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SPECIATIONAL HISTORY OF AUSTRALIAN GRASS FINCHES (*POEPHILA*) INFERRED FROM THIRTY GENE TREES*

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Abstract.—Multilocus genealogical approaches are still uncommon in phylogeography and historical demography, fields which have been dominated by microsatellite markers and mitochondrial DNA, particularly for vertebrates. Using 30 newly developed anonymous nuclear loci, we estimated population divergence times and ancestral population sizes of three closely related species of Australian grass finches (*Poephila*) distributed across two barriers in northern Australia. We verified that substitution rates were generally constant both among lineages and among loci, and that intralocus recombination was uncommon in our dataset, thereby satisfying two assumptions of our multilocus analysis. The reconstructed gene trees exhibited all three possible tree topologies and displayed considerable variation in coalescent times, yet this information provided the raw data for maximum likelihood and Bayesian estimation of population divergence times and ancestral population sizes. Estimates of these parameters were in close agreement with each other regardless of statistical approach and our Bayesian estimates were robust to prior assumptions. Our results suggest that black-throated finches (*Poephila cincta*) diverged from long-tailed finches (*P. acuticauda* and *P. hecki*) across the Carpentarian Barrier in northeastern Australia around 0.6 million years ago (mya), and that *P. acuticauda* diverged from *P. hecki* across the Kimberley Plateau–Arnhem Land Barrier in northwestern Australia approximately 0.3 mya. Bayesian 95% credibility intervals around these estimates strongly support Pleistocene timing for both speciation events, despite the fact that many gene divergences across the Carpentarian region clearly predated the Pleistocene. Estimates of ancestral effective population sizes for the basal ancestor and long-tailed finch ancestor were large (about 521,000 and about 384,000, respectively). Although the errors around the population size parameter estimates are considerable, they are the first for birds taking into account multiple sources of variance.

Key words.—Ancestral population size, anonymous loci, coalescent theory, historical demography, multiple loci, population divergence time.

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Phylogeographers have often used gene divergence or coalescent time in mitochondrial DNA (mtDNA) between closely related species (usually sister pairs) as a proxy for population divergence or speciation time (see Klicka and Zink 1997). Because of its small effective population size and high substitution rate relative to autosomal nuclear DNA, mtDNA harbors a disproportionate amount of information regarding speciational history for a single locus (Moore 1995; Avise 1998). Still, estimates of population divergence derived from such single-locus datasets are expected to suffer from at least two problems. First, because gene divergence usually precedes population divergence, these studies could overestimate population divergence due to polymorphisms in the ancestral species, yet the extent of overestimation is difficult to determine from one locus (Edwards and Beerli 2000; Beerli and Edwards 2002). Secondly, the variance around single-locus estimates of gene divergence time is large because of the stochastic nature of lineage sorting (Neigel and Avise 1986; Moritz et al. 1987; Edwards and Beerli 2000) and nucleotide substitution (Wilson et al. 1987; Hillis et al. 1996); indeed, some sources of variance in divergence time, such as the among-locus variance, cannot be estimated from mtDNA alone.

Ancestral polymorphisms may not only cause discrepancies between gene and population divergence time estimates, they may also lead to conflicts between reconstructed gene and species trees (Hudson 1983; Tajima 1983; Rosenberg 2002; Felsenstein 2004). Although this latter phenomenon is often troubling for molecular phylogeneticists, such retained polymorphisms can be a fruitful source of information about historical demography of populations. For example, if we assume that the topology of a three-species gene tree is phylogenetically reconstructed without error, and that hybridization or intralocus recombination has not altered the tree, then for neutral alleles the probability of recovering a topology that is incongruent with the species tree becomes a simple function of the time between speciation events (i.e., internode length) relative to the size of the effective population size of the ancestor (Hudson 1983; Saitou and Nei 1986; Nei 1987; Wu 1991; Hudson 1992; Yang 2002). This probability increases as internode time decreases relative to the ancestral population size; for many scenarios we expect a mixture of such trees, although empirical examples have been few.

Nei (1987) proposed a method for estimating ancestral population sizes that capitalizes on gene tree–species tree conflicts in multilocus datasets. These so-called tree mismatch methods (Yang 2002) have not only become popular for estimating ancestral population sizes (Takahata 1986; Nei 1987; Wu 1991; Hudson 1992; Chen and Li 2001; Yang 2002; Rannala and Yang 2003; Wall 2003), but have been expanded further to include multilocus estimation of species phylogenies (Saitou and Nei 1986; Pamilo and Nei 1988; Wu 1991; Ruvolo 1997) and speciation times (Yang 2002; Rannala and Yang 2003; Wall 2003). Recently, Yang (2002) and Rannala

*We dedicate this paper to Ernst Mayr for his contributions to speciation research in Australian birds.

and Yang (2003) further refined these methods by using information from branch lengths in each gene tree and adding the Markov chain Monte Carlo algorithm to help accommodate uncertainty in gene tree reconstructions.

Nuclear DNA Haplotypes and Their Value to Phylogeographic Studies

Until recently, phylogeographers have employed mtDNA as their marker of choice not only for history-of-the field reasons (Avise 2000), but also due simply to the lack of nuclear markers. Microsatellites suffer from high mutation rates, contain insufficient information for their gene trees to be reconstructed, and are afflicted with ascertainment bias. On the other hand, single nucleotide polymorphisms (SNPs) or nuclear DNA polymorphisms determined by resequencing represent important markers that may help refine comparative phylogeography and historical demography of populations (Brumfield et al. 2003). The promise of nuclear haplotypes is partly due to their lower mutation rates (relative to microsatellites), ease of reconstructing the gene tree of a given locus when recombination is negligible, and their ability to be harvested in unlimited quantities with minimal ascertainments bias (Karl and Avise 1993; Hare et al. 1996; Hare and Avise 1998; Hare 2001; Brumfield et al. 2003). The lower mutation rates of nuclear loci are generally thought to compromise their use in phylogeography; however, even incompletely resolved nuclear gene trees, when summed over multiple loci, can provide a strong signal for inference of demographic history (Hare et al. 2002).

Australian Phylogeography

Australian birds have captivated biogeographers for several decades. Careful study of present-day species' distributions in Australia by Keast (1961) revealed the existence of multiple codistributed songbird species, which implicated vicariance as the primary mechanism responsible for speciation. Consequently, the geographic junctures between putative sister species pairs may mark the locations of important geological barriers of the past (Keast 1961; Schodde 1982; Schodde and Mason 1999). Cracraft (1986) garnered support for this proposition using cladistic analysis of morphology and plumage for a number of bird clades. Two putative barriers in northern Australia, the Carpentarian Barrier and Kimberley Plateau–Arnhem Land Barrier, may have played a key role in bird diversification in the northern Australian tropics (Keast 1961; MacDonald 1969; Ford 1978; Cracraft 1986). Keast (1961) first hypothesized that the arid country located at the southern terminus of the Gulf of Carpentaria helped isolate the northern tropical biotas from those found on the Cape York Peninsula and east coast. Ford (1978) later proposed that the river valleys (Ord, Victoria, and Daly Rivers) located between the Kimberley Plateau region of Western Australia and the Arnhem Land region of the Northern Territory subdivided tropical biotas east and west of this region.

Several closely related species of Australian grass finches in the genus *Poephila* nicely illustrate both of these classic northern Australian biogeographic patterns (Keast 1961; Cracraft 1986). Black-throated finches (*P. cincta*) are distributed to the east of the Carpentarian Barrier, whereas its closest relatives, two species of long-tailed finches, are found to the west. Long-tailed finches occur as two geographical and morphologically differentiable populations separated by the Kimberley Plateau–Arnhem Land Barrier, that are variably treated as differentiated populations or full species (Keast 1958; Mayr et al. 1968; Harrison 1974; Zann 1976; Ford 1978; Immelmann 1982; Cracraft 1986; Boles 1988; Schodde and Mason 1999). Although there are no detailed population genetic studies, morphological (Cracraft 1986) and mtDNA (M. Sorenson, pers. comm. 2004) evidence supports an (*acuticauda*, *hecki*) grouping with *cincta* as their closest living relative.

Despite these studies, outstanding questions relating to Australian bird speciation remain untackled, particularly with regard to time: previous studies of differentiation have been unable to reveal the timing of diversification, although such timing has been inferred for some mammals (Wilmer et al. 1999) and extensively for the biota in the Wet Tropics in Australia's northeast (Schneider et al. 1998). For example, did speciation in Carpentaria occur during Pleistocene times as many North American passerines seem to have done (Johnson and Cicero 2004), or did it happen earlier? Did speciation occur for all codistributed species simultaneously? And what can the timing of Carpentarian diversification tell us about the environmental factors contributing to differentiation? Here, we apply new multilocus coalescent-based methods to the problem of Australian grass finch speciation. Specifically, we present for the first time population divergence time and ancestral population size estimates for birds using a large sample of anonymous nuclear loci. In addition to adding to the small database bearing on the role of the Pleistocene in Southern Hemisphere continents (Brumfield and Capparella 1996; Garcia-Moreno et al. 1998), these data may help contribute to a better understanding of the importance of the Carpentarian and Kimberley Plateau–Arnhem Land Barriers to avian speciation in Australia.

Materials and Methods

Sampling

Like recent studies from primates (Saitou and Nei 1986; Takahata et al. 1995; Chen and Li 2001), allelic data were obtained from only one individual per population of *Poephila acuticauda*, *P. hecki*, and *P. cincta*. We also included sequences from a distant relative, the zebra finch (*P. guttata*), which we used in a number of analyses to help us assess the validity of our assumptions of no intralocus recombination, correct root location in each reconstructed gene tree, and no among-locus mutation rate variation (see below). Localities and museum accession numbers of these four individuals are: *Poephila hecki* (UWBM60707-female) Australia: Queensland; Doomadgee; Nicholson River; 18°00′S 138°51′E. *Poephila acuticauda* (UWB60742-male) Australia: Western Australia; Fitzroy Crossing, 34.2 km N, 2.4 km E Leopold Downs; 17°53′S 125°36′E. *Poephila cincta* (UWBM57525-male) Australia: Queensland; Chillagoe, 38 km N, 39 km W; Nolan Creek, Cape York. *Poephila guttata* (UWBM57692-male) Australia: New South Wales; Menindee, 15 km N, 4 km W, Lake Panamaroo. Clearly, our sample size of individuals indicates we are not concerned here with current in-

traspecific phylogeography and population parameters; rather, our primary concern is with ancestral, not current, population sizes. We chose to focus our efforts on the number of loci because this strategy should lead to better divergence time estimates and is not greatly improved by sampling multiple individuals within species (Edwards and Beerli 2000). This sampling strategy also highlights the diversity of questions that can be addressed with but a single sampled allele per species. Finally, on a practical note, the maximum likelihood program we used to estimate divergence times (see below) only accommodates a three-tip tree. Other available software for performing multilocus estimates of population parameters can accommodate multiple alleles per species but are generally restricted to analysis of two species at a time.

Molecular Data

Development of anonymous nuclear loci.—We elected to use multiple anonymous loci as the molecular markers in our study (Karl and Avise 1993; Hare et al. 1996; Hare and Avise 1998). Because each locus is presumably from a random location in the genome, we assume that loci recombine among themselves freely (Edwards and Beerli 2000; Arbogast et al. 2002). *Poephila hecki* individual number UWB60707 was used to construct a small-insert genomic library from which our genetic markers were produced. Genomic DNA was extracted from frozen heart, liver, or muscle tissues using standard phenol/chloroform methods (Maniatis et al. 1982). A cup horn sonicator was used to shear the DNA followed by a ''blunting'' treatment of fragment ends consisting of (1) incubating a mixture of sonicated DNA, mung bean nuclease, and enzyme buffer (New England Biolabs, Ipswich, MA) at 37°C for 30 min; (2) one phenol-chloroform extraction followed by ethanol precipitation of DNA; (3) resuspension of DNA in water followed by the addition of T4 polymerase and enzyme buffer (New England Biolabs) incubated at 16°C for 2 h. Sheared repaired DNA was then electrophoresced through a 1% agarose gel so fragments suitable for plasmid cloning could be selectively excised from the gel. We selected fragments in the 0.7–1.5 kb range to maximize sequence available for gene tree reconstructions and minimize the number of sequencing reactions needed to sequence each locus. End repaired fragments (20.5 ng) were then ligated into pUC19 plasmid vectors (50 ng) before being transformed into chemically-competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA). Clones were then plated on agar plates containing ampicillin. Clones were picked at random with a toothpick, plasmids were purified using a mini-prep kit (Qiagen, Valencia, CA), and sequenced using vector primers from both ends. We then designed polymerase chain reaction (PCR) primers nested within each finch-insert sequence. The inserts in 33 clones were fully sequenced. Although we expect our random genomewide cloning approach to yield mostly sequences of noncoding loci, we nevertheless conducted BLAST (basic local alignment search tool) analysis of each clone sequence to determine whether any of our sequences are homologous with coding DNA and tested each sequence for an open reading frame using the software Se-Al (Rambaut 1995). As in many human resequencing studies, we decided to skip a hybridization step for confirming the unique sequence status of each locus employed in some protocols (Karl and Avise 1993) because such a step does not eliminate the possibility of simultaneous amplification of paralogs (Hare et al. 1996), and because we reasoned that amplified paralogous or repeated copies of noncoding autosomal loci would be easily detected during PCR or sequencing.

Determination of haplotype sequences.—The Elongase enzyme system (Invitrogen) was used in all PCR reactions to help minimize polymerase-induced base errors in PCR products. Allele sequences (haplotypes) for each PCR product were obtained using the TOPO TA cloning and sequencing kit (Invitrogen); we usually cloned between three and six alleles per amplicon. All DNA sequences are deposited in GenBank (accession nos. DQ129747–DQ129863).

Data Analyses

Evaluation of intralocus recombination.—An important assumption in our analyses is that multiple nucleotide sites on a given locus are linked to the extent that each site shares the same genealogical history (Wakeley and Hey 1997; Wall 2003; Felsenstein 2004). Although available evidence suggests that intralocus recombination may be high in birds (Smith and Burt 1998; Edwards and Dillon 2004), the incidence of recombination among a large sample of small (<1 kb) anonymous loci has not yet been studied. We therefore assessed the prevalence of historical intralocus recombination events by employing the ''four-gamete test'' (Hudson and Kaplan 1985) as implemented in the software DNAsp version 4 (Rozas and Sanchez 2003). Although this procedure only identifies the minimum number of intralocus recombination events, it nevertheless is a sensitive indicator of the presence of recombination, which is our primary concern. For any locus flagged by the four-gamete test as having undergone past recombination event(s), we discarded the sequence to the left or right of the leftmost or rightmost hypothesized recombination event(s). We then retained the longest contiguous and presumably unrecombined block for further analyses.

Estimation of gene trees.—Gene trees were reconstructed using maximum likelihood (ML) with a Jukes-Cantor substitution model (Jukes and Cantor 1969) as implemented in the program PAUP* 4.0.0d64 (Swofford 2000). Our choice of a Jukes-Cantor model is reasonable given that the three sequences are from closely related species (Yang 2002) and nuclear genes evolve slower than mtDNA. Because of the small number of taxa, exact tree searches could be performed. Trees were first rooted using a molecular clock with the three ingroup taxa, a format that is required for the ML analysis of population parameters. Internode support in each gene tree was assessed using nonparametric bootstrapping (Felsenstein 1985; Hillis and Bull 1993) as implemented in PAUP* 4.0.0d64 (Swofford 2000). We consider bootstrap proportions of 70% or greater as constituting strong support for existence of a particular clade following Hillis and Bull (1993). We evaluated the robustness of these clock root locations by comparing the topologies of clock-rooted trees to trees rooted with the zebra finch outgroup. Specifically, three additional analyses using an outgroup were conducted: (1) ingroup + outgroup with clock enforced using ML; (2) in-

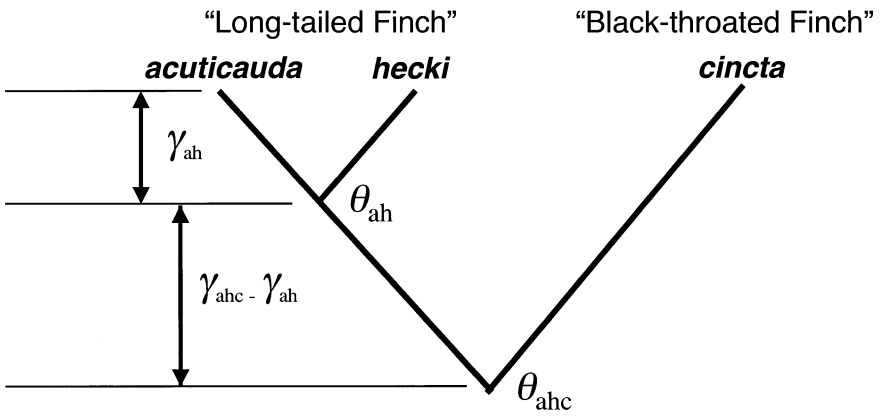

FIG. 1. Assumed species tree and parameters of interest in present study. Lowercase letters a, h, and c represent abbreviated species' names *acuticauda*, *hecki*, and *cincta*.

group + outgroup with clock not enforced using ML; and (3) ingroup + outgroup estimated using maximum parsimony. All phylogenetic analyses were run using PAUP* 4.0.0d64 (Swofford 2000).

Evaluation of intralocus, among-lineage rate variation.—The quality of our clock-constrained gene tree reconstructions depends on whether the molecular clock assumption was met for each locus. We used a method by Tajima (1993; his one-degree-of-freedom [1D] method) to test each locus individually for its adherence to a molecular clock. This test is tailored to three-tip trees and has power comparable to the relative rates and likelihood ratio tests but is preferable to these alternative methods because it does not call for assumptions about the patterns of nucleotide substitution within or across sites (Tajima 1993). The only assumption of Tajima's 1D test is that the population history (species tree) of the three species is justified, which is the case here (see below). We rejected the clock hypothesis for any locus showing statistically significant chi-squared values at the 5% level of significance (Rohlf and Sokal 1981).

Justification for finch species tree.—Although significant phylogenetic evidence already exists supporting the ((*acuticauda*, *hecki*), *cincta*) species tree (Cracraft 1986; M. Sorenson pers. comm. 2004), we nevertheless sought to verify this proposition by using our multilocus data to infer the species tree for these taxa because our molecular clock analyses and estimates of divergence times and ancestral population sizes depend on the assumption that we know the populational history of these species. One way to estimate a species tree from multiple gene trees is via a majority-rule criterion, whereby the gene tree whose topology is found most frequently is presumed to reflect the topology of the species tree (Saitou and Nei 1986; Pamilo and Nei 1988; Maddison 1997). Another method for estimating the species tree from multilocus data involves using ML to estimate divergence times and ancestral population sizes for each of the three possible tree topologies and then designating the tree with the highest likelihood value as the most probable species tree (J. Wakeley, pers. comm. 2004).

Maximum likelihood estimation of divergence times and ancestral population sizes.—We used the maximum likelihood program Ne3sML (Yang 2002) to estimate two population divergence time ($\gamma_{ahc\text{-}ah}$, γ_{ah}) and two ancestral population size (θ_{ah}, θ_{ahc}) parameters. Throughout, subscripts a, h, and c represent the three finch species *acuticauda*, *hecki*, and *cincta*, respectively (Fig. 1). Note, each γ parameter represents a time interval *between* speciation events (in mutation units), as opposed to indicating nodal ages (although, corresponding to the root of the species tree, γ_{ah} is also a nodal age). This method assumes no gene flow after population divergence, free recombination among loci but not within them, and random mating within ancestral populations. We initially conducted analyses assuming a constant mutation rate across all loci (one-rate model). However, among-locus rate variation can bias estimates of ancestral population size (Takahata and Satta 1997; Yang 1997, 2002; Wall 2003). We therefore also performed likelihood analyses that incorporated information about locus-specific mutation rates (variable-rate model). Locus-specific rates were estimated by first calculating average outgroup-to-ingroup distances, then standardizing the estimate for each locus using the mean of all locus-specific distances (see Yang 2002).

The program yields maximum likelihood estimates (MLE) in units of substitutions per site. To convert these estimates to absolute values of speciation times in years (τ) and ancestral effective population sizes (N_a), we assumed that generation time for these species is one year and that our sequences have been evolving at a neutral autosomal mutation rate (μ) of 3.6×10^{-9} substitutions/site/year. We note that our generation time, which is based on zebra finches, may represent a rough estimate owing to the highly unpredictable nature of adult survivorship in this species (Zann 1996). The mutation rate is a recent calibration for autosomal nuclear genes based on divergences between the chicken and turkey lineages (Axelsson et al. 2004). The latter two assumptions allowed us to obtain τ and N_a via the equations $\gamma = \tau\mu$ and $\theta = 4N_a\mu$.

Bayesian analyses of divergence times and ancestral population sizes.—We also analyzed our multilocus data using a Bayes Markov chain Monte Carlo method implemented in the MCMCcoal program (Yang 2002; Rannala and Yang 2003). As in the maximum likelihood program, the Bayesian program uses multiple independent loci, with the same assumptions associated with the maximum likelihood method,

to estimate population divergence times and ancestral population sizes. In contrast to the likelihood program, however, independent gamma prior probability distributions (not to be confused with divergence time γ) must be specified for each of the four parameters. Specifically, values for the α and β hyperparameters must be selected, which in turn determine the mean (α/β) and variance (α/β^2) of each gamma distribution (Yang 2002). The priors therefore represent both a strength and a weakness of this approach, depending on how informative (or misleading) a given set of priors are relative to how much information exists in the data (likelihood). As is likely to be the case with this type of study, we did not have good prior information about our parameters. Faced with such a problem, Bayesians often employ the use of ''vague'' prior distributions (Broemeling 2002), a strategy that reduces the prior's influence on the resulting posterior probability distributions, thereby enabling one to still exploit the Bayesian statistical machinery. Assuming the data contain some useful signal, the resulting mean of the posterior distribution should closely resemble the MLE of the same data (Yang 2002).

Rannala and Yang (2003) recommend using a minimum α value of 1. We therefore specified exponential priors for each parameter (i.e., $\alpha = 1$). Although the exponential is an extremely vague prior, we nevertheless performed a sensitivity analysis to assess the signal strength in our data. The priors used in our first analysis (analysis 1) were set so that both divergence time parameters ($\tau_{ahc\text{-}ah}$ and τ_{ah}) had a mean value of 1.0 million years (my), and ancestral effective population sizes (N_{ah} and N_{ahc}) at 50,000. In analysis 2, the prior means were increased an order of magnitude to 10.0 my for $\tau_{ahc\text{-}ah}$ and τ_{ah}, and 500,000 for N_{ah} and N_{ahc}. Our rationale is that if our data contain sufficient signal then changing the prior should have little effect on the posterior results and our posterior means should also approximate the MLE in both cases. Bayesian runs were continued for 10^6 generations with a burn-in of 10^4 generations. Each analysis was run at least twice and with different random number seeds to check for convergence.

Parameter variance versus number of loci.—We evaluated the hypothesis that increases in the number of loci causes a decrease in parameter variance (Pluzhnikov and Donnelly 1996) by plotting the Bayesian 95% credibility interval (CI) for each parameter versus number of loci. To guard against biases due to sampling artifacts, we performed each analysis using five random subsets of our data and then plotted mean values for the upper and lower CI. Moreover, we elected to use the priors in analysis 2 so that the prior would exert less influence over the posterior.

RESULTS

Data Characteristics and Gene Tree Reconstructions

A total of 30 anonymous loci were developed, ranging in size from 216 to 825 bp (average size 553 bp; total of 16,598 nucleotide sites), a descriptive summary of which is presented in Table 1. Intralocus variability across all three species ranged from 0 to 5.6% of the sites (Table 1). In addition to the site polymorphisms at variable nucleotide sites, Fig. 2A), another type of sequence variability was evident in the form of allele length polymorphisms due to indel sites not shared between amplified alleles (Fig. 2B,C). Cloning of such PCR products resolved both SNP and allele length heterozygotes, thereby yielding the actual PCR allele sequences (Fig. 2A–C). Only three loci had homologous matches with the GenBank database according to our BLAST results. Locus Pa-01 matched an olfactory receptor gene in *Gallus gallus* ($P < 6.0 \times 10^{-68}$, 248 bp alignment), Pa-14 matched a sacsin gene in *Gallus gallus* ($P = 0.0$, 648 bp alignment); and Pa-25 matched sequences with several species of *Amazona* β-fibrinogen intron 7 region ($P < 4.0 \times 10^{-4}$, 169 bp alignment; coincidentally a locus of interest in avian systematics; Prychitko and Moore 1997). Open reading frames were only found in the two putatively coding loci (Pa-01 and Pa-14), confirming that the vast majority of our loci are noncoding as expected. We also observed low (<50%) GC content in most of our presumably noncoding loci (Table 1), consistent with findings from at least some songbirds (Prychitko and Moore 1997; Gasper et al. 2001; Drovetski 2002). We were able to determine outgroup sequences for 27 of the 30 loci. The overall incidence of intralocus recombination in our data appears uncommon, as four-gamete tests suggested only eight historical recombination events dispersed among six loci (Table 1). The molecular clock hypothesis was rejected for only three loci (Table 1), a result that may in part be due to low power of rejection for our short sequences. Consistent with this result is the fact that 26 of the 27 gene trees for which there were outgroups were rooted at the same place using clock or outgroup methods (Table 2). For the two unresolved loci, the clock-root location in locus Pa-29 conflicted with the outgroup-chosen location, whereas locus Pa-30 contained no polymorphic sites (Table 2). The topologies of 21 gene trees were well supported as evidenced by their high bootstrap values (Fig. 3). There was considerable variation in coalescent times among the trees, which also reflected all three possible topologies (Fig. 3).

Sixteen gene trees in Figure 3 matched the putative species tree ((a, h), c), whereas the other topologies were found with a frequency of seven ((a, c), h), and five ((h, c), a). Thus, by the majority-rule criterion (Saitou and Nei 1986; Pamilo and Nei 1988) these results suggest that topology ((a, h), c) represents the species tree assuming a pure species isolation model. Moreover, our coalescent-maximum likelihood analysis of the complete dataset is weakly consistent with that the assumed species tree being correct, because the three possible tree topologies yielded ML values of -1379.9, -1381.6, and -1381.5 for topologies ((a, h), c), ((a, c), h), and ((h, c), a), respectively.

Maximum Likelihood Estimates of Divergence Times and Ancestral Population Sizes

Taking into account substitution rate parameters as outlined in the methods, our maximum likelihood analyses of a one-rate model suggest that the long-tailed finches (*acuticauda* and *hecki*) split from each other across the Kimberley Plateau–Arnhem Land Barrier approximately 0.34 mya and that black-throated finches diverged from the long-tailed finches across the Carpentarian Barrier around 0.22 my earlier (Table 3). Ancestral effective population sizes seem to have

TABLE 1. Descriptive statistics for the 30 loci used in this study. Sequence length includes alignment gaps. Each locus was tested for its adherence to a molecular clock using Tajima's 1D test (Tajima 1993). Nonsignificant test results are indicated by ns; asterisks indicate statistical significance at the $*P < 0.05$ and $**P < 0.005$ levels, respectively. Minimum numbers of historical intralocus recombination events were ascertained using four-gamete tests (Hudson and Kaplan 1985).

Locus	Sequence length in base pairs	No. of variable sites (%)	% GC	Molecular clock test	Minimum number of recombination events
Pa-01	577	11 (1.9)	50	ns	0
Pa-02	613	13 (2.1)	38	ns	0
Pa-03	317	10 (3.2)	32	ns	0
Pa-04	512	3 (0.6)	34	ns	0
Pa-05	590	4 (0.7)	41	ns	0
Pa-06	544	2 (0.4)	33	ns	0
Pa-07	500	18 (3.6)	43	ns	0
Pa-08	573	9 (1.6)	38	ns	0
Pa-09	659	14 (2.1)	45	ns	0
Pa-10	454	14 (3.1)	40	ns	0
Pa-11	681[1] (253)	17 (2.5)	38	ns	2
Pa-12	825[2] (797)	17 (2.1)	49	ns	0
Pa-13	520[1] (379)	4 (0.8)	36	ns	1
Pa-14	648	1 (0.2)	42	ns	0
Pa-15	468	1 (0.2)	39	ns	0
Pa-16	481	6 (1.2)	37	ns	0
Pa-17	216	2 (0.9)	35	ns	0
Pa-18	620	16 (2.6)	38	**	0
Pa-19	608	6 (1.0)	38	*	0
Pa-20	339[1] (134)	19 (5.6)	54	ns	2
Pa-21	591	14 (2.4)	41	ns	0
Pa-22	640	11 (1.7)	37	ns	0
Pa-23	657	9 (1.4)	40	ns	0
Pa-24	561[1] (318)	6 (1.1)	37	ns	1
Pa-25	618	6 (1.0)	54	ns	0
Pa-26	540[1] (333)	5 (0.9)	46	ns	1
Pa-27	405	8 (2.0)	33	**	0
Pa-28	613	10 (1.6)	42	ns	0
Pa-29	657[1] (443)	16 (2.4)	41	ns	1
Pa-30	571	0 (0.0)	44	ns	0

[1] Sequence length in parentheses represents a presumed single nonrecombined block (see Materials and Methods).
[2] Sequence length in parentheses after sites 776–803 was omitted due to questionable sequence alignment involving an indel region.

been quite large, on the order of several hundred thousand (Table 3). The relative or locus-specific mutation rates estimated for the 27 loci with outgroup sequences available vary minimally (Fig. 4). Only locus Pa-20 stands out as an outlier, exhibiting a far higher mutation rate than the other loci (Fig. 4), perhaps because it is a pseudogene or, less likely, due to paralogous relationships among amplified sequences. Regardless, MLEs of the four parameters with these locus-specific rates incorporated were very similar to those with the one-rate model (Table 3) and our results are unaffected by the outlier locus.

Bayesian Estimates of Divergence Times and Ancestral Population Sizes

Results of analysis 1 with the first set of priors suggest that the two long-tailed finches (*acuticauda* and *hecki*) diverged from each other around 0.61 mya, and the black-throated finches (*cincta*) diverged from the long-tailed finch lineage 0.10 my earlier (Table 4). In analysis 2, with the larger and broader priors, these divergence times were found to be 0.31 my and 0.29 my, respectively (Table 4) thereby reflecting the MLEs (Table 3 and Fig. 5A,B). Despite the use of prior means differing by 9 my, the resulting posterior means in each analysis remained within approximately 0.3 my of each other (Table 4, Fig. 5A,B). The ancestral population sizes estimated in analysis 1 were substantially smaller than those in analysis 2, most likely because the prior means and variances in analysis 1 were smaller than in analysis 2 (Table 4). Figure 5C clearly shows that the posteriors are tracking the priors for the N_{ah} parameter. Despite the fact that the posterior distribution is nearly centered over the MLE for this parameter, the Bayesian estimates of N_{ah} should remain questionable. The Bayesian estimates of the other ancestral population size, N_{ahc}, appear to be less biased by the prior, perhaps because the data contain more signal for estimating N_{ahc} (Fig. 5D).

Parameter Variance versus Number of Loci

Our analyses show that the variance for both divergence time parameters undergoes a steep decrease from two to 10 loci, before leveling out and remaining constant thereafter (Fig. 6A,B). Indeed, both divergence time estimates experienced nearly a threefold reduction in variance when as few as 10 loci are sampled (Fig. 6A,B). Oddly, such a variance reduction was not observed for one of the ancestral population size parameters (N_{ah}), because the variance remained constant regardless of numbers of loci analyzed (Fig. 6C). Note also that the posterior mean value stays very close to the prior mean value (about 500,000), which suggests that the prior may have driven the result owing to lack of signal

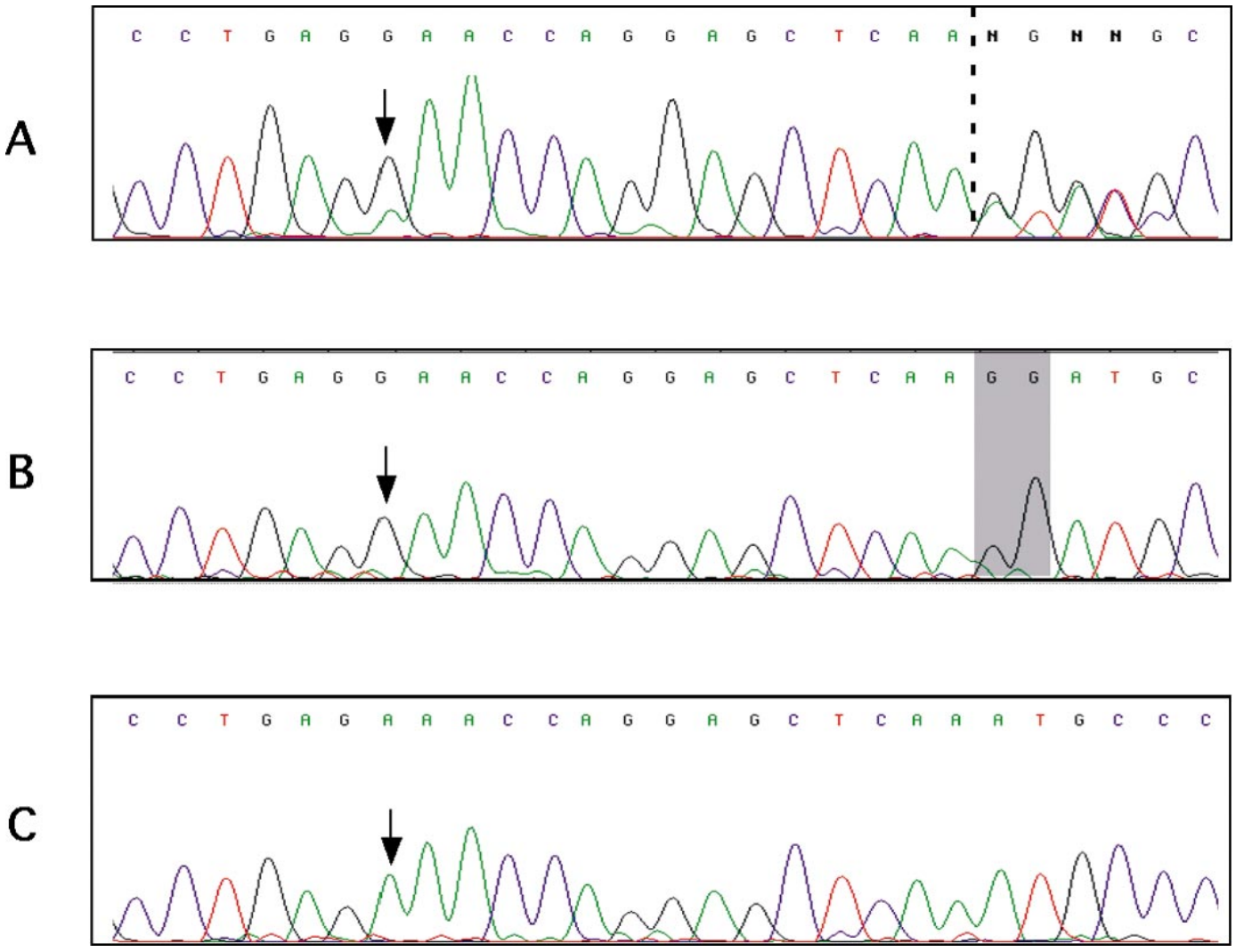

FIG. 2. Two types of DNA sequence polymorphism illustrated in chromatograms of the same nuclear locus. (A) Partial chromatogram generated from a directly sequenced polymerase chain reaction (PCR) product showing it to be heterozygous for a nucleotide site (arrow) and also for an indel (to the right of the vertical dashed line). Cloning and sequencing of this PCR product confirms the existence of G and A nucleotides (at arrows) as well as a GG found in the shaded region of (B) but missing in (C), thereby explaining the length differences between alleles.

in the data (also see Fig. 5C). In contrast, the variance around the other ancestral population size parameter (N_{ahc}) underwent a gradual narrowing with increasing numbers of loci culminating in a twofold reduction with 30 loci (Fig. 6D). Although the posterior mean of N_{ahc} stabilized to values very close to the prior mean beginning at 10 loci (Fig. 6D), this estimate nonetheless agrees with the MLE of this parameter (Table 3), suggesting that the prior may not have unduly influenced the posterior (see also Fig. 5D) as it did with N_{ah} (see also Fig. 5C).

Gene Divergence Times versus Population Divergence Times

We used the Jukes-Cantor distances, *D*, of our ML gene trees from the root to the tips (Fig. 3) to approximate coalescent times (*D*/2). Although this approach is not the most efficient estimate of coalescent time, it is known to be unbiased (Tavare et al. 1997). We contrasted gene divergence (coalescent) times (*D*/2) with inferred population divergence times (γ). A striking level of discordance is seen when we compare average gene divergence with the maximum likelihood and Bayesian estimates of population divergence (Fig. 7). All population divergence estimates including their error bars indicate that speciation occurred entirely within the Pleistocene as does the gene divergence for the *acuticauda* versus *hecki* split. By contrast, gene divergence for the *cincta* versus (*acuticauda*, *hecki*) is inferred to have occurred somewhere between the late Pliocene and early Pleistocene, over 1.3 my earlier than the corresponding estimated population divergence (Fig. 7).

DISCUSSION

We used a multilocus coalescent approach to infer basic details of speciation in *Poephila* finches, a classic example

TABLE 2. Hypothesized root locations in each of the 30 gene trees using four different rooting methods. The first analysis used the clock rooting method, whereas the other three analyses consisted of variations of the outgroup rooting method. Taxon names in table are the ingroup branches where the root is hypothesized to be located on each tree. Optimality criterion used in each analysis is shown in parentheses. ML represents maximum likelihood optimality criterion, u represents unresolved root location, and asterisks denote cases in which an outgroup sequence was unavailable. *Poephila cincta* (shown in bold) represents the presumed root location in the species tree. See Figure 3 for illustrations of each reconstructed gene tree showing root locations.

Locus	Clock rooting (ML-clock enforced)	Outgroup rooting (ML-clock enforced)	Outgroup rooting (ML-no clock enforced)	Outgroup rooting (maximum parsimony)
Pa-01	***cincta***	***cincta***	***cincta***	***cincta***
Pa-02	***cincta***	***cincta***	u	u
Pa-03	***cincta***	***cincta***	***cincta***	***cincta***
Pa-04	***cincta***	***cincta***	u	u
Pa-05	***cincta***	u	u	u
Pa-06	***cincta***	***cincta***	***cincta***	***cincta***
Pa-07	***cincta***	***cincta***	***cincta***	***cincta***
Pa-08	***cincta***	***cincta***	***cincta***	***cincta***
Pa-09	***cincta***	***cincta***	***cincta***	***cincta***
Pa-10	***cincta***	*	*	*
Pa-11	***cincta***	***cincta***	***cincta***	***cincta***
Pa-12	***cincta***	***cincta***	u	u
Pa-13	u	***cincta***	***cincta***	***cincta***
Pa-14	***cincta***	*	*	*
Pa-15	***cincta***	***cincta***	u	u
Pa-16	***cincta***	u	u	u
Pa-17	*hecki*	*hecki*	*hecki*	*hecki*
Pa-18	*hecki*	*hecki*	*hecki*	*hecki*
Pa-19	*hecki*	*hecki*	*hecki*	*hecki*
Pa-20	*hecki*	*hecki*	*hecki*	*hecki*
Pa-21	*hecki*	*hecki*	*hecki*	*hecki*
Pa-22	u	*hecki*	*hecki*	*hecki*
Pa-23	*hecki*	*hecki*	*hecki*	*hecki*
Pa-24	*acuticauda*	*acuticauda*	*acuticauda*	*acuticauda*
Pa-25	*acuticauda*	u	u	u
Pa-26	*acuticauda*	*acuticauda*	*acuticauda*	*acuticauda*
Pa-27	*acuticauda*	*acuticauda*	*acuticauda*	*acuticauda*
Pa-28	*acuticauda*	*	*	*
Pa-29	*acuticauda*	***cincta***	***cincta***	***cincta***
Pa-30	u	u	u	u

of divergence across the Carpentarian Barrier. We also investigated the significance of another northern Australian barrier, the lesser-known Kimberley Plateau–Arnhem Land Barrier. Speciation patterns across northern Australia have hitherto been studied with allozymes, chromosome variation, mtDNA or microsatellites, and ours is the first to use a multiple gene tree approach for species in this area (Edwards 1993; Wilmer et al. 1999). Moreover, our large sample of gene trees afforded us an opportunity to empirically evaluate the efficacy of multilocus estimation of the *Poephila* species tree, population divergence times, and ancestral population sizes using recently developed statistical tools.

Gene Tree Reconstructions, Multiple Nuclear Loci, and Historical Demography

In this and in similar recent studies (e.g., Chen and Li 2001) locus sizes have been in the 200–800 bp range. Although the majority of our loci evidently had sufficient information to permit robust reconstructions of gene tree topologies, considerable error in branch lengths due to low number of variable sites probably contributed to the large variances, particularly around our ancestral population size estimates. Indeed, errors around estimates of ancestral θ in recent studies have tended to be large (Yang 2002; Rannala and Yang 2003; this study). Why ancestral population sizes of extant species are more difficult to estimate accurately and precisely than current population sizes is not yet known (Wall 2003). One possible remedy would be to increase the length of each locus, which could then improve gene tree reconstructions. Based on a simulation study, Rannala and Yang (2003) observed that increasing the number of sites on each locus resulted in twofold reductions in Bayesian posterior credibility intervals of ancestral population size parameters and even more impressive variance decreases associated with divergence times. However, increasing locus lengths will also tend to increase the chances of including sites that have undergone recombination thereby confounding multiple genealogical histories (see Felsenstein 2004, pp. 464–465). Intralocus recombination is expected to reduce the coalescent variance leading to underestimation of ancestral population sizes (Wall 2003). The severity of the problem of intralocus recombination will probably vary among taxa (Hare 2001; Wall 2003); in *Drosophila*, such recombination is often high, necessitating the use of methods that incorporate this force (Hey and Kliman 2002). The situation may be similar in passerines, particularly when population sizes are large, providing abundant opportunities for recombination (Edwards and Dillon 2004). However, in the present study we found

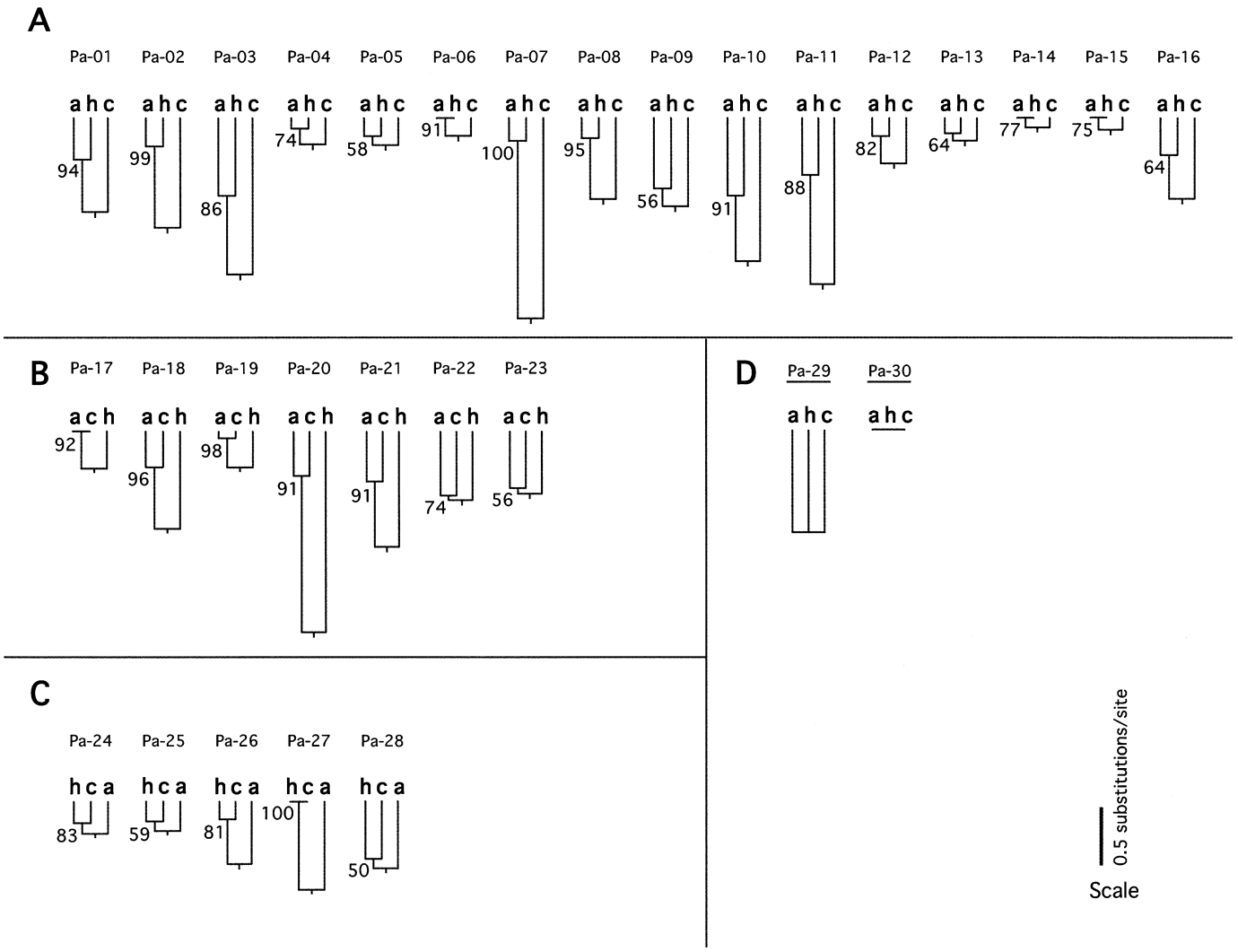

FIG. 3. Variation in topology and coalescent times for 30 gene trees. Root locations were determined via molecular clock; in most cases these root locations were confirmed by an outgroup (see Table 2). (A) Topologies of 16 gene trees that are congruent with the presumed species tree ((a, h), c). (B) Seven gene trees with topology ((a, c), h) that are incongruent with the species tree. (C) Five gene trees with topology ((h, c), a) that are incongruent with the species tree. (D) Two unresolved gene trees. Lowercase letters a, h, and c represent abbreviated species' names *acuticauda*, *hecki*, and *cincta*. Ultrametric trees were reconstructed from 30 independent nuclear loci using maximum likelihood and a Jukes-Cantor substitution model under a molecular clock. Numbers adjacent to each internal branch are bootstrap proportions. Each locus identifier is above its respective tree and further descriptive information about each locus can be found in Table 1. Branch lengths are drawn to same mutational scale.

TABLE 3. Maximum likelihood estimates (MLE) of divergence times and ancestral effective population sizes. One-rate model assumes homogeneous among-locus mutation rate variation, whereas variable-rate model incorporates information on relative rates estimated using outgroup sequences. Relative rates were not available for three loci (Pa-10, Pa-14, and Pa-28); therefore, these loci were excluded from the variable-rates analysis. A one-rate analysis was conducted on the 27-locus dataset for comparative purposes with the variable rates results. Parameters γ and θ were converted to τ and N_a using equations $\gamma = \tau\mu$ and $\theta = 4N_a\mu$, respectively. Generation time is assumed to be one year and neutral autosomal mutation rate to be $\mu = 3.6 \times 10^{-9}$ substitutions/site/year. my represents units of a million years. Subscripted letters a, h, and c refer to species *acuticauda*, *hecki*, and *cincta*.

Parameter	MLE one-rate model (30 loci)	MLE one-rate model (27 loci)	MLE variable-rate model (27 loci)
$\gamma_{ahc\text{-}ah}$ ($\tau_{ahc\text{-}ah}$)	0.00080 (0.22 my)	0.00050 (0.14 my)	0.00050 (0.14 my)
γ_{ah} (τ_{ah})	0.00123 (0.34 my)	0.00156 (0.43 my)	0.00181 (0.50 my)
θ_{ah} (N_{ah})	0.00554 (384,653)	0.00312 (216,681)	0.00282 (195,951)
θ_{ahc} (N_{ahc})	0.00751 (521,347)	0.00740 (514,076)	0.00939 (652,250)

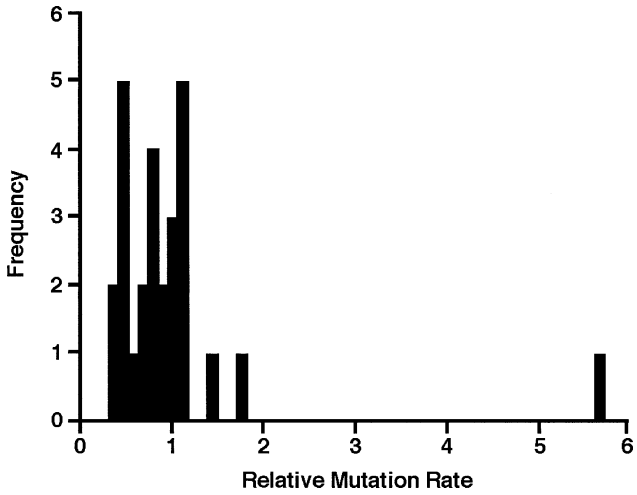

FIG. 4. Relative or among-locus mutation rate variation for 27 loci. Shown is a frequency distribution of among-locus relative rates derived from average genetic distances between ingroup and outgroup sequences scaled by the average among-locus rate (for details see Yang 2002).

recombination rates within our set of anonymous loci to be sufficiently low as to have negligible effects on our parameter estimates. Among-locus mutation rate variation is another factor that can negatively affect estimates of ancestral population sizes because it inflates coalescent variance, thereby causing an upward bias in estimates (Takahata and Satta 1997; Yang 1997, 2002). Fortunately, however, multilocus studies have so far found among-locus rate variation to be sufficiently low as to not adversely affect estimates of divergence time and ancestral population size (Yang 2002; Wall 2003; this study). Elucidating the factor(s) responsible for the large variances of ancestral population sizes remains a challenge and further insights into this problem may come from simulation studies under a variety of demographic and genetic situations.

Given that large numbers of anonymous nuclear loci could potentially be developed from a single genome, one of the key questions emerging from multilocus population genetics is how many loci are needed for parameter estimation? This question is of great practical importance because increasing the number of independent loci should generally lead to a reduction in variance associated with coalescent estimates (Pluzhnikov and Donnelly 1996; J. Felsenstein, unpubl. ms.). Indeed, the present study supports this notion, as our divergence time parameters underwent parallel threefold reductions in variances when going from a two-locus dataset to a 10-locus dataset. The variances then remained at a constant level regardless whether 10 or 30 loci were analyzed. Likewise, the ancestral population size parameter θ_{ahc} also experienced an appreciable decrease in variance when larger datasets were analyzed. In contrast to the divergence times, however, the decrease in variance associated with θ_{ahc} occurred more gradually with increasing numbers of loci, suggesting that datasets containing more than 30 loci may provide further improvements in precision. Surprisingly, the variance of the other ancestral population size parameter, θ_{ah}, remained the same regardless whether two or 30 loci were analyzed. It is not clear to us why we obtained such a result, but the fact that the priors we used in the Bayesian analyses had a large influence over the posteriors in this analysis suggests that our data contained insufficient information to estimate this parameter. Clearly, the methods we employed are more useful for estimating divergence times than ancestral population sizes as recent similar studies have found (Yang 2002; Rannala and Yang 2003). However, these observations may be contingent on the particular population history we

TABLE 4. Results of Bayesian Markov chain Monte Carlo analyses. In analysis 1, prior means were set to about 1.0 million years (my) for speciation times (τ) and 50,000 for ancestral effective population sizes (N_a) after converting γ and θ parameters to τ and N_a using the equations $\gamma = \tau\mu$ and $\theta = 4N_a\mu$, respectively. We assumed generation time to be one year and $\mu = 3.6 \times 10^{-9}$ substitutions/site/year. In analysis 2 prior means were increased 10-fold to assess sensitivity of posterior results to specified priors. In all analyses, exponential gamma priors were used for each parameter with the prior mean = α/β and prior variance = α/β^2. Approximations of prior densities were generated from the Bayes program. For clarity, γ and θ values are in bold with the corresponding values of τ and N_a immediately below. Subscripted letters a, h, and c refer to species *acuticauda*, *hecki*, and *cincta*.

Parameter	(α, β)	Prior mean (95% interval)	Posterior mean (95% interval)
		Analysis 1	
$\gamma_{ahc\text{-}ah}$	**(1, 278)**	**0.00355 (0.00010, 0.01301)**	**0.00036 (0.00002, 0.00122)**
$\tau_{ahc\text{-}ah}$		0.99 my (0.03 my, 3.61 my)	0.10 my (0.01 my, 0.34 my)
γ_{ah}	**(1, 278)**	**0.00346 (0.00009, 0.01273)**	**0.00218 (0.00124, 0.00310)**
τ_{ah}		0.96 my (0.03 my, 3.54 my)	0.61 my (0.35 my, 0.86 my)
θ_{ah}	**(1, 1389)**	**0.00073 (0.00002, 0.00266)**	**0.00142 (0.00018, 0.00396)**
N_{ah}		50,694 (1,597, 184,792)	98,889 (12,222, 275,278)
θ_{ahc}	**(1, 1389)**	**0.00075 (0.00003, 0.00269)**	**0.00537 (0.00347, 0.00776)**
N_{ahc}		52,014 (1,944, 186,667)	373,056 (240,625, 538,611)
		Analysis 2	
$\gamma_{ahc\text{-}ah}$	**(1, 28)**	**0.03647 (0.00105, 0.13380)**	**0.00103 (0.00007, 0.00249)**
$\tau_{ahc\text{-}ah}$		10.13 my (0.29 my, 37.17 my)	0.29 my (0.02 my, 0.69 my)
γ_{ah}	**(1, 28)**	**0.03560 (0.00092, 0.13010)**	**0.00113 (0.00010, 0.00231)**
τ_{ah}		9.89 my (0.25 my, 36.14 my)	0.31 my (0.03 my, 0.64 my)
θ_{ah}	**(1, 139)**	**0.00701 (0.00021, 0.02599)**	**0.00917 (0.00117, 0.02604)**
N_{ah}		486,597 (14,236, 1,805,069)	636,528 (81,389, 1,808,056)
θ_{ahc}	**(1, 139)**	**0.00739 (0.00019, 0.02695)**	**0.00741 (0.00461, 0.01105)**
N_{ahc}		513,194 (13,472, 1,871,319)	514,583 (320,278, 767,639)

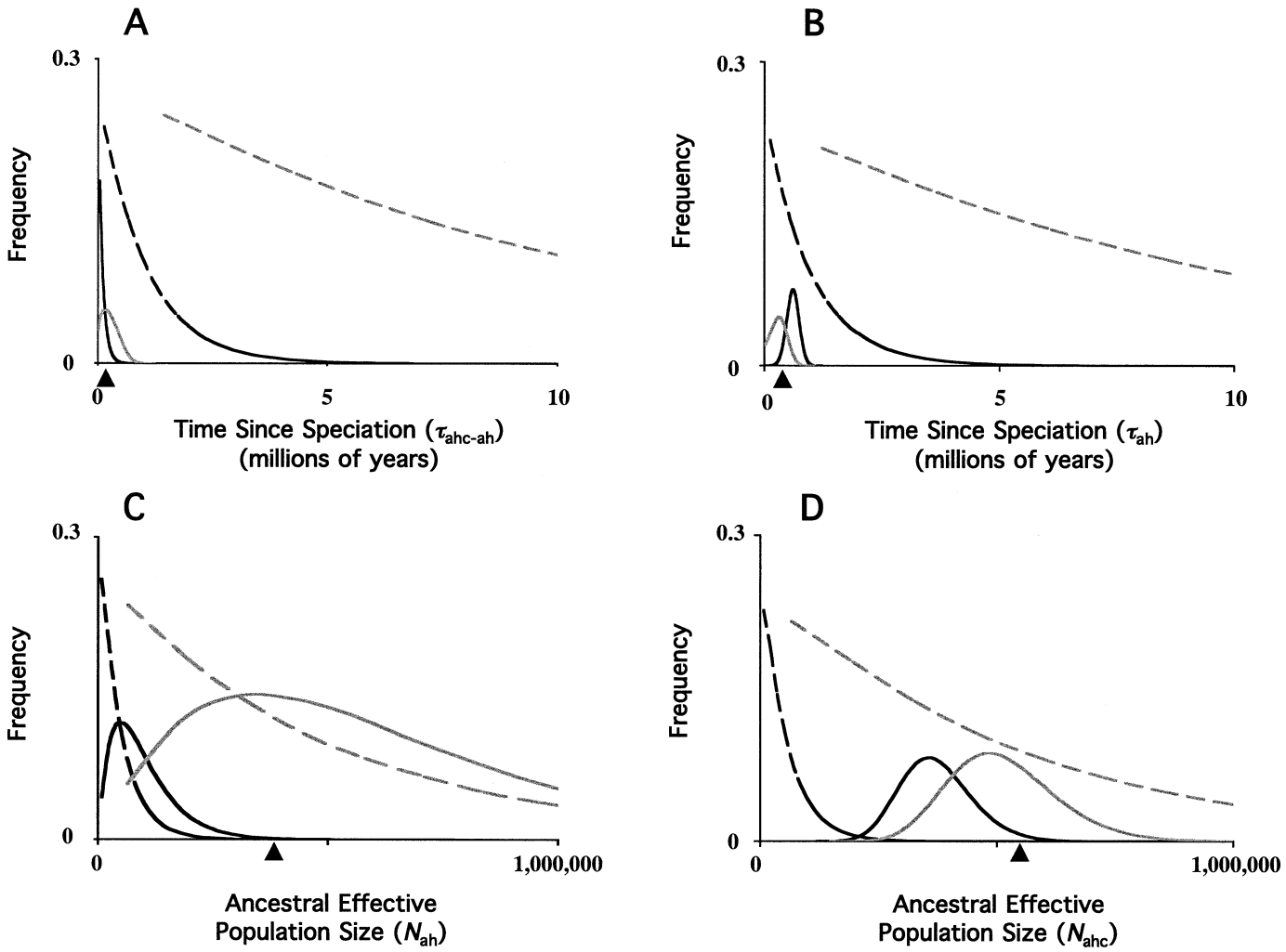

FIG. 5. Prior and posterior probability distributions of parameters in the Bayes analyses. Dashed curves represent prior probability distributions and solid curves are the posterior distributions. Black curves are results of priors used for analysis 1 and gray curves are results of priors used in analysis 2. (A) Time elapsed between speciation events (τ_{ahc-ah}). (B) Time since most recent speciation event (τ_{ah}). (C) Ancestral effective population size of long-tailed finches (N_{ah}). (D) Ancestral effective population size of the three finch species (N_{ahc}). See Table 4 for listing of prior and posterior means and 95% intervals. Approximations of prior densities were generated from the Bayes program. Solid black triangles located below the abscissa in each graph indicate the corresponding maximum likelihood estimate based on a one-rate mutation model for all loci (see Table 3).

studied, and more or fewer loci may be required in other speciation scenarios.

Species Tree, Speciation Times, and Ancestral Population Sizes

Although our analyses are based on an assumed species tree topology previously supported by morphological and mtDNA studies, results of our coalescent-based approach to estimating the species tree adds strong corroborating evidence that this assumption is well founded. Our results indicate that the two types of long-tailed finches (*acuticauda* and *hecki*) last shared a common ancestor 0.31 mya with a 95% CI of 0.03–0.64 mya. Additionally, the time elapsed between the two speciation events was estimated to be only 0.29 my with a 95% interval of 0.02 to 0.69 my. Combining the divergence times, the population split that gave rise to black-throated (*cincta*) and long-tailed finches (*acuticauda* and *hecki*) occurred only 0.6 mya. Such a young estimate for the speciation time is not surprising, as these species were previously characterized on morphological grounds as possible ''examples of the final stage in the speciation process, and not just unusually distinctive isolates.'' (Keast 1961, p. 394) Even after the error around our divergence time estimates is considered, our data suggest that the oldest timing for this event was 1.33 mya, which is still within Pleistocene epoch (i.e., 1.81–0.01 mya). The robustness of our estimate would appear to rest largely on our assumed mutation rate of 3.6×10^{-9} substitutions/site/year (Axelsson et al. 2004). Given our estimates for both divergence (γ) parameters, a mutation rate of 2.65×10^{-9} substitutions/site/year would be required to enlarge our divergence time estimates enough to entirely predate the Pleistocene. Although calibrations for neutral autosomal loci in birds are rare, Axelsson et al. (2004) noted the striking concordance between their calibration (3.6

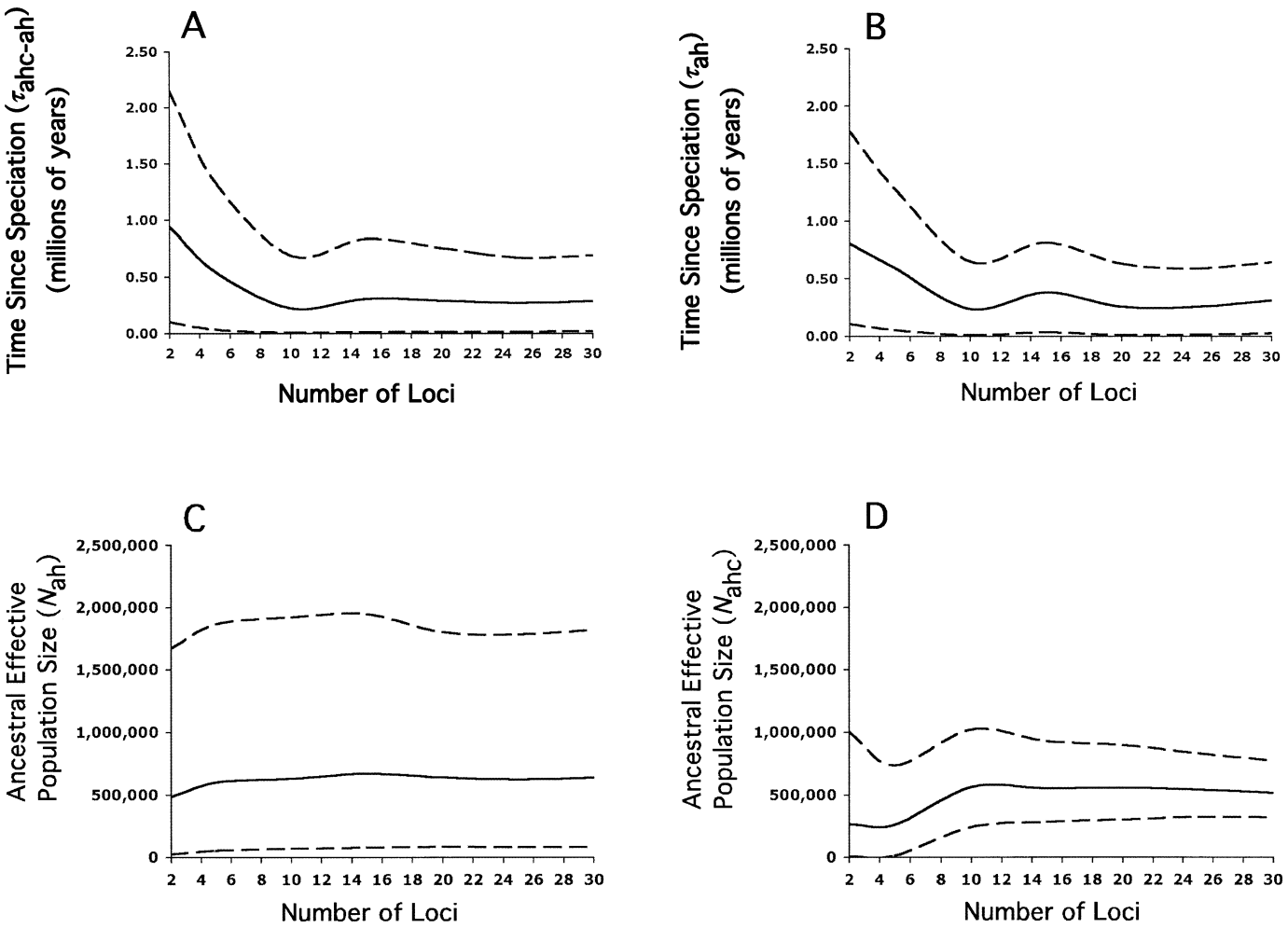

FIG. 6. Parameter variance as a function of numbers of independent loci. In each graph the mean of a Bayesian posterior probability distribution (solid line) along with the 95% credibility interval (between upper and lower dashed lines) is shown. Datapoints represent averages of five analyses involving randomly generated subsets of the data (see Materials and Methods). (A) Time in millions of years between speciation events (τ_{ahc-ah}). (B) Time since most recent speciation event (τ_{ah}), which gave rise to *acuticauda* and *hecki* lineages. (C) Effective population size in the long-tailed finch ancestor (N_{ah}). (D) Effective population size in the ancestor to all three species of finches (N_{ahc}). Subscripted letters a, h, and c represent species names as before.

$\times 10^{-9}$ substitutions/site/year), based on 33 autosomal intron sequences, with previously published rates of 3.4×10^{-9} and 4.0×10^{-9} substitutions/site/year for the same taxa but which were based on restriction site mapping and fossils (Helm-Bychowski and Wilson 1986). Generational time differences between our study organisms versus the calibrated galliform divergences may also contribute to some bias in our results, although in our study generation time only informs estimation of population sizes, not divergence times (Yang 2002). Although our data indicate that the ancestral population sizes of these birds were large, on the order of several hundred thousand each, the estimated size of the long-tailed finch ancestor was earlier questioned on statistical grounds. However, the estimate for the basal ancestor has not only proven robust to our assumptions about the priors in the Bayesian analysis, but also to intralocus recombination and among-locus rate variation. This suggests that the latter population size estimate may be reasonable provided that our assumed generation time of one year is correct (e.g., a longer generation time would result in a smaller ancestral population size estimate).

Although we have found evidence for detectable temporal divergence between *acuticauda* and *hecki* in our study, our use of a single individual per taxon makes inferences of gene flow and reproductive isolation challenging. On the one hand, the finding of a temporal split between these populations adds support to the idea that present-day contact between them represents an instance of secondary contact after a period of isolation possibly arising as a result of the postulated Kimberley Plateau–Arnhem Land Barrier. Despite the lack of reciprocal monophyly between these populations—by no means a requirement for evidence of population isolation in the recent past—the existence of genetic divergence (this study) as well as population-specific variation in bill color (Keast 1958; Harrison 1974; Boles 1988; Schodde and Mason 1999) and male song (Zann 1976) are consistent with a hypothesis in which the two populations have recently become reproductively isolated from each other (Irwin et al. 2001).

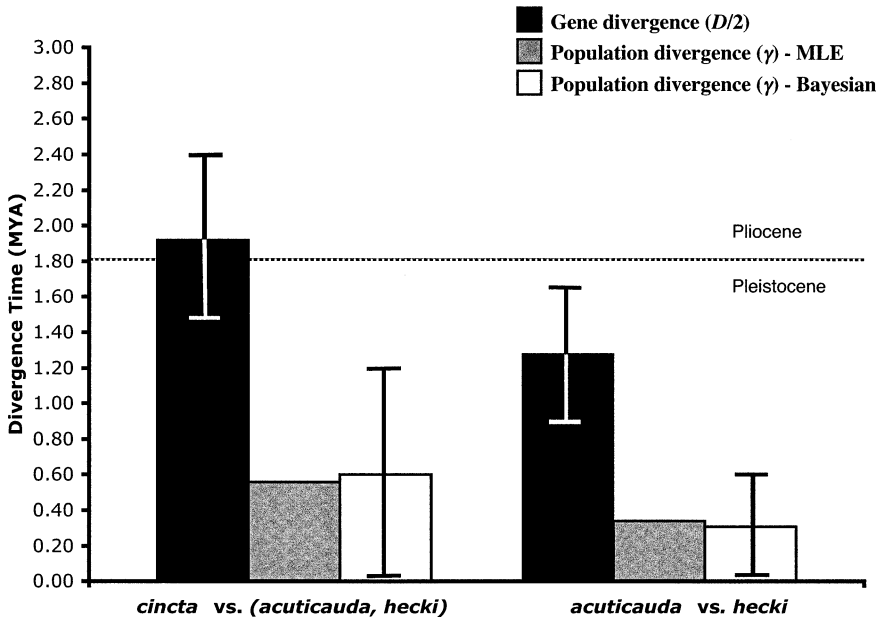

FIG. 7. Comparison of three methods for estimating population divergence time between black-throated and long-tailed finches (left three bars) and between long-tailed finch species (right three bars). Black bars, gene divergence (*D*/2) as a proxy for population divergence time estimated via average pairwise (Jukes-Cantor) distances across loci; gray bars, population divergence (γ) estimated via maximum likelihood estimation (MLE); white bars, population divergence (γ) estimated via Bayesian method. Because the maximum likelihood and Bayesian methods estimates speciation intervals (as opposed to strictly nodal ages), their values in the *cincta* versus (*acuticauda*, *hecki*) comparisons are composite values constructed using the sum of the two speciation intervals (i.e., $\tau_{ahc\text{-}ah} + \tau_{ah}$) taken from Tables 3 and 4. Approximate 95% confidence limit bars for gene divergence were estimated in a standard way, whereas the error bars for the Bayesian estimate were approximated by summing the two upper-bound and two lower-bound values from each speciation interval's 95% CI from Table 4. The Bayesian means and CI were selected from analysis 2 in Table 4 because the priors seem to have a less adverse influence on the posteriors than the priors in analysis 1. Confidence limits for the maximum likelihood method are difficult to estimate and are therefore not shown.

Extensive gene flow across the Kimberley Plateau–Arnhem Land or the Carpentarian Barriers would have resulted in a more even distribution of the three gene trees recovered. Still, our sample sizes for individuals are too small to rule out alternative explanations, and additional studies are needed to ascertain whether hybridization is occurring and whether song variation in male long-tailed finches does in fact represent a prezygotic (behavioral) isolating barrier between the two populations (Coyne and Orr 2004).

Implications for Comparative Phylogeographic Studies

Comparative phylogeography is a powerful approach to studying the importance of geographical barriers to speciation (Bermingham and Moritz 1998; Moritz and Faith 1998). This approach has been fruitfully applied to biogeographic studies of Wet Tropics rainforests in eastern Australia, in which the importance of barriers to vertebrate (Joseph et al. 1993, 1995; Joseph and Moritz 1994; Schneider et al. 1998) and invertebrate (Hugall et al. 2002) speciation has been convincingly demonstrated. Once it has been established that a particular barrier sundered the populations of multiple unrelated organisms, it is then of interest to know whether such sundering of populations occurred simultaneously or at different times. In principle, comparative phylogeography can address this question as well, although previous efforts have focused primarily on single-locus estimates of gene divergence and no direct estimates of population divergence times. However, comparisons of single-gene divergence times across multiple codistributed taxa may yield spurious conclusions because gene divergence almost always precedes population divergence and because different gene divergence times will not necessarily reflect different speciation times across the barrier of interest. Indeed, although the population divergence times we obtained using coalescent-based methods strongly suggest that both speciation events occurred entirely within the Pleistocene, gene divergence estimates would have, on average, drastically overestimated these speciation times, in some cases placing them in the Pliocene or earlier (Fig. 7). This result shows just how important ancestral polymorphisms can be in estimates of population divergence times, which have rarely been examined in empirical studies (Melnick et al. 1993; Edwards and Beerli 2000; Arbogast et al. 2002).

Building on earlier anonymous locus work (Karl and Avise 1993), our study has illustrated the utility of multilocus approaches to avian phylogeography. Streamlining of molecular protocols, particularly approaches that would obviate the need to clone each locus from each individual, will help efforts to scale up multilocus comparative phylogeography in several dimensions. Our results, which we have demonstrated to be robust to many of our assumptions, provide preliminary evidence that the Pleistocene may have played an important role in Australian songbird speciation, which if true would parallel the history of songbird speciation in North America (Johnson and Cicero 2004). However, the true sig-

nificance of the Pleistocene as well as the Carpentarian Barrier and Kimberley Plateau–Arnhem Land Barrier to songbird speciation in Australia will not be understood until many similar multilocus based studies are done.

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