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CHAPTER 2

MOLECULAR ADVANCES IN THE STUDY OF GEOGRAPHIC VARIATION AND SPECIATION IN BIRDS

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Abstract.—Problems in deciphering the patterns and causes of geographic variation and speciation in birds occupied Ned Johnson (e.g., Johnson 1980, Cicero and Johnson 1998, Johnson and Cicero 2002) and many other ornithologists for much of their lives, but the recent onslaught of molecular studies and associated analytical methods are providing breakthroughs in understanding these evolutionary phenomena. In particular, coalescent theory and Markov chain Monte Carlo (MCMC) applications have shown that bird species are sometimes strongly structured into well-differentiated populations by historical subdivision, high philopatry, and small effective population sizes, whereas other species that have recently recolonized parts of their range are effectively panmictic. These are the sorts of results that were impossible to obtain from studies of geographic variation in phenotypic characters alone. Recovery of well-supported species trees from gene trees is much more likely when multiple genes are sequenced, and provides the means for inferring divergence times and patterns and processes of evolution in birds. As in other vertebrates, patterns of cladogenesis in large clades of birds correlate with major paleoenvironmental changes and associated adaptive radiations, reminding us that much of current biodiversity on the planet had its genesis in the distant past.
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Resumen.—Ornitólogos como Ned Johnson, entre muchos otros, han dedicado gran parte de sus carreras a analizar las causas y patrones en la variación geográfica y la especiación en aves (e.g. Johnson 1980, Cicero and Johnson 1998, Johnson and Cicero 2002). Recientemente, los estudios moleculares y los métodos analíticos asociados han provisto de las herramientas necesarias para entender estos fenómenos evolutivos. En particular, el uso de la teoría de coalescencia y los algoritmos asociados a las cadenas de Markov Monte Carlo han demostrado que algunas especies de aves presentan una estructura poblacional muy marcada, producto de subdiviciones históricas, filopatría y tamaños poblacionales efectivos reducidos, mientras que otras especies que recientemente han recolonizado una parte de su área de distribución son panmíticas. Estos resultados son difíciles de obtener estudiando únicamente la variación geográfica de caracteres fenotípicos. Al igual que en otros vertebrados, los patrones de cladogénesis en grandes clados de aves se correlacionan con eventos paleoambientales y radiaciones adaptativas asociadas, recordándonos que gran parte de la biodiversidad del planeta tuvo su génesis en un pasado lejano.

Ned Johnson had a consuming interest in the study of geographic variation and its connection to speciation in birds and was a strong advocate for the biological species concept (e.g., Johnson et al. 1999). He and his students and postdocs embraced new methods of detecting and quantifying variation within and among species as the way to make groundbreaking progress in this ever-evolving field and, thus, his contributions were many and varied. A landmark paper from Johnson's laboratory on the nature of genic variation in birds established the importance of neutrality of molecular markers in analyzing genetic structure in avian species (Barrowclough et al. 1985). Subsequent use of putatively neutral markers elucidated some of the major forces acting on avian populations, including founder effects and bottlenecks, genetic drift, gene flow, and geographic

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isolation (e.g., Baker and Moeed 1987, Wenink et al. 1996, Clegg et al. 2002). Although ornithologists have used molecular methods to elucidate the timing, geography and ecology of speciation, the molecular and behavioral mechanisms of speciation await thorough investigation in birds (for an excellent review, see Edwards et al. 2005). The roles of genetic incompatibilities and behavioral mechanisms in speciation, including sexual selection, song learning, imprinting, and reinforcement, are currently hot topics (e.g., Irwin et al. 2001, Sorenson et al. 2003).

As a tribute to the achievements of Johnson and his collaborators and the impact they have had on the field, it is appropriate to highlight some of the advances being made in the study of population structure and modes of speciation in birds, especially with respect to molecular tools and their applications. I have focused on a very selective set of topics, with exemplars mainly from my own laboratory. To make some specific points, I outline a few examples of molecular population structure in bird species, discuss the roles of isolation and philopatry in geographic variation and speciation, and point out the role of ancient paleoenvironmental changes in generating species radiations that are reflected in extant avian biodiversity.

Population Genetic Structure in Birds

A Need for Multiple Markers

Although it has long been believed that populations of most bird species may be panmictic or only weakly structured (e.g., Zink 1997) because of homogenizing gene flow mediated by the dispersal power of flight, molecular assays have revealed increasingly that many widely distributed populations are subdivided (e.g., Wenink et al. 1996, Irwin et al. 2001, Griswold and Baker 2002, Zink 2004, Baker et al. 2005, Brito 2005). Use of DNA-sequence data sets has not only provided genetic markers for detecting population structure in avian species, but has also catalyzed analytical advances via mathematical models (e.g., coalescent theory) that give exact probabilities of the data as a function of the parameters that underlie population history (Hein et al. 2004).

This approach is illustrated below for an ancestral population that split into two populations, some time in the past, which potentially exchange migrants (Nielsen and Wakeley 2001). Control-region (CR) sequences of 403 base pairs (bp) (Baker et al. unpubl. data) obtained from samples of two putative subspecies of the Purple Martin from east (*Progne subis subis*; n = 71) and west (*P. s. arboricola*; n = 50) of the Rocky Mountains were analyzed using the program MDIV (Nielsen and Wakeley 2001), which implements this basic non-equilibrium model. Modal values of the population mutation parameter (θ = 5.95 substitutions locus^{-1}), time of population divergence (*tpop* = 2.72), time to the most recent common ancestor (TMRCA) and scaled migration rate (M = 0.13) were obtained from the posterior distributions generated by the MCMC procedure after 2 million generations and a burn-in period of 500,000 generations (Fig. 1). To convert these values to time in years, we estimated generation time using the equation (Sæther et al. 2005) $g = \alpha \times (s/1 - s)$, where α is age at first breeding and s is annual

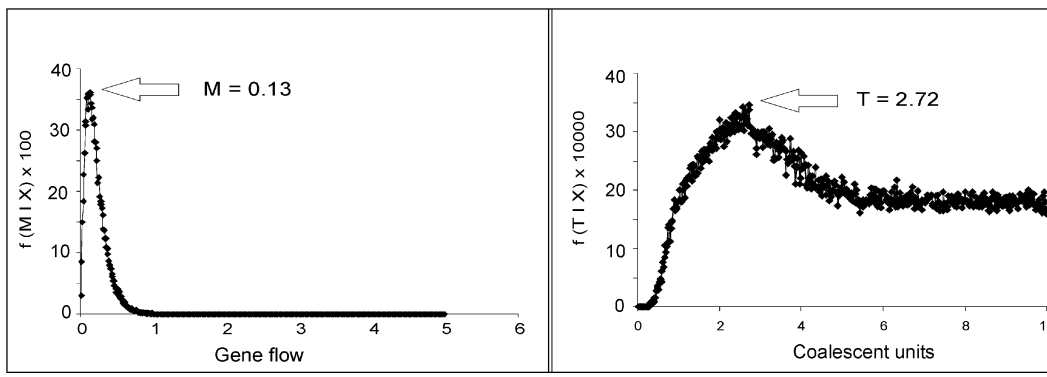

Fig. 1. Posterior distributions of scaled migration rate (M) and population divergence time (*Tpop*) generated in MDIV for eastern and western subspecies of Purple Martins.

survival (0.7). This yielded a generation time of 3.3 years for Purple Martins. We also estimated mutation rate (μ) for the CR sequences using a range of calculated mutation rates for the CR in birds (5%, 10%, and 20% per million years; Brito 2005) because a specific rate for Purple Martins is not available. For example, a rate of 10% per million years converts to 10^{-7} substitutions per site per year and a per-gene rate of $3.3 \times 403 \times 10^{-7}$ substitutions per generation. Population divergence time can be calculated with the expression $([tpop \times \theta]/2) \times 1/\mu = 60{,}800$ generations, or ~200,400 years before present (ybp). Similarly, mutation rates of 5% per million years and 20% per million years translate to a range of about 400,000 to 100,000 ybp for the population splitting time. Using a mutation rate of 10% per million years, for example, the TMRCA of the CR sequences in the gene tree is 3.16, which translates to ~230,000 ybp. Because the TMRCA can be used to approximate the age of the ancestral population, we can infer that migration between the two descendant subspecies populations probably ceased shortly after their historical separation.

However, mitochondrial DNA (mtDNA) is only one gene and, thus, the 95% confidence intervals on demographic parameters are substantial. Recently, major concerns have been raised about inferences of historical demography based solely on this genome, owing to its unusual evolutionary rules and the influence of selection (Ballard and Whitlock 2004, Bazin et al. 2006). For example, the discovery of low mtDNA diversity and higher nuclear-DNA diversity in warblers of the genus *Phylloscopus* suggests that maternally inherited genes were affected by a selective sweep (Bensch et al. 2006).

One solution to these potential problems is to assay molecular variation at multiple unlinked nuclear loci to provide independent estimates of the within-species genealogy. This enables checking of inferences made from mtDNA sequences alone and provides reduced confidence intervals around demographic parameters. Even a comparison of phylogeographic patterns based on mtDNA sequences versus microsatellites often can be instructive, as for example in the Brown Kiwi (*Apteryx australis*) in New Zealand (Burbidge et al. 2003; see Fig. 2). At least in this case, the genealogies appear to be very similar, and thus the CR sequences and the nine microsatellite loci could be combined in a coalescent program such as IM (Hey and Nielsen 2004) to improve the inference of demographic parameters for South Island versus Stewart Island. A recent innovative example in Jennings and Edwards (2005) showed how a multilocus coalescent approach can make advances in understanding the evolutionary connection between geographic variation and speciation. They analyzed variation in 30 anonymous nuclear loci of three closely related species of Australian grass-finches (*Poephila* spp.) and, despite different topologies and coalescent times for each gene tree, they had enough information to estimate population divergence times and ancestral population sizes.

Determining the Number of Populations

The advent of molecular analyses of samples collected over large stretches of geography have forced investigators to confront again the problem faced earlier by ornithologists studying geographic variation in classical phenotypic traits: how do we determine the number of populations represented by a number of samples? Often, the conventional solution to this problem has been to use the sampling locale as the "population" for analysis, but newer methods, such as those in the programs BAPS (Corander et al. 2003) and STRUCTURE (Pritchard et al. 2000), can infer population structure using multilocus data. For example, Given (2004) assayed variation in 10 microsatellite loci from samples of Silver Gulls (*Larus novaehollandiae*) collected at nine locales in mainland New Zealand ($n = 224$) and one locale on sub-Antarctic Campbell Island ($n = 24$). Analysis with both clustering methods placed all New Zealand samples in one population and Campbell Island in another population (Fig. 3). Nevertheless, distance between sampled colonies and their genetic differentiation (*Fst*) were positively correlated (Mantel test; $Z = -209.18$, $r^2 = 0.46$, $P = 0.004$; Given 2004). This indicates that gene flow between colonies fits an isolation-by-distance model and is sufficient to prevent significant population structure in the New Zealand mainland but not between New Zealand and geographically remote Campbell Island.

Caution is warranted, however, in using multilocus data to interpret the biological meaning of statistically significant population structure in birds, as illustrated in the analysis of 157

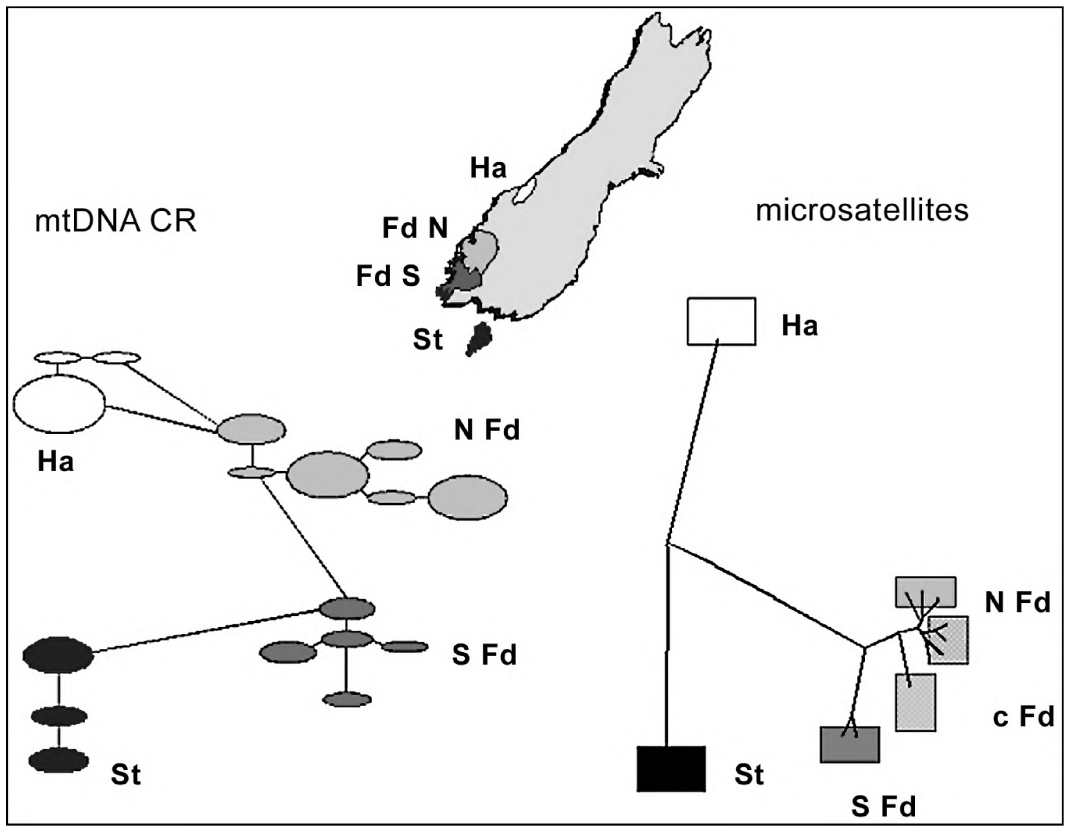

FIG. 2. Genetic divergence in mtDNA CR sequences and nine microsatellite loci among four regional populations of *Apteryx australis* in the South Island of New Zealand (Burbidge et al. unpubl. data).

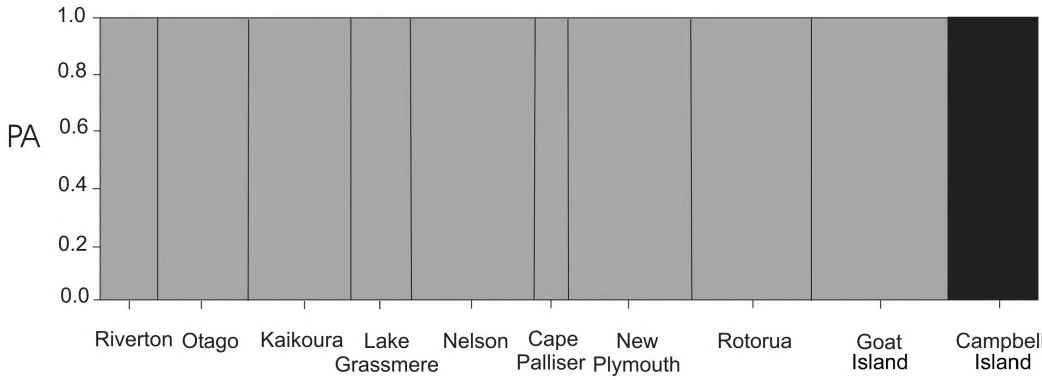

FIG. 3. Identification of populations of *Larus novaehollandiae* in the New Zealand region based on the estimated proportion of ancestry (PA) of individuals using 10 microsatellite loci with the program STRUCTURE. From Given (2004).

polymorphic nuclear loci identified by amplified fragment-length polymorphisms in populations of House Finches (*Carpodacus mexicanus frontalis*) in North America (Wang et al. 2003). Analysis of this data set with STRUCTURE diagnosed three almost completely nonoverlapping populations that might be assigned naively to three species under a version of the phylogenetic species concept, one in their native range in the western United States and two others introduced separately to Hawaii and the eastern United States within the past 100 years. Instead, this example indicates that significant shifts in allele frequencies can occur rapidly through genetic drift and selection.

Geographic Variation and Speciation

When pronounced patterns of geographic variation are detected in samples, it is often difficult to decide whether they represent population structure within a species or whether they are sufficiently discrete to be considered different species. This is especially true in analyzing patterns of morphological variation and is further accentuated in extinct organisms, for which it is impossible to judge the degree of genetic incompatibility among well-differentiated samples. A telling example is provided by the extinct moas of New Zealand, which are known from a large number of subfossil remains recovered from swamps, caves, and middens. The complex nature of morphological variation led to the naming of at least 64 species and 20 genera of moa by early workers, which was whittled down to 11 species in 6 genera in the 1990 *Checklist of New Zealand Birds* (Turbott 1990). However, when it proved possible to amplify nuclear genes for molecular sexing from ancient DNA extracted from within the cortex of fossil bones, an unexpectedly large degree of sexual dimorphism was discovered in most genera, with smaller bones belonging to males and larger ones to females (with some overlap at intermediate sizes; Bunce et al. 2003, Huynen et al. 2003). Males and females from one species of the giant *Dinornis* had been split into two species using bone size alone, because they differed in size by up to 50%.

To test the prevailing taxonomy and to attempt to partition morphological variation into geographic variation within versus among species, 125 specimens in museum collections were typed genetically with a 658 bp mtDNA CR sequence (Baker et al. 2005). Bayesian analysis of these sequences recovered 14 monophyletic lineages, 9 of which are currently recognized, plus 5 new lineages that may warrant species status. When exemplars of these 14 lineages were sequenced for a total of 2,814 bp from 10 mtDNA genes (including the CR sequences), a strongly supported phylogeny was derived. Molecular dating with a relaxed molecular clock to account for evolutionary-rate variation among lineages indicated that the most recent common ancestor of moas can be traced back to just after the Oligocene "drowning" of New Zealand, when previous populations likely underwent severe reductions. Phylogeographic patterns in gene trees can be highly informative with respect to the mode of speciation, because they usually bear the signature of the geographic and population-demographic or selective events that accompany speciation (Avise 2000). Much of the cladogenesis in the CR gene tree is concentrated in the period around 4–10 mya, which corresponds to the time when the landmass was fragmented by tectonic and mountain-building events as well as a globally cooling climate (Fig. 4). Lineages therefore became isolated geographically and probably speciated allopatrically with concomitant ecological specialization.

The discovery of the moa lineages using ancient DNA provided an opportunity to test the efficacy of DNA barcoding with the standard cytochrome oxidase I (COI) sequence. This technique has been criticized because it needs to be evaluated in a phylogenetic framework in the limiting case of sister-species comparisons (Moritz and Cicero 2004) or it fails on different clades of organisms (e.g., Meier et al. 2006) or in broad parameter space (Hickerson et al. 2006). Twelve of the 14 lineages that were known at the time of testing had DNA barcodes that differed by >2.7% (Lambert et al. 2005), which exceeds the 10× threshold for within-species differences calibrated for North American birds (Hebert et al. 2004). Subsequently, the two new lineages of *Dinornis* discovered in the South Island of New Zealand were found to have nearly identical COI sequences but were easily distinguished by their CR sequences and had been accorded separate taxonomic status in the past on the basis of skeletal differences. Given the recency of their divergence times, the slow rate of mtDNA evolution in moas, and the correlation done of

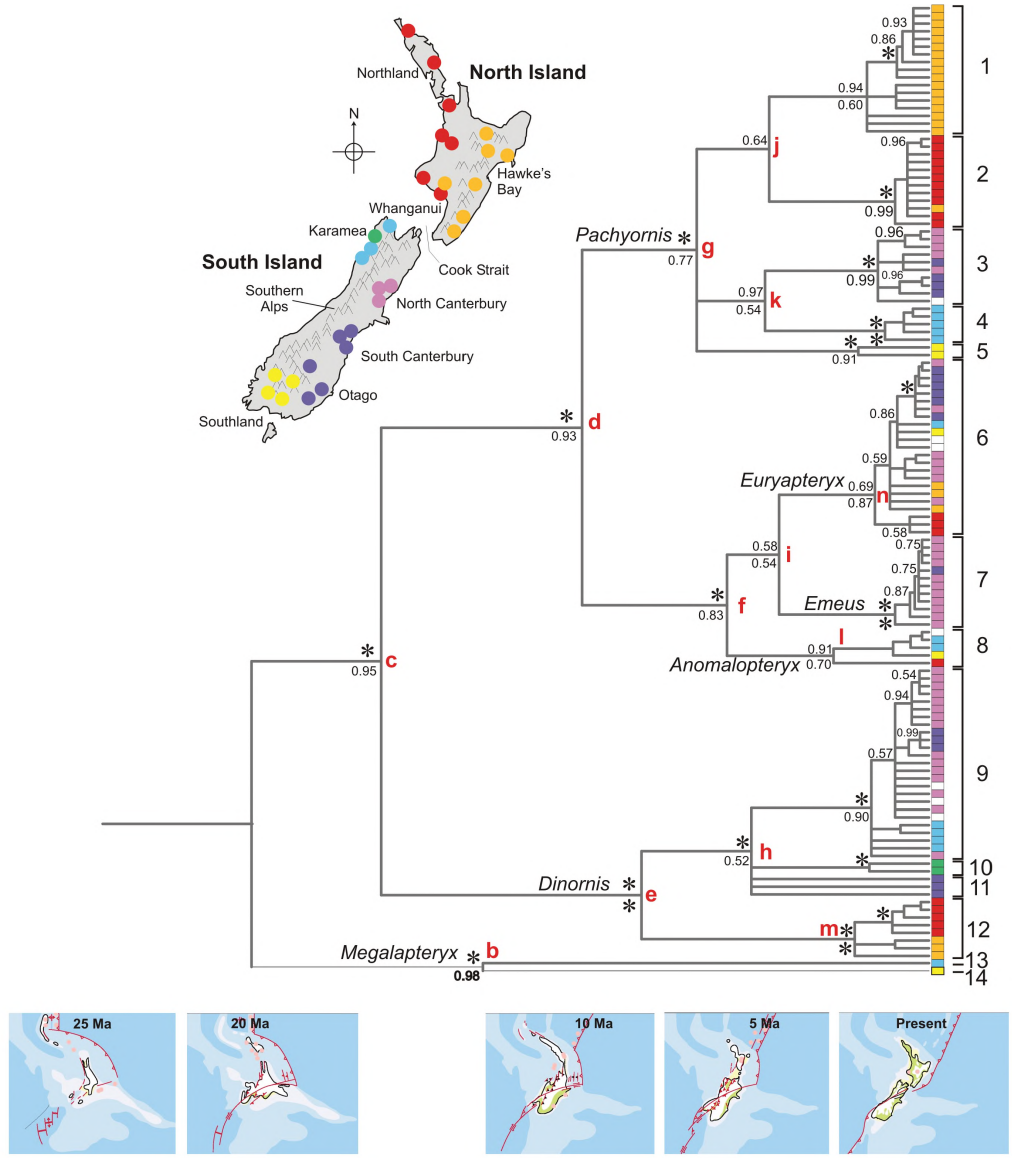

FIG. 4. Bayesian tree constructed with 658-bp CR sequences from 125 moa specimens under a GTR+I+G model of evolution. Numbers at the branch tips identify the 14 major lineages as follows: (1) *Pachyornis mappini*, (2) *P. n.sp.A*, (3) *P. elephantopus*, (4) *P. australis*, (5) *P. n.sp.B.*, (6) *Euryapteryx geranodies*, (7) *Emeus crassus*, (8) *Anomalopteryx didiformis*, (9) *Dinornis robustus*, (10) *D. n.sp.A*, (11) *D. n.sp.B*, (12) *D. novaezealandiae*, (13) *Megalapteryx didinus*, and (14) *M. benhami*. Specimens are color-coded according to geographic locations plotted along with place names on the map. From Baker et al. (2005). Samples of lineages (1) and (2) previously referred to *P. mappini* were used for coalescent modeling with the program IM.

percentage of divergence of COI barcodes with time of lineage-splitting (Tavares and Baker unpublished data), it may be more appropriate to barcode this clade of birds with the central conserved domain of the CR.

The geographic partitioning of the two lineages of *Pachyornis mappini* in the eastern (n = 17) and western (n = 12) regions of the North Island of New Zealand (Fig. 4) suggests that they were effectively isolated a long

time ago. To estimate their time of splitting, a coalescent analysis was run in IM (Hey and Nielsen 2004), which relaxes the assumption of equal population size in the ancestral and two daughter populations and of symmetric gene flow between populations inherent in MDIV. Two runs of IM were conducted, each for 7 million generations with priors for maximum effective population size (N_1 and N_2) and "population" splitting time (t) set to 30, and maximum migration rates (m_1 and m_2) between the two populations set to 10. Convergence of the four chains in the MCMC was monitored by autocorrelations (<0.03), and effective sample-size estimates (>235) were generated by the program for all the parameters. Similar estimates were obtained in both runs, with scaled population-size estimates for the two populations (q_1 = 56.95, q_2 = 24.45), and ancestral population size q_A = 26.99), coalescent time (t = 12.00), and migration rates into population 1 (m_1 = 0.005) and population 2 (m_2 = 0.005). To convert these parameter values to effective population sizes of females, time in years, and gene flow per generation, two estimates of mutation rate were used for the conserved central domain of the CR of these moas. Rates of molecular evolution in ratites are generally slower than in other birds (Pereira and Baker 2006), so it may be appropriate to use rates of 2.5% and 5% per million years as observed in some slower-evolving Neoaves (Table 1).

The low levels of migration and the potentially deep time of population splitting between these lineages indicate that they evolved in isolation, on opposite sides of the North Island separated by mountain barriers. Effective population sizes of females are moderate, and assuming a 1:1 sex ratio and that census population size was ~5× larger (as in the Brown Kiwis), the two populations of moa could have comprised 200,000–400,000 individuals. These rough estimates support the claim by Gemmell et al. (2004) that moa populations in New Zealand were large. However, demographic parameters estimated with the single CR locus also need to be estimated with multiple nuclear genes to improve confidence and to set narrower 90% bounds on the posterior distributions. For example, estimation of 90% highest posterior density interval (90% HPD) values for ancestral effective population size was not possible, but given the wide 90% HPD values for N_1 and N_2 that overlap the modal estimate for N_A, this suggests that modeling the moa sequences under constant population size (as was done in this example) is reasonable. Coalescent modeling of the sequences in DNAsp (Rozas et al. 2003) also failed to detect population growth (Fs = –2.52, P = 0.19).

Influence of Paleoevironmental Changes on Speciation in Birds

The blossoming of studies of variation in DNA sequences has not only clarified the nature of population structure in bird species but also has opened new vistas in avian evolution. In particular, phylogenetic studies of major clades of birds have shown that patterns of cladogenesis and speciation have their genesis in deep time (e.g., Haddrath and Baker 2001, Paton et al. 2002, Pereira and Baker 2006, Tavares et al. 2006) and, thus, may be connected causally to major paleoenvironmental changes in the past. This perspective has emerged because investigators have increasingly employed large DNA-sequence data sets from multiple genes from nuclear and mtDNA genomes, and also because advances have been made in fitting separate

Table 1. Estimates of demographic quantities and their 90% highest posterior density intervals (90% HPD) for two lineages of *P. mappini* using a per-locus mutation rate of 5% per million years. Halving this rate will double estimates of effective population sizes and population divergence time.

Demographic parameter	Mode	Lower 90% HPD	Upper 90% HPD
N_1	43,278	23,518	83,870
N_2	18,578	7,625	42,204
N_A	20,511	–	–
t (years)	364,589	131,762	902,279
$2N_1m_1 = \theta_1 m_1/2$	0.14	0.14	3.27
$2N_2m_2 = \theta_2 m_2/2$	0.06	0.06	2.88

models of sequence evolution to each gene partition or codon position. Bayesian methods (e.g., Ronquist and Hulsenbeck 2003) have speeded this transition and have facilitated the use of relaxed molecular clocks (e.g., Thorne and Kishino 2002) that accommodate variation in evolutionary rate among species and account for other major sources of phylogenetic uncertainty. This has allowed estimation of clade and species divergence times and their 95% confidence intervals, thereby enabling comparisons with the onset of well-documented paleoenvironmental changes.

Apart from the moa example outlined above, penguins and Neotropical parrots provide two additional examples of the influence of large-scale paleoenvironmental changes. A strongly supported phylogeny constructed from 2,802 bp of the nuclear gene RAG-1 and 2,899 bp of the mitochondrial genes 12S, 16S, cytochrome *b*, and COI was used to derive a Bayesian estimate of the ancestral area for modern penguins in Antarctica (Baker et al. 2006). To explain the current circumpolar distribution and restriction of penguins to the southern oceans, a chronogram was produced that depicts estimates of divergence times at the nodes and associated 95% credibility intervals (Fig. 5). Major global cooling events and the formation of ice sheets in Antarctica were shown to coincide with two bouts of cladogenesis in penguins. The first cooling period (about 34–25 mya) coincided with the divergence of three genera (*Spheniscus*, *Eudyptes*, and *Eudyptula*) with temperate-latitude distributions from older Antarctic genera. Their dispersal northwards out of Antarctica was probably via the newly formed circumpolar current. A second bout of diversification leading to the rise of multiple species of extant penguins dated from 12–14 mya at the time of the middle Miocene climate transition (MMCT), when surface waters in the southern oceans cooled further and ice volume in Antarctica increased again. Because the younger species resulting from this climate-induced expansion out of Antarctica are distributed today on isolated islands and the tips of southern continents, we can infer that they probably speciated allopatrically. Only the species in the genera *Aptenodytes* and *Pygoscelis* stayed in Antarctica and adapted to cooler conditions, but they too may have left the continent during glacial maxima (Baker et al. 2006).

A second example is nicely demonstrated by Neotropical parrot genera (Tavares et al. 2006). Using 6,388 bp from RAG-1 and seven mtDNA genes, they constructed a strongly supported phylogeny of 29 species in 25 of the 30 genera. Amazons and their allies were resolved as a sister clade to macaws, conures, and allies, which are jointly sister to parrotlets (Fig. 6). Molecular dating with relaxed clocks placed the divergence of Neotropical and Australian parrots between 51 and 66 mya, thus indicating that ancestral parrots were widespread in Gondwanaland before fragmentation by continental drift. The three major clades of Neotropical parrots were estimated to have diverged between 41 and 57 mya, but patterns of speciation are quite different within these clades.

Genera of amazons and allies diversified steadily between 46 and 16 mya, probably in South America, as forests expanded and contracted in response to global shifts in temperature and sea levels that periodically fragmented the biota. By contrast, macaws, conures, and their relatives radiated much later, beginning ~28 mya, when ecological speciation would have been mediated by the uplift of the Andes and by the formation of dry grassland habitats that they invaded from the forests. The two parrotlet genera may be ancient relicts that originated from an ancestral stock in Antarctica or the southern cone of South America and were driven northward by the formation of ice sheets in Antarctica.

Conclusions

The age of population genomics is just dawning, and we can look forward to many more developments in the near future as molecular technologies continue to advance. Already, new sequencing technologies have demonstrated how even highly fragmented ancient DNA in Neanderthals and mammoths can be retrieved and possibly used to reconstruct whole genomes (Green et al. 2006, Poinar et al. 2006). Genome-sequencing costs are likely to decrease significantly in the near future. This will enable targeted approaches on a multitude of genes, leading not only to much more reliable estimates of population divergence times, gene flow, and effective population size, but also a new age in phylogenomics and genetic mechanisms of speciation in birds.

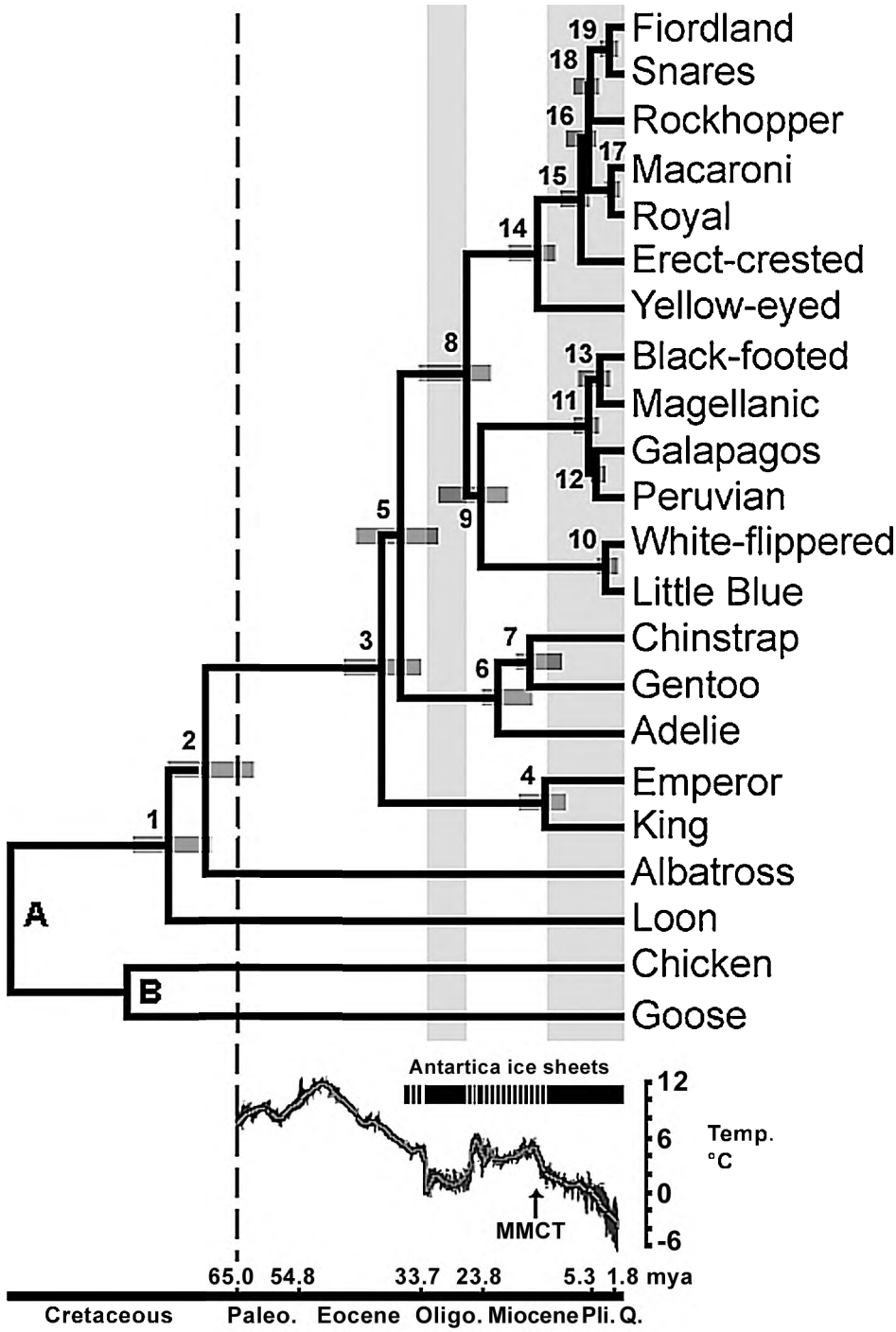

FIG. 5. Chronogram of penguin diversification from Baker et al. (2006). Nodes A and B were fixed at 104 and 90 mya. Gray bars at numbered internal nodes indicate credibility intervals (95%). Vertical dashed line indicates the Cretaceous–Tertiary boundary. Periods when Antarctica was ice-covered are projected as shaded gray rectangles in the chronogram. Ocean temperature is based on high-resolution deep-sea oxygen isotope records. The Middle Miocene Climate Transition (MMCT) is indicated by an arrow.

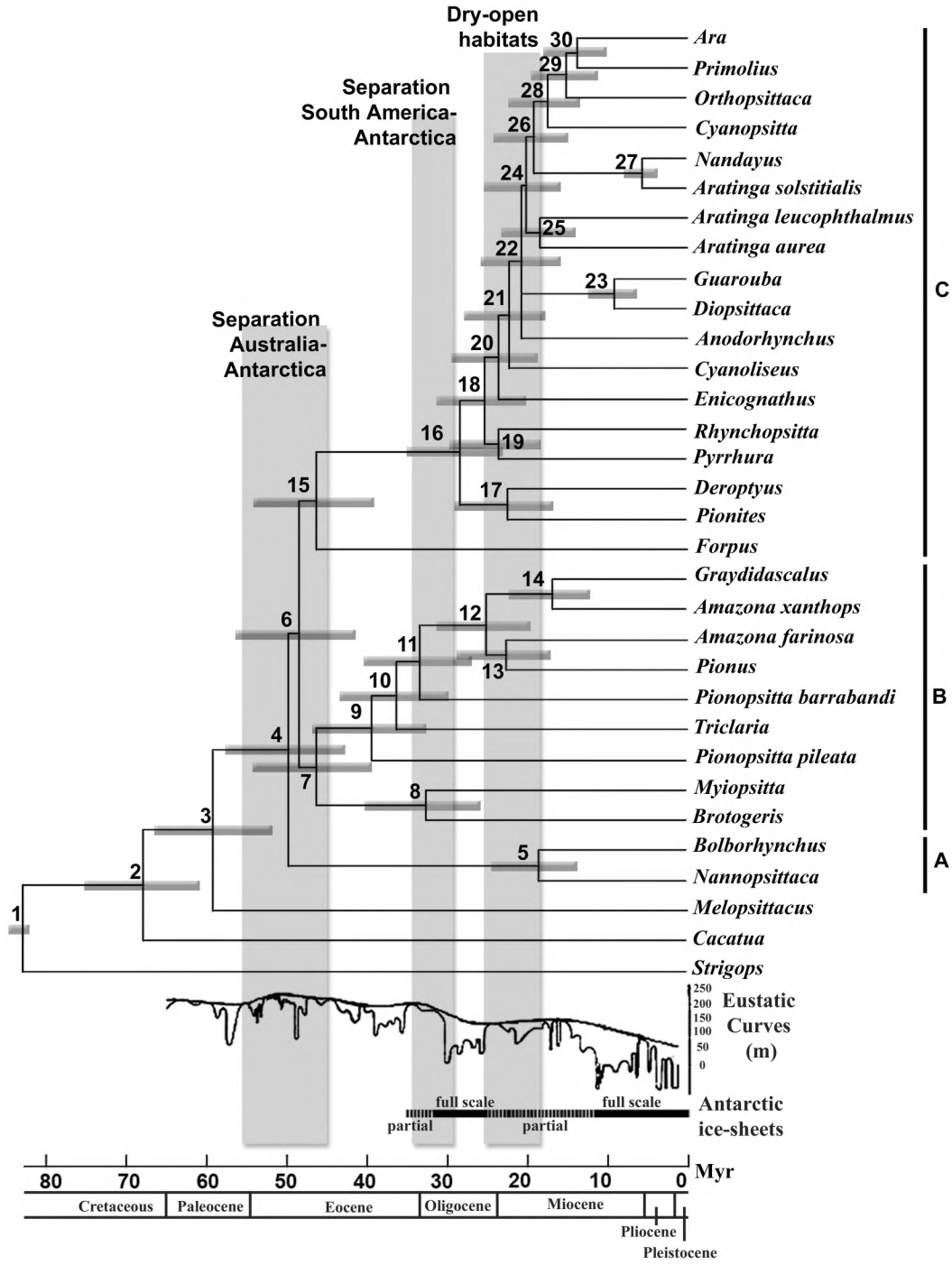

FIG. 6. Chronogram showing divergence times among the parrot genera and paleoevents possibly related to the Neotropical diversification. Numbers on nodes correspond to the estimated divergence times of lineages as given in table 3 of Tavares et al. (2006). Horizontal bars at nodes are 95% credibility intervals of divergence times. Clades are defined as A, parrotlets; B, amazons and allies; and C, macaws, conures, and allies. Eustatic curves of sea level and the extent of the Antarctic ice sheets are shown below the chronogram.

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