date for the *Branta–Anser* split, and modern models of mtDNA evolution, and estimated a similar mean substitution rate of 2.1% for the cytochrome-*b* and ATPase8 genes.

Hawaiian honeycreepers.—Avian mitochondrial clocks have been used most extensively in studies of passerine birds. But perhaps because passerines are poorly represented in the fossil record and are often difficult to identify to species when they do occur, rate calibrations have been conducted for only one passerine clade. The ingenious set of studies by Robert Fleischer and colleagues has employed calibration dates derived from the dates of emergence of the volcanic Hawaiian Islands. Tarr and Fleischer (1993) first calibrated mitochondrial substitution rates as part of their investigation

of phylogenetic relationships in the Hawaiian honeycreeper genus *Hemignathus*. Taxa in this "amakihi complex" are known from all of the large Hawaiian islands and two species are present on Hawaii and Kauai. Tarr and Fleischer (1993) estimated both inter-island divergence and within-island diversity via mitochondrial RFLP. Their calibrations of mtDNA substitution rates were based on the geological ages of the most recently formed islands (Hawaii and Maui), which place upper bounds on the potential ages of the bird populations found on them and hence lower bounds on their rates of mitochondrial evolution. Several potential variables complicated this calibration: the ancestral sources of immigrants to Hawaii and Maui were equivocal, the amahiki populations on Hawaii and Maui could each be considerably younger than those islands, and the presence of within-population polymorphism in several of the extant populations demonstrated that substantial mitochondrial divergence could arise in an ancestral population prior to a colonization event. Depending on the assumptions made to minimize those potential biases, Tarr and Fleischer (1993) generated minimum rate calibrations of 2.0%, 2.4%, 2.7%, and 5.0% Ma^{-1}, but felt their most reliable estimate of the minimum substitution rate was likely to be "~2% Ma^{-1}."

Fleischer et al. (1998) subsequently used partial cytochrome-*b* sequences from the amakihi complex and the Maui Alavahio (*Paroreomyza montana*) and Akikiki (*Oreomystis bairdii*) to generate three calibration points, at approximately 0.5, 1.5, and 3.75 mya. In a careful analysis that included gamma-corrected divergence estimates and controls for intrapopulation variation, they found that those calibration points supported a linear substitution rate of 1.6% to 1.9% Ma^{-1}, depending on the distance metric employed.

Cranes.—Krajewski and King (1996) used cytochrome-*b* sequence data from all extant crane species to conduct a nested set of four rate calibrations that spanned a wide range of evolutionary divergence (among subspecies, sister species, species groups, and subfamilies), estimated times of divergence (0.5–20 Ma), and levels of sequence differentiation (1% to 15%). For each comparison, calibration dates were estimated from the fossil record, and pairwise nucleotide divergences were calculated from the associated cytochrome-*b* sequences. Calibrations based on the midpoint of the range of fossil dates and on the mean pairwise distance for each lineage produced estimates of 1.5% Ma^{-1} among gruine subspecies, 1.5% Ma^{-1} among gruine sister species, 1.3% Ma^{-1} among gruine species groups, and 0.9% Ma^{-1} between the gruine and balearcine subfamilies. However, the range of estimates that incorporate both the range in the estimated fossil dates and the variation in pairwise divergences within each category are much more variable, spanning 0.6% to 4.1% Ma^{-1}.

Procellariiforms.—Nunn et al. (1996) calibrated the rate of cytochrome-*b* sequence evolution as part of a phylogenetic study of albatrosses. Calibration points were provided by fossil evidence, which suggests that *Phoebastria* and *Diomedea* split ~15 mya and that *Thalassarche* and *Pheobetria* split ~10 mya, resulting in rate estimates of 1.58% Ma^{-1} for *Phoebastria–Diomedea* and 2.86% Ma^{-1} for *Thalassarche–Pheobetria*. Although those rates are superficially similar to (and have been frequently cited in support of) the standard 2% Ma^{-1} value, Nunn et al. (1996) considered only changes at third codon positions in performing those calibrations. Because third codon position sites are much more variable than first or second position sites, rates of change at third positions will be much greater than rates calculated across all codon positions.

Nunn and Stanley (1998) used an expanded cytochrome-*b* data set to perform three additional rate calibrations in which they divided the maximum sequence divergence within the Diomedeidae, Procellariidae, and Oceanitinae by the date of the first fossil appearance of each of those clades. They found uncorrected rates of 0.62%, 0.78%, and 0.92% Ma^{-1}, respectively, using uncorrected distances, and 0.88%, 0.90%, and 1.29% using distances calculated with Kimura's two-parameter distance metric. These apparent rate differences among taxa were correlated with the body sizes of those groups (Nunn and Stanley 1998).

Partridges.—Randi (1996) examined phylogenetic relationships among seven species of *Alectoris* partridges, using complete cytochrome-*b* sequences. He calibrated an absolute rate of sequence divergence by assuming that the ancestral *Alectoris* lineage separated from the *Gallus* lineage at 20 mya. Randi based his rate calibration only on third-position transversion substitutions, and hence, like the Nunn et

al. (1996) calibration, it is not directly comparable to the other published cytochrome-*b* calibrations. He estimated a rate of 2.2 third-position transversions per million years for the 1,143 bp cytochrome-*b* gene, which corresponds to a rate of 0.19% Ma^{-1} for this class of substitutions alone. Assuming his empirical average transition–transversion ratio of 6.6 (Randi 1996), the overall rate of divergence is ~1.4% Ma^{-1}.

Ratites.—Using nearly the entire protein-coding mtDNA genome from a number of extant and extinct ratite lineages, Cooper et al. (2001) based a rate calibration on the assumption of a split between the moas and other ratites associated with the geological isolation of New Zealand at 82–85 mya. On the basis of that Cretaceous vicariant event, they report a resulting estimate of mean substitution rate of 0.27% Ma^{-1}. Although that value is appropriate for comparison to other ratite lineages of equivalent age, its ancient calibration point is likely to incur severe saturation biases that will be difficult to correct, and its extension to much more recent divergences is difficult.

Prospects for Improved mtDNA Clock Calibrations

Despite the lack of strong support for the 2% rule, mitochondrial clock calibrations will continue to play a central role in interpreting the recent evolutionary history of birds. What can be done to improve the support for the avian mitochondrial clock and to increase the utility of the existing panel of rate calibrations?

Encourage new rate calibrations.—There have been notably few new rate calibrations since the late 1990s, perhaps because the avian phylogenetics community considers this a largely resolved issue. Each new estimate, however, is a highly valuable contribution given the heterogeneity of the small number of previous calibrations. Furthermore, rates of molecular evolution apparently differ across avian groups (e.g. Nunn and Stanley 1998), and therefore it is best to employ calibrations derived from lineages allied as closely as possible to the group under investigation. This problem is particularly acute for the passerines, for which we have rate calibrations for only a single clade that represents a few dozen of the more than 5,700 extant species.

In seeking calibration opportunities, avian molecular phylogeneticists could almost certainly be more proactive in seeking input from our paleontologist colleagues to identify new calibration points supported by the fossil record. Additional opportunities to perform calibrations in the absence of fossils could come from other oceanic islands with known dates of emergence (e.g. Barbados; Lovette et al. 1999), from vicariant events or the opening of dispersal corridors of known age (e.g. the closure of the Central American land-bridge at ~3 mya or the isolation of various land-bridge islands by rising Pleistocene sea levels), and from "molecular fossil" mitochondrial pseudogene sequences that are preserved in the nuclear genome (Bensasson et al. 2001).

Revisit existing calibrations with new DNA data.—As noted above, the initial goose, honeycreeper, and procellariiform calibrations have been recalculated using DNA sequence data with a higher information content and more sophisticated evolutionary models. This is an important undertaking that greatly improves the comparability of those calibrations to other sequence-based data sets. Additional power could come from increasing the nucleotide sampling in these and other calibrations, as an increase in the amount of sequence being compared reduces the error associated with estimates of sequence divergence. A second reason to expand nucleotide sampling is that many avian studies are now employing mtDNA genes in addition to cytochrome *b*, and these additional markers may have somewhat different rates of change.

Revisit existing calibrations with new analytical methods.—In recent years there has been constant improvement in models of the DNA substitution process. It is now possible, for example, to conduct rate calibrations for clades with heterogeneous underlying substitution rates. Methods for correcting for saturation effects have similarly become increasingly sophisticated; this is especially important for the majority of calibration points that involve relatively deep divergence points (especially those >8% to 10% pairwise divergence). Use of these improved techniques can have large effects on rate calibrations; for example, Arbogast and Slowinski (1998) reanalyzed Randi's (1996) data on partridges using a gamma-corrected substitution model and found a 2-fold increase in the apparent substitution rate. As molecular

studies increasingly employ these more sophisticated methods, the previously published clock calibrations will become less directly applicable, requiring the recalculation of calibrations using concordant distance metrics.

In summary, although the 2% rule for avian mitochondrial evolution is in widespread use, this standard rate is supported by only a small number of calibration studies that show substantial heterogeneity. Given the many important conclusions that rest upon dates derived from mitochondrial data, expanding and refining our understanding of rates of mitochondrial evolution should be a high priority for the users of avian mitochondrial clocks.

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