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Phylogenetic Relationships and Historical Biogeography of Neotropical Parrots (Psittaciformes: Psittacidae: Arini) Inferred from Mitochondrial and Nuclear DNA Sequences

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Abstract.—Previous hypotheses of phylogenetic relationships among Neotropical parrots were based on limited taxon sampling and lacked support for most internal nodes. In this study we increased the number of taxa (29 species belonging to 25 of the 30 genera) and gene sequences (6388 base pairs of RAG-1, cyt b, NADH2, ATPase 6, ATPase 8, COIII, 12S rDNA, and 16S rDNA) to obtain a stronger molecular phylogenetic hypothesis for this group of birds. Analyses of the combined gene sequences using maximum likelihood and Bayesian methods resulted in a well-supported phylogeny and indicated that amazons and allies are a sister clade to macaws, conures, and relatives, and these two clades are in turn a sister group to parrotlets. Key morphological and behavioral characters used in previous classifications were mapped on the molecular tree and were phylogenetically uninformative. We estimated divergence times of taxa using the molecular tree and Bayesian and penalized likelihood methods that allow for rate variation in DNA substitutions among sites and taxa. Our estimates suggest that the Neotropical parrots shared a common ancestor with Australian parrots 59 Mya (million of years ago; 95% credibility interval (CrI) 66, 51 Mya), well before Australia separated from Antarctica and South America, implying that ancestral parrots were widespread in Gondwanaland. Thus, the divergence of Australian and Neotropical parrots could be attributed to vicariance. The three major clades of Neotropical parrots originated about 50 Mya (95% CrI 57, 41 Mya), coinciding with periods of higher sea level when both Antarctica and South America were fragmented with transcontinental seaways, and likely isolated the ancestors of modern Neotropical parrots in different regions in these continents. The correspondence between major paleoenvironmental changes in South America and the diversification of genera in the clade of amazons and allies between 46 and 16 Mya suggests they diversified exclusively in South America. Conversely, ancestors of parrotlets and of macaws, conures, and allies may have been isolated in Antarctica and/or the southern cone of South America, and only dispersed out of these southern regions when climate cooled and Antarctica became ice-encrusted about 35 Mya. The subsequent radiation of macaws and their allies in South America beginning about 28 Mya (95% CrI 22, 35 Mya) coincides with the uplift of the Andes and the subsequent formation of dry, open grassland habitats that would have facilitated ecological speciation via niche expansion from forested habitats. [Biogeography; divergence times; mitochondrial DNA; molecular clock; molecular phylogeny; Neotropical parrots; nuclear DNA; tribe Arini.]

Neotropical parrots (Psittaciformes: Psittacidae: Arini) occur from Mexico to the extreme south of South America (Forshaw, 1989). They comprise the largest group within the order, numbering 30 genera and 149 of the total 330 recognized species of Psittaciformes (Collar, 1997; Rowley, 1997). They vary greatly in body mass, habitat preference, geographic distribution, and behavior (Forshaw, 1989). The systematics of Neotropical parrots has had a checkered history. Earlier workers, based on external morphology (Salvadori, 1891; Boetticher, 1943, 1959), anatomy (Verheyen, 1956), or behavior (Brereton, 1963), usually split them into two groups with similar composition, sometimes including African taxa (Salvadori, 1891; Boetticher, 1943, 1959). Verheyen (1956) suggested that the two groups Amazoninae and Arinae, which includes only Neotropical parrots, share a combination of anatomical characters that separates them from the other non-Neotropical parrots but did not suggest a category to group them. In a comprehensive review of the Psittaciformes, Smith (1975) rejected previous groupings of Neotropical genera because none of the characters were informative and placed all the Neotropical taxa in the tribe Arini (family Psittacidae) on the basis of two exclusive characters (e.g., chicks hatching with an imperforate ear canal, and copulatory stance on one leg). Smith's proposal has been accepted in all further classifications (e.g., Forshaw, 1989; Collar, 1997). The monophyly of Neotropical parrots was supported by DNA-DNA hybridization studies (Sibley and Alquist, 1990), but with limited taxon sampling, and by DNA sequences from an intron of the spindlin gene located on the avian sex chromosomes (de Kloet and de Kloet, 2005).

Additionally, an analysis of 1771 base pairs (bp) of mitochondrial genes from nine genera of the Arini recovered two monophyletic groups (Miyaki et al., 1998), and referred to them as short-tailed and long-tailed groups, in accordance with an identification key (Sick, 1997). Branch support was generally low, except for the monophyly of Neotropical parrots (Miyaki et al., 1998). Later, this data set was increased to 3245 bp for 13 parrots, but the analyses still did not provide good overall support for the phylogenetic relationships among genera (Tavares et al., 2004). Despite efforts to understand the phylogenetic relationships among Neotropical parrot taxa using molecular data, the relationships among most genera remain unresolved or are not strongly supported in existing phylogenies. These studies have shown polytomies and lack of support at some nodes because of low phylogenetic signal in the DNA sequences analyzed. The recommended strategy to improve tree resolution is to increase the number of taxa and characters sampled for phylogenetic analysis (Poe and Swofford, 1999; Haddrath and Baker, 2001; Slowinski, 2001; Paton

et al., 2002; Pereira et al., 2002). Moreover, several studies indicated that *Aratinga*, *Amazona*, and *Pionopsitta* were not monophyletic genera (Tavares et al., 2004; Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005).

Estimates of divergence times assuming a time-constrained tree, and 100 million years ago (Mya) for the divergence of Galliformes and Psittaciformes, indicated the split between the Australian parakeet *Melopsittacus undulatus* and some Neotropical parrots occurred about 76 Mya, which suggests that continental drift could have been responsible for the isolation of these groups of parrots (Miyaki et al., 1998). The divergences between some Neotropical parrot genera were estimated to have occurred in the late Oligocene and the Miocene (between 27 and 16 Mya) and were likely associated with environmental changes such as sea level regression, orogenic movements in the Andes and expansion of forest/riparian habitat (Miyaki et al., 1998; Tavares et al., 2004). Divergence times between congeneric species of parrots were estimated between 0.5 and 1.3 Mya using the standard mitochondrial rate of 1.6–2.0%/Myr (Tavares et al., 2004; Eberhard and Bermingham, 2004, 2005; Ribas and Miyaki, 2004; Ribas et al., 2005).

In an attempt to better understand the evolution of this group, we obtained a large data set of mitochondrial and nuclear DNA sequences totaling 6388 bp for most genera of the tribe Arini, including representatives of putatively nonmonophyletic genera. We inferred their phylogenetic relationships based on several tree-building methods and checked the phylogenetic utility of morphological characters, some of which were used previously to define groups within Neotropical parrots. As the hypothesis of rate constancy was rejected for Neotropical parrot genera and the method of linearized tree does not account for rate variation of DNA substitution among lineages, divergence times were estimated using methods that accommodate rate variation as Bayesian technique (Thorne et al., 1998; Kishino et al., 2001) and penalized likelihood (Sanderson, 2002). Therefore, the dates estimated were used to help construct a biogeographic hypothesis for the diversification of the group.

Materials and Methods

Taxon Sampling

We obtained samples from 29 species representing 25 of the 30 genera of the tribe Arini (Table 1). As previous studies suggested that the genera *Amazona*, *Aratinga*, and *Pionopsitta* are not monophyletic (Russello and Amato, 2004; Tavares et al., 2004; Ribas and Miyaki, 2004; Ribas et al., 2005), we sampled two or three species from each of these genera to represent the distinct lineages that have been reported. We chose the Australian *Melopsittacus undulatus* (tribe Platycercini), *Cacatua goffini* (family Cacatuidae), and the New Zealand *Strigops habroptilus* (tribe Strigopini, Genbank accession no. NC_005931, Harrison et al., 2004) as representatives of non-Neotropical parrots because each one belongs to a major group of parrots (de Kloet and de Kloet, 2005). *Falco peregrinus* (Genbank accession nos. NC_000878, Mindell et al., 1998; AY461399, Griffiths et al., 2004), and *Gallus gallus* (Genbank accession nos. X52392, Desjardins and Morais, 1990; AF143730, Groth and Barrowclough, 1999) were selected as more distant outgroups to root the topology. The classification of parrots adopted here follows Collar (1997), except for *Propyrrhura*, which was referred to as *Primolius* following Penhallurick (2001) (Table 1).

DNA Extraction and Sequencing

DNA was extracted from blood (except *Brotogeris chiriri*, *Nannopsittaca dachilleae*, and *Pionopsitta barrabandi*, which were from muscle) in a solution containing 0.1% SDS, 100 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA, and 10 mg/ml proteinase K kept overnight at 55°C. DNA purification was performed by standard phenol-chloroform-isoamyl alcohol method (Bruford et al., 1992). Polymerase chain reaction (PCR) amplifications were performed according to Hagelberg (1994) using 25-μl reactions, with a buffer solution containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, and 160 μg/ml bovine serum albumin (BSA), 0.4 mM dNTPs, 0.2 μM of each primer, 1 U *Taq* polymerase Platinum (Gibco), and 25 to 50 ng DNA. PCR cycle conditions for RAG-1 amplifications follow Groth and Barrowclough (1999). Mitochondrial genes were amplified in 36 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 1 min, with an initial denaturation of 94°C for 5 min and a final extension at 72°C for 7 min. Amplified segments were purified by excising bands from agarose gels and centrifuging each through a filter tip. Sequences were obtained on an Li-Cor 4200 bidirectional automated DNA sequencer (Li-Cor Biotechnology) or ABI3100 (Applied Biosystems) according to the manufacturers' suggested protocols. Sequences were obtained from the nuclear gene RAG-1, and the mitochondrial genes cyt b, NADH2, ATPase 6, ATPase 8, COIII, 12S rDNA, and 16S rDNA.

The primers used to amplify RAG-1 were R13, R17, R18, R21, R24, and R2B (Groth and Barrowclough, 1999). Additional primers used only in sequencing reactions were R1, R11, R14, R19, R20, and R22 (Groth and Barrowclough, 1999). The primers used to amplify cytochrome b were b1, b52, and b6 (Kocher et al., 1989). The following mitochondrial primers were developed by Oliver Haddrath (personal communication): for NADH2 amplifications the external primers used were MetL (AAG CTA TCG GGC CCA TAC CCG) and ASNH (GAT CRA GGC CCA TCT GTC TAG), and sequencing primers were ND2H (CCT TGA AGC ACT TCT GGG AAT CAG A) and ND2-54H (GGG GTG GTG AGA TTT TGC GA). For a segment including ATPase8, ATPase6, and COIII the primers used in amplifications were LysL (CAG CAC TAG CCT TTT AAG CT), and COIIIRH (ATT ATT CCG TAT CGN AGN CCY TTT TG), and sequencing primers were A5ATP6 (TAG GAG TGT

TABLE 1. Taxa sampled for this study. Ratio of wing length:tail length and museum skins used to verify the states of the shape of the tail, nostrils and periophthalmic ring are also given.

Species	Sample number[a]	Wing/tail[b]	Skin examined MZUSP[c]	Shape of the tail[d]	Nostrils[f]	Periophthalmic ring[g]
Amazona farinosa	3683	1.9	6731	R	E	N
Amazona xanthops	1876	2.6	4330	R	E	N
Anodorhynchus hyacinthinus	5281*	0.8	32290	C	H	N
Ara ararauna	13	0.8	13992	C	E	N
Aratinga aurea	346	1.2	14893	C	E	N
Aratinga leucophthalmus	2090*	1.2	10653	C	E	N
Aratinga solstitialis	2042	1.1	6490	C	E	N
Bolborhynchus lineola	4393	1.9	13080	C	E	F
Brotogeris chiriri	5486*	1.3	74601	C	E	N
Cacatua goffini	4582	—	—	—	—	—
Cyanoliseus patagonus	4967	1.0	2272	C	E	F
Cyanopsitta spixii	409	0.8	76154	C	E	N
Deroptyus accipitrinus	395	1.4	44059	R	E	N
Diopsittaca nobilis	973	1.2	15897	C	E	N
Enicognathus leptorhynchus	5173	1.2	21754	C	H	F
Forpus crassirostris	501	2.1	39569	R	E	F
Graydidascalus brachyurus	1624*	2.9	22573	R	E	F
Guarouba guarouba	69	1.4	43982	C	E	N
Melopsittacus undulatus	4999	—	—	—	—	—
Myiopsitta monachus	2821	1.1	2277	C	H	F
Nandayus nenday	143	1.2	13083	C	E	N
Nanopsittaca dachilleae	5495*	2.3	—	R[e]	E[e]	F[e]
Orthopsittaca manilata	1852	1.1	38318	C	E	N
Pionites leucogaster	2377	2.1	12226	C	E	N
Pionopsitta pileata	3467	2.1	69441	R	E	N
Pionopsitta barrabandi	389693*	2.4	3501	R	E	N
Pionus maximiliani	1219	2.2	14015	R	E	N
Primolius auricollis	1742	1.1	44077	C	E	N
Pyrrhura leucotis	3921	1.0	34495	C	E	N
Rynchopsitta pachyryncha	5237	1.6	—	C[e]	H[e]	N[e]
Triclaria malachitacea	415	1.4	28102	R	E	N

[a]Catalogue number at the genetic collection, LGEMA, Universidade de São Paulo, except 389693 from Field Museum of Natural History, 5495 from Museu Paraense Emílio Goeldi (voucher MPEG52710), 5486 at Museu de Zoologia da Universidade de São Paulo; [b]ratio obtained from males, measures from Forshaw (1989); [c]voucher number at the skin collection of the Museu de Zoologia da Universidade de São Paulo; [d]R, rounded; C, cunneated; [e]data from the literature (Salvadori, 1891; Forshaw, 1989; Collar, 1997); [f]E, exposed; H, hidden; [g]N, naked and evident; F, feathered; *wild-caught birds, otherwise captive.

GCT TGG TGT GCC ATT) and ATP6L (AAA YAT YTA ATG GCA CAC CAA GC). To amplify the rDNAs 12S and 16S the primers used were L1537 (AAT CTT GTG CCA GCC ACC GCG G) and 12Send (GTG CAC CTT CCG GTA CAC TTA CC), and 16Sa (AAG CCW ANC GAG CYG GGT GAT AGC TGG) and 16Se (GCA CGG TTA GGA TAC CGC GGC CG). Sequencing primers for the 16S segment were 16Sb (CAT AGA TAG AAA CCG ACC TGG) and 16Sc (TTC TTC AAG GTC GCC CCA ACC). The DNA sequences are deposited in GenBank under accession numbers: DQ143208 to DQ143324 and DQ150989 to DQ150996. NADH2 sequences were obtained from GenBank for *Amazona farinosa* (AY194461, Eberhard and Bermingham, 2004), *Pionopsitta barrabandi*, *Pionopsitta pileata*, and *Triclaria malachitacea* (AY669468, AY669481, and AY669486; Ribas et al., 2005), as well as cyt b for *Diopsittaca nobilis* (AF370769; Tavares et al., 2004).

Sequence Analysis

Sequences generated by the automated sequencers were checked for ambiguities using Sequencher 4.1.2 (GeneCodes Corp., Ann Arbor, Michigan). Alignments of coding genes were verified visually in MacClade 4.0 (Maddison and Maddison, 2000). Indels, ambiguously aligned sites, and overlapping bases between ATPase 8 and ATPase 6 and ATPase 6 and COIII were removed from phylogenetic analyses. Base composition, transition and transversion rates, and genetic distances among nucleotide sequences were calculated in PAUP* 4.0b10 (Swofford, 2002). The degree of sequence saturation was evaluated for each gene by plotting the number of transitions and transversions against the corrected pairwise distances.

The variable sites in each gene alignment were tested for stationarity in base composition using TREEPUZZLE 5.0 (Schmidt et al., 2002) because nonstationarity in base composition can bias methods of tree inference and result in erroneous tree topologies (Penny et al., 1990; Lockhart et al., 1994; Foster and Hickey, 1999; Chang and Campbell, 2000; Haddrath and Baker, 2001; Paton et al., 2002). The invariable sites were excluded since they can hinder detection of compositional bias among taxa (Foster and Hickey, 1999). Symmetrical directional mutation pressure acting on all coding gene sequences was estimated with DMP 2.0 (Jermiin et al., 1996) and was

compared with the G+C content in the third codon positions where bias is expected to be greatest.

Phylogenetic Analysis

The best-fit models of nucleotide evolution for each partition and for the combined data set were selected with a hierarchical likelihood-ratio test in ModelTest 3.7 (Posada and Crandall, 1998). Initial Bayesian analyses with Markov chain Monte Carlo (MCMC) sampling was performed with MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) for each gene separately, for the mitochondrial and nuclear partitions independently, and for the concatenated data set to evaluate whether mitochondrial and nuclear partitions were congruent and whether partitioning of genes with different models improved the fit to the data. The final Bayesian analysis was conducted for the combined data set using a partitioned likelihood approach (one partition for each gene), in which parameters were estimated separately for each data partition. Four different runs were executed, each for two million generations, with one cold and four heated chains, sampling once every 1000 trees and with a burn-in time determined by the time to convergence of the likelihood scores. The posterior probabilities of each node were computed for all Bayesian analyses across the sampled trees after burn-in.

The parameters of the models of DNA substitution were estimated in ModelTest 3.7 (Posada and Crandall, 1998) and used as initial estimates for a maximum likelihood (ML) search in PAUP* 4.0b10 (Swofford, 2002), performed with a heuristic search, stepwise addition (five random taxa additions), and tree bisection reconnection (TBR) algorithm. Support at the nodes was estimated by the nonparametric bootstrap (Effron, 1979; Felsenstein, 1985) with 1000 replicates and random addition of taxa in PHYML (Guindon and Gascuel, 2003), using the same parameter values as in the ML search. We compared the resulting tree with that obtained with an ML analysis under a nonhomogeneous model of substitution using the program NHML 3.0 (Galtier and Gouy, 1998), with a correction for rate variation among sites (HKY+Γ). The high number of terminal taxa ($n = 32$) precluded a tree search in NHML; thus, a topological constraint was used on the nodes supported by at least 90% bootstrap support in the homogeneous ML analysis.

Comparison of the Tree Topologies

An approximately unbiased (AU) test (Shimodaira, 2002) implemented in CONSEL 0.1f (Shimodaira and Hasegawa, 2001) was used to compare distinct tree topologies. The AU test uses a multiscale bootstrap technique, where several sets of bootstrap replicates are generated by changing the sequence length, which may differ from that of the original data. The number of times the hypothesis is supported by the replicates is counted for each set to obtain bootstrap probabilities (BP) values along the matrices of different sequence lengths (Shimodaira, 2002).

Mapping and Comparing Morphological, Behavioral, and Sex-Linked Minisatellite Characters

Eight characters that were used to group or diagnose Neotropical parrot genera in previous classifications (Salvadori, 1891; Brereton and Immelmann, 1962; Forshaw, 1989), that were considered in systematic studies (Smith, 1975), or that were suggested to be exclusive to a group (Miyaki et al., 1997) were mapped onto the Bayesian consensus topology using Mesquite 1.05 (Maddison and Maddison, 2004), under likelihood method and Markov k-state one-parameter model to evaluate whether they were phylogenetically informative (assuming that the DNA sequence tree is correct). The characters selected and their states were (1) the length of the tail (Salvadori, 1891); as the definition of the states of this character is not clear, we assumed two states: long (ratio of wing length: tail length lower than 1.7) or short (ratio of wing length: tail length higher than 1.8). The definition of these cutoffs was based on the fact that in the distribution of ratios, no values between 1.6 and 1.9 were observed (Table 1); (2) the shape of the tail: cuneated (feathers are gradually longer from the edges towards the center) or rounded (all the feathers have approximately the same size) (Salvadori, 1891); (3) morphological sexual dimorphism: present or absent (Smith, 1975); (4) bicolored 'pericyclic' iris: present or absent (Smith, 1975); (5) scratching the head with a foot: by pulling the leg 'directly' forward under the wing, or lifting the foot 'indirectly' over the wing (Brereton and Immelmann, 1962); (6) nostrils: exposed or hidden (Salvadori, 1891); (7) periophthalmic ring: naked and evident or feathered (Forshaw, 1989); (8) minisatellites linked to the W chromosome: present or absent (Miyaki et al., 1997). The latter character was used in the comparison but its ancestral states were not reconstructed because not all the taxa sampled here were included in Miyaki et al. (1997); therefore, the presence or absence of the W-linked minisatellite in these taxa is unknown. The lengths of the tail and the wing followed Forshaw (1989; obtained from museum skins). The states of shape of the tail, nostrils, and periophthalmic ring were also determined from an examination of museum skins (Table 1).

Divergence Times

The assumption of rate constancy of DNA substitution through time within the Neotropical parrots was tested using a likelihood ratio test comparing the ML topology obtained with and without a clock constraint in PAUP*4.0b10 (Swofford, 2002). The difference in likelihood values was compared with a chi-square distribution, with the number of taxa minus 2 as the number of degrees of freedom. Estimates of divergence times were obtained using two methods that do not assume constant rates of evolution along the lineages: a penalized likelihood (PL) method and Bayesian inference (Thorne et al., 1998; Kishino et al., 2001).

PL was performed in the program r8s 1.5 (Sanderson, 2003), which assumes a parametric model with a

different substitution rate along each branch and a nonparametric roughness penalty that costs the model more if rates change too quickly between branches (Sanderson, 2002). Prior to the estimation of divergence times, a cross-validation analysis (Sanderson, 2002) was performed in r8s to determine the best smoothing parameter for the data. The branch-lengths used were from the partitioned likelihood Bayesian tree topology including the same taxa used in phylogeny reconstruction, but only with the mitochondrial genes as RAG-1 sequences were not available for *Strigops*.

The Bayesian inference for divergence time estimation was performed in MULTIDIVTIME (MULTIDISTRIBUTE package; http://statgen.ncsu.edu/thorne/multidivtime.html), which uses a probabilistic model to describe the change in evolutionary rate over time. It also uses the Markov chain Monte Carlo (MCMC) procedure to derive the posterior distribution of rates (Thorne et al., 1998; Kishino et al., 2001) and times, and in a multigene data set allows each gene to be analyzed with an independent model (Thorne and Kishino, 2002). Initially, the baseml program in PAML 3.14 (Yang, 1997) was used to obtain estimations of transition/transversion rates, values of substitution parameters under the model HKY85 plus Γ, and a value of the α parameter for Γ distribution for each gene. These data were converted in paml2modelinf of the MULTIDISTRIBUTE package to a file recognized by estbranches, which estimates branch lengths and their variance-covariance matrix. MCMC analyses are conducted in MULTIDIVTIME to approximate the posterior distribution of substitution rates and divergence times. The gamma prior distribution for the expected time between the tip and the ingroup (rtrate) and its standard deviation (SD) were set to 90 and 20 Myr, respectively; rate of root node (rtrate) and its SD were both set 0.094 substitutions/site/unit time as estimated from the median of all tip-to-root of the ingroup branch lengths divided by rttime; rate of change between the ancestral and descendent node (brownmean) and its SD were set such that rtrate times brownmean = 1. A large SD was chosen because it allows a gene to have a large variation in rate change over time and because a priori information for rate change is unknown (Thorne and Kishino, 2002). Multiple runs with different initial states were performed to check for the convergence of the MCMC algorithm by comparing each run's posterior distribution of divergence times, branch lengths, and the proportion of successful changes of those parameters along the Markov chain. The data set used in these estimates was the same used to infer phylogenetic relationships among taxa. As this method allows incomplete taxon sampling across genes, RAG-1 was included in the analysis even though we did not have a sequence for *Strigops*.

Several independent studies of molecular dating of divergence times in birds support the origin of the Orders of the Neoaves around 90 Mya, in the early-mid Cretaceous (Cooper and Penny, 1997; van Tuinen and Hedges, 2001; Paton et al., 2002). This evidence is in agreement with a hypothesis of a vicariant origin for the parrots and other bird lineages mediated by the breakup of Gondwanaland (Cracraft, 1973, 2001), and it is also corroborated by high-level phylogenetic inferences among parrot lineages (Barrowclough et al., 2004; de Kloet and de Kloet, 2005). These analyses suggest that the large New Zealand parrots are sister to all the other parrots (Barrowclough et al., 2004; de Kloet and de Kloet, 2005), and the next most basal monophyletic clade is the traditional Cacatuidae, including *Nymphicus* (Barrowclough et al., 2004). To test if the time of isolation of New Zealand (82 to 85 Mya; Cooper and Millener, 1993) is a reasonable constraint to estimate divergence times in parrots, a data set including the same genes and taxa used in phylogenetic estimates and additional taxa of other bird orders were run in MULTIDIVTIME to independently estimate the age of the node that separates *Strigops* from the other parrots. The taxa added in this analysis were *Rhea americana* (Genbank assession nos. NC_000846; Harlid et al., 1998), *Anas platyrhynchos* (Genbank assession nos L16770, Liu et al., 1996; L22476, Ramirez et al., 1993; AF059082 and AF059142, Johnson and Sorenson, 1998), *Aythya americana* (Genbank assession no. NC_000877; Mindell et al., 1999), and *Anseranas semipalmata* (Genbank assession no NC_005933; Harrison et al., 2004). The topology was constrained to match well-supported phylogenetic relationships in other studies (Groth and Barrowclough, 1999; Livezey and Zusi, 2001; Paton et al., 2002; García-Moreno et al., 2003). Prior information assumed was (1) minimum age of 65 Myr for the common ancestor of screamers and true ducks (Clarke et al., 2005; Pereira and Baker, 2006); (2) the separation of Galloanseres and Neoaves at a lower limit of 90 Mya (Haddrath and Baker, 2001) and an upper limit of 130 Mya (van Tuinen and Hedges, 2001); and (3) a lower limit of 65 Mya for the split of the Psittaciformes and other Neoaves (Cooper and Penny, 1997). The estimated divergence time for the node that separates *Strigops* from the other parrots was 116 (95% CrI 98,127 Mya), which is older than the separation of New Zealand from Antarctica. We therefore calibrated the estimates of divergence times among the genera within the tribe Arini by fixing the age of the node that separates the New Zealand taxon *Strigops* from the other parrots at 85 to 82 Mya, because this split could have predated the vicariant event. As the fossil record for parrots and allies is scanty, and the phylogenetic placement of fossils has not been determined in a cladistic framework, we decided not to use them as minimum age constraints in our analyses.

RESULTS

Sequence Statistics

The PCR amplifications resulted in single products of about 800 to 1300 bp, which are larger than the size of most putative mitochondrial genes translocated to the nuclear genome of chicken (Pereira and Baker, 2004). Additionally, the translation of the protein-coding genes sequenced did not indicate unexpected stop codons or frameshift mutations, and codon positions showed the

expected levels of variation. Given the paucity of nuclear pseudogenes of mitochondrial origin in birds, it is extremely unlikely that any were amplified in our study.

The sequences obtained for each sample totaled 6388 base pairs (bp) long, composed of 2703 bp of nuclear DNA and 3685 bp of mitochondrial DNA (mtDNA). Sequence alignment and phylogenetic trees obtained were deposited in TreeBase (Study accession number: S1522, Matrix accession number: M2730). Substitutions accumulated linearly against corrected distances for RAG-1, 12S, and 16S rDNAs. Transitions at third codon positions were saturated for the remaining genes in comparisons between Neotropical parrots and outgroups, as were transitions at third codon position of ATPase6, ATPase8, and COIII among Neotropical parrots. The average uncorrected pairwise distances calculated separately for each gene show that RAG-1 was the slowest evolving gene (p-distances among Neotropical parrots in the range 0.04% to 4%), as expected for an exon of a nuclear coding gene. Among the mitochondrial genes, the most slowly evolving genes were 12S rDNA (467 bp) and 16S rDNA (483 bp), with p-distances in the range 1.1% to 9.2%, followed by the protein-coding genes COIII (191 bp) and cyt b (888 bp) with p-distances 3.1% to 16.3%, and NADH2 (894 bp) with p-distances 5% to 20%. The most rapidly evolving genes were ATPase 6 (673 bp) and ATPase 8 (89 bp), with p-distances ranging from 4.3% to 26.1%. The transition/transversion ratio for the Neotropical parrots was 3.15, 4.33, and 4.26 for RAG-1, the combined mitochondrial genes, and the concatenated sequences from both genomes, respectively.

The percent base composition for the Neotropical parrot sequences was A = 31.6–31.9, C = 19.7–20.2, T = 24.4–24.9, G = 23.5–23.8 for RAG-1, and A = 29.8–32.5, C = 31.2–34.6, T = 21.2–24.5, G = 12.3–14.2 for the mitochondrial genes combined. These values are in agreement with the expected ranges for nuclear and mitochondrial genes observed in other birds (e.g., Pereira et al., 2002). Significant variation in nucleotide composition ($P < 0.05$) was detected in third codon positions of mitochondrial coding genes of 14 taxa (Table 2), whereas RAG-1 sequences did not have a bias in base composition among taxa. Significant symmetrical directional mutation pressure ($P < 0.05$) towards G+C content was detected for six taxa and towards A+T content for one taxon. Variation in the G+C content was expressed mostly at synonymous sites (Table 2). The concatenated nuclear and mtDNA sequences also were not stationary, but heterogeneity in base composition was reduced from

TABLE 2. Symmetrical directional mutation pressure (μ_D) for the mitochondrial protein coding genes. $\mu_D < 0.5$ and $\mu_D > 0.5$ represent greater A+T and greater G+C contents, respectively. The values represent the GC% observed in the total sequence (Pobs) at synonymous (Psyn) sites, nonsynonymous (Pnon) sites, and third codon positions (P_3). Significant variation in base composition is indicated by $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Taxon	μ_D	Pobs	Pnon	Psyn	P_3
Ara[a]	0.519	0.460	0.412	0.534	0.474
Primolius	0.523	0.460	0.410	0.537	0.479
Orthopsittaca	0.540	0.472	0.418	0.554	0.489
Cyanopsitta	0.539	0.466	0.409	0.553	0.491
Nandayus	0.508	0.452	0.406	0.524	0.463
Aratinga solstitialis[b]	0.502	0.447	0.402	0.517	0.459
Aratinga leucophthalmus	0.533	0.467	0.414	0.548	0.493
Aratinga aurea	0.525	0.460	0.408	0.540	0.475
Guarouba	0.519	0.454	0.400	0.535	0.467
Diopsittaca	0.522	0. 458	0.406	0.537	0.470
Cyanoliseus[b]	0.502	0.448	0.403	0.517	0.462
Anodorhynchus[c]	0.529	0.462	0.408	0.543	0.479
Enicognathus	0.541	0.468	0.410	0.559	0.489
Rhynchopsitta[a,b]	0.485	0.444	0.406	0.500	0.435
Pyrrhura[a]	0.572**	0.481	0.413	0.585	0.523
Deroptyus	0.556**	0.473	0.410	0.570	0.501
Pionites	0.563***	0.475	0.409	0.577	0.513
Forpus[d]	0.514	0.455	0.406	0.529	0.458
Graydidascalus[a]	0.540	0.465	0.407	0.555	0.487
Amazona xanthops	0.535	0.467	0.412	0.549	0.480
Amazona[b]	0.563***	0.474	0.406	0.576	0.512
Pionus	0.551**	0.473	0.413	0.565	0.491
Pionopsitta barrabandi	0.519	0.457	0.407	0.534	0.478
Triclaria[b]	0.520	0.460	0.412	0.535	0.466
Pionopsitta pileata	0.528	0.463	0.411	0.543	0.479
Myiopsitta[a]	0.470**	0.434	0.402	0.483	0.417
Brotogeris[b]	0.565***	0.475	0.408	0.578	0.513
Bolborhynchus	0.541	0.469	0.412	0.555	0.486
Nannopsittaca	0.537	0.466	0.411	0.551	0.477
Cacatua[e]	0.540	0.470	0.414	0.555	0.486
Melopsittacus[d]	0.511	0.455	0.409	0.526	0.457
Falco	0.500	0.454	0.415	0.515	0.455

[a]ATPase 6; [b]cyt b; [c]ATPase 8; [d]NADH2; [e]COIII.

14 to 10 taxa in which the mtDNA bias was evident. The four taxa that have base compositional heterogeneity in mitochondrial sequences, but reached stationarity in the combined data set, were *Anodorhynchus*, *Ara*, *Melopsittacus*, and *Triclaria*.

Phylogenetic Analyses

Bayesian analysis using only the RAG-1 gene or only the combined mitochondrial genes resulted in similar tree topologies (Fig. 1). The RAG-1 tree has higher posterior probabilities at most of the deeper nodes, but lacks resolution in shallower nodes that were better resolved with the mitochondrial data set. The taxa with significantly biased mtDNA base compositions (Table 2) are in the same positions in both trees, suggesting that the bias in base composition is not negatively affecting tree inference (Fig. 1). This conclusion is also reinforced by the fact that the ML analyses with a non-homogeneous evolution model recovered basically the same topology (not shown), differing only in the position of nodes that form a polytomy in the mitochondrial Bayesian consensus tree. Therefore, we opted to use concatenated sequences from both genomes and discuss our results based on this combined data set. Evolutionary models used were NST = 2 plus Γ for Rag1 and NST = 6 plus Γ and proportion of invariable sites (I) for each one of the mitochondrial genes.

The partitioned Bayesian analysis of the combined nuclear and mtDNA data sets resulted in a consensus topology very similar to the one obtained with the mitochondrial genes. In all trees three major clades were recovered (Fig. 2): clade A, parrotlets (*Bolborhynchus* and *Nannopsittaca*); clade B, amazons and allies (*Amazona*, *Pionus*, *Graydidascalus*, *Pionopsitta*, *Triclaria*, *Myiopsitta*, and *Brotogeris*); and clade C, macaws, conures, and allies (*Ara*, *Primolius*, *Orthopsittaca*, *Cyanopsitta*, *Diopsittaca*, *Guarouba*, *Nandayus*, *Aratinga*, *Anodorhynchus*, *Cyanoliseus*, *Enicognathus*, *Rhynchopsitta*, *Pyrrhura*, *Deroptyus*, *Pionites*, and *Forpus*). The node between clade B and clade C was not highly supported (52% and 0.87, ML bootstrap and posterior probabilities of Bayesian analysis, respectively). However, when only *Falco* is used as an outgroup the support of this node is increased to a posterior probability of 0.96.

A nonpartitioned ML search using starting parameters from ModelTest 3.6 recovered a similar topology to the partitioned Bayesian tree (Fig. 2), except that *Pyrrhura* was a sister genus to all other genera in clade C (excluding a clade containing *Deroptyus* and *Pionites*). Both bootstrap and Bayesian posterior probabilities provided good support for most of the clades. However, some nodes with low bootstrap support values in ML analysis have high posterior probabilities. For example, the sister-group relationship between *Forpus* and the rest of the macaws and conures clade had 78% bootstrap support and a posterior probability of 1.0. The homogeneous ML and nonhomogeneous ML searches recovered the same topology, differing only in branches with low posterior probabilities or bootstrap support. These relatively minor topological differences between the trees from the three methods were not significant at the 5% level in the AU test.

Mapping Morphological, Behavioral, and Sex-Linked Minisatellite Characters on the Molecular Tree

Morphological and behavioral characters used in previous classifications were inferred to be homoplasious when mapped onto our phylogenetic hypothesis (Table 1, Fig. 3). Additionally, the minisatellites linked to chromosome W are present in taxa from macaws, conures, and allies, excluding the basal genera *Pionites*, *Deroptyus*, and *Pyrrhura*, and are absent in other genera surveyed within amazons and allies (Fig. 3h). However, the state is not known in the parrotlet clade, in two taxa from amazons and allies, and in four taxa from macaws, conures, and allies. Thus, a wider survey of taxa to investigate the presence of such minisatellites is needed to better evaluate phylogenetic utility of these markers.

Divergence Times

Rate constancy among the Neotropical parrot sequences was rejected by the likelihood ratio test between trees with and without an enforced molecular clock, including the outgroups *Falco* and *Gallus* (log-likelihood of clock tree = −39,016.92; log-likelihood of nonclock tree = −38,962.05; $2\Delta = 109.73$, df = 33; $P < 0.001$), and excluding them (log-likelihood of clock tree = −33,714.15; log likelihood of non-clock tree = −33,664.22; $2\Delta = 99.88$; df = 30; $P < 0.001$). Cross-validation indicated a nonparametric rate-smoothing value of 3.16 to estimate divergence times under the PL method. Divergence times estimated with the PL and Bayesian approach were similar, and thus we discuss the events based on the results of the Bayesian approach as it accounts for uncertainty of branch lengths and time estimates (Table 3, Fig. 4).

Based on 95% credibility intervals (95% CrI), the divergence between the Neotropical and Australian parrot lineages was estimated to have occurred around 66 to 51 Mya, in the Early Eocene. The three extant major lineages of the Arini started their diversification with the separation of the lineage leading to the small parrotlets (clade A) around 57 to 42 Mya (Fig. 4, Table 3), followed by the separation of amazons and allies (clade B) and the macaws, conures, and allies (clade C) at 56 to 41 Mya. The pattern of cladogenesis was distinctively different in clades B and C. Genera of amazons and allies (clade B) radiated in a continuous fashion from as early as 54 Mya to as late as 12 Mya. In contrast, in clade C the separation of the *Forpus* lineage occurred around 54 to 39 Mya, but further cladogenesis of genera followed later from 35 to 4 Mya, after a 20-Myr gap.

DISCUSSION

Phylogenetic Relationships and Implications for Parrot Systematics

In the present study, we sampled most genera of Neotropical parrots and gathered more mitochondrial and nuclear sequence data than previous studies of

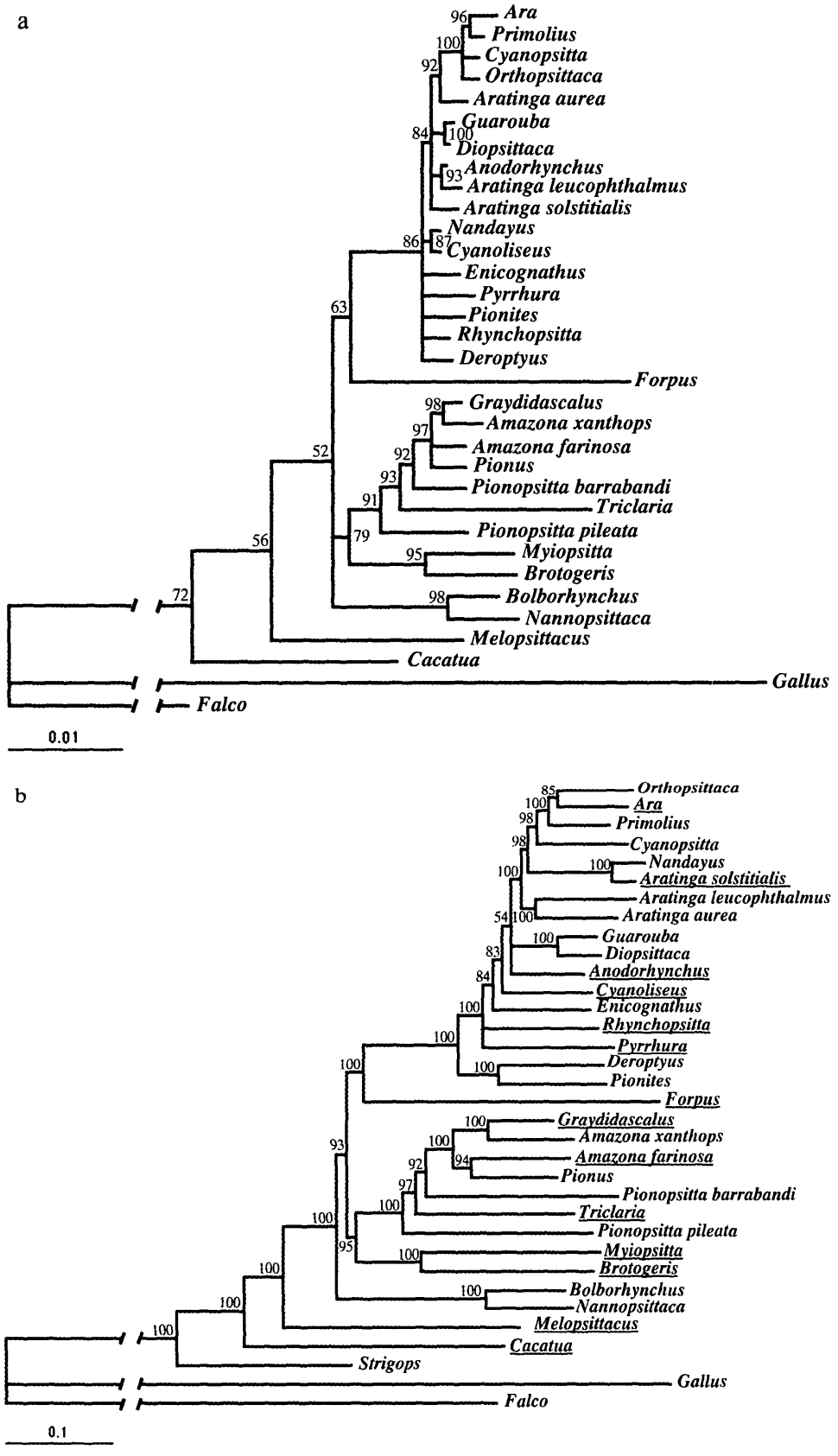

FIGURE 1. Bayesian analysis of 29 Neotropical parrots taxa based on: (a) 2703 base pairs of nuclear RAG-1 sequences; and (b) 3685 base pairs of mitochondrial sequence. Taxa with significant bias in base composition in the mitochondrial genes are underlined. Numbers at the nodes are Bayesian posterior probabilities. Scale bar corresponds to number of expected substitutions per site.

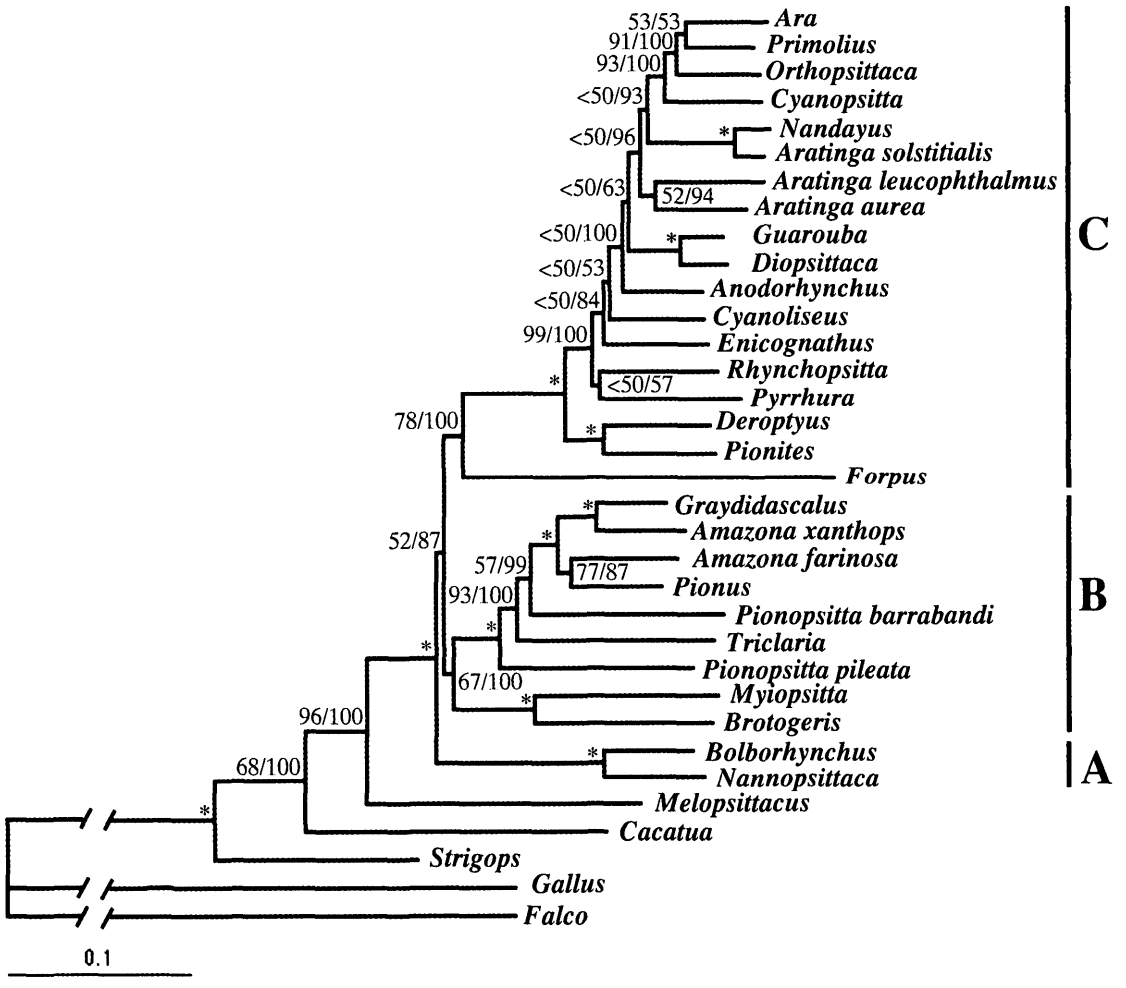

FIGURE 2. Bayesian analysis of 29 Neotropical parrots taxa (representing 25 of the 30 recognized genera) based on combined analyses of 6388 base pairs of nuclear and mitochondrial sequences. Numbers on the nodes are ML bootstrap percentages/Bayesian posterior probabilities, which are represented as asterisks when 100/100. Three major clades are indicated: A, parrotlets; B, amazons and allies; and C, macaws, conures, and allies. The scale bar depicts expected number of substitutions per site.

phylogenetic relationships of Psittaciformes at the genus level (Miyaki et al., 1998, Tavares et al., 2004, de Kloet and de Kloet, 2005). Branch support and resolution were improved by this study as a result of a more complete taxon sampling and the combined analysis of mitochondrial and nuclear sequences (Poe and Swofford, 1999; Haddrath and Baker, 2001; Slowinski, 2001; Paton et al., 2002). Also, it has been shown previously that the combined analysis of the mitochondrial and nuclear sequences provides resolution in different parts of the tree, complementing each other and resulting in a more robust topology (e.g., Pereira et al., 2002).

We detected three major clades within Neotropical parrots: parrotlets of the genera *Bolborhynchus* and *Nannopsitta* (clade A), amazons and allies of the genera *Amazona, Pionus, Graydidascalus, Pionopsitta, Triclaria, Myiopsitta,* and *Brotogeris* (clade B), and macaws, conures, and relatives in the genera *Ara, Primolius, Orthopsittaca, Cyanopsitta, Nandayus, Aratinga, Guarouba, Diopsittaca, Anodorhynchus, Cyanoliseus, Rhynchopsitta, Enicognathus, Pyrrhura, Pionites, Deroptyus,* and *Forpus* (clade C). The inclusion of *Forpus* in clade C is well supported in both Bayesian and ML trees (1.00 and 78%, respectively), but it is on a long branch and its placement may be phylogenetically problematical. Although clades B and C have been recovered in other studies using less dense taxon sampling (Miyaki et al., 1998; Tavares et al., 2004; de Kloet and de Kloet, 2005), a novel proposal in our results is the suggestion that the parrotlets (clade A) are a sister group of all the other taxa. The nonmonophyly of *Aratinga, Amazona,* and *Pionopsitta* was also confirmed as previously suggested (Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005).

Morphological, Behavioral, and Sex-Linked Minisatellite Characters Mapped on the Molecular Tree

None of the morphological or behavioral characters analyzed were congruent with our molecular phylogenetic hypothesis. The size and shape of the tail were the main characters adopted in a previous classification to

define two groups that includes the Neotropical parrots (Salvadori, 1891). Furthermore, the importance of the size and shape of the tail was raised again in a more recent molecular study, where character state variation agreed with the inferred phylogenetic relationships among nine taxa sampled (Miyaki et al., 1998). However, meager taxon sampling may have led to the erroneous conclusion that size or shape of the tail could be phylogenetically informative. The use of these characters as diagnostic descriptors in systematic studies of Neotropical parrots (e.g., Ribas and Miyaki, 2004; Russello and Amato, 2004; Ribas et al., 2005; de Kloet and de Kloet, 2005) should be discontinued.

Divergence Times and Reconstruction of Historical Biogeography

Previous estimates of divergence times among parrots have been proposed based on methods that do not account for rate variation in DNA substitution among lineages. For example, Miyaki et al. (1998) and Tavares et al. (2004) used the linearized tree method (Takezaki et al., 1995), which detects taxa that are evolving at rates that are significantly different from the average and excludes them from the analysis until a set of taxa evolving at a similar rate remains. They then assumed that Galliformes and Anseriformes separated from other Neognath birds around 100 Mya to calibrate their clock. Tavares et al. (2004) also applied the 'standard' mitochondrial rate of DNA substitution of 2.0%/Myr to a set of clock-like sequences. This procedure has also been applied to closely related species of parrots, based on rates of 1.6–2.0%/Myr (Ribas and Miyaki, 2004; Eberhard and Bermingham, 2004, 2005; Ribas et al., 2005). In general, these studies resulted in dates that are younger than the estimates obtained in this study. We believe that our approach to estimate divergence times is more appropriate because we relaxed the assumption of a strict molecular clock by using methods that accounted for rate variation among lineages within Neotropical parrots, accounted for uncertainty in branch lengths and time estimates by a Bayesian approach, and used a hypothesis for the phylogenetic relationships among genera that was better supported at the genus level than previous hypotheses (Miyaki et al., 1998; Tavares et al., 2004). Moreover, the use of an independent geological calibration point

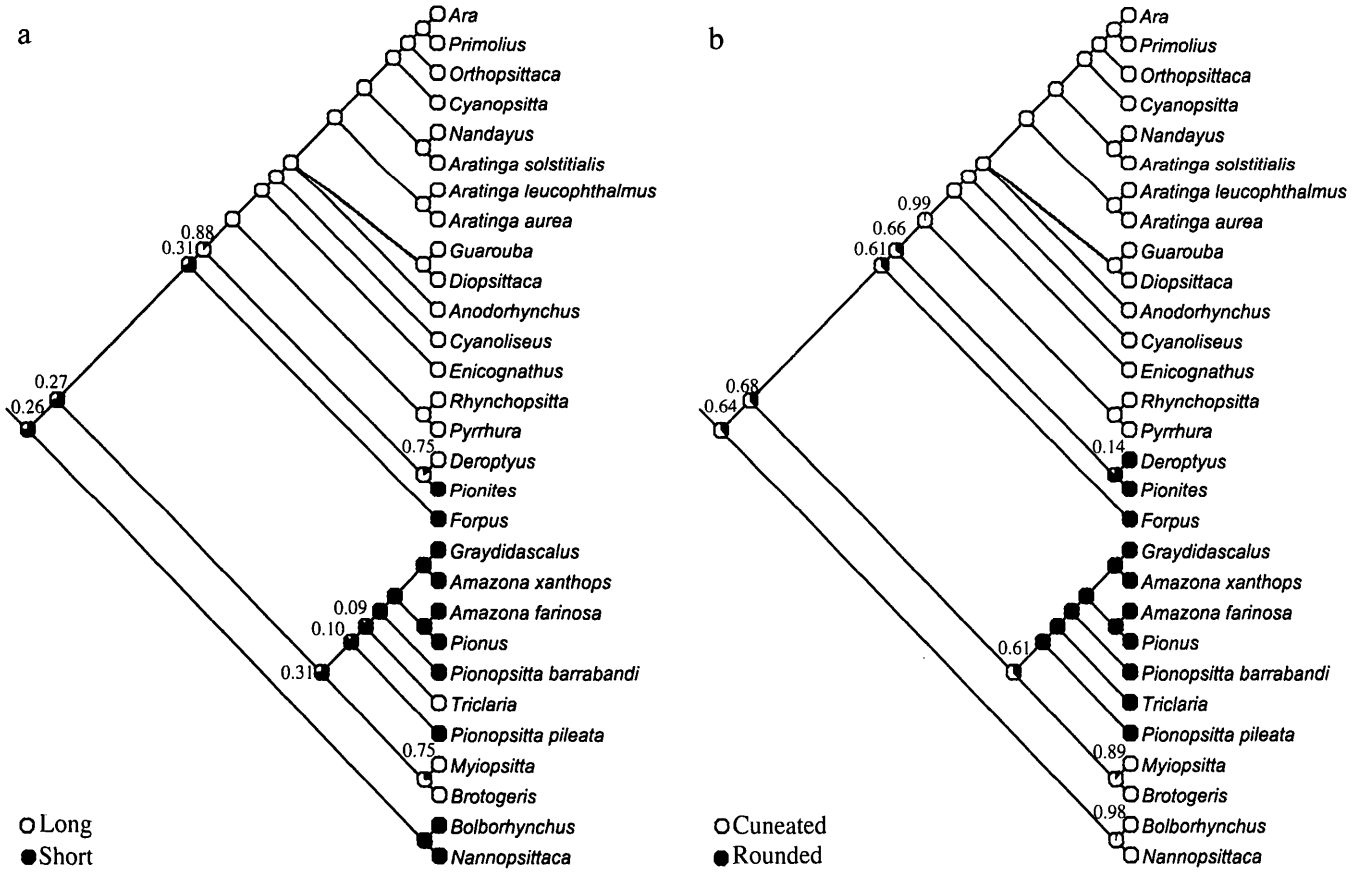

FIGURE 3. Mapping of characters on the molecular phylogeny. (a) Length of the tail; (b) shape of the tail; (c) morphological sexual dimorphism; (d) bicolored pericyclic iris; (e) scratching the head with a foot; (f) nostrils; (g) periophthalmic ring; and (h) minisatellites linked to the W chromosome. The circles at terminal nodes represent the observed character state for the corresponding species to the right. Pie charts at internal nodes represent proportions of the marginal probabilities for the reconstructed character states at that node. Numbers above the charts (a–g) are corresponding proportions of the states represented in white along the characters, when these values are not 0 or 1. The circles on the terminals are states in extant taxa. *(Continued)*

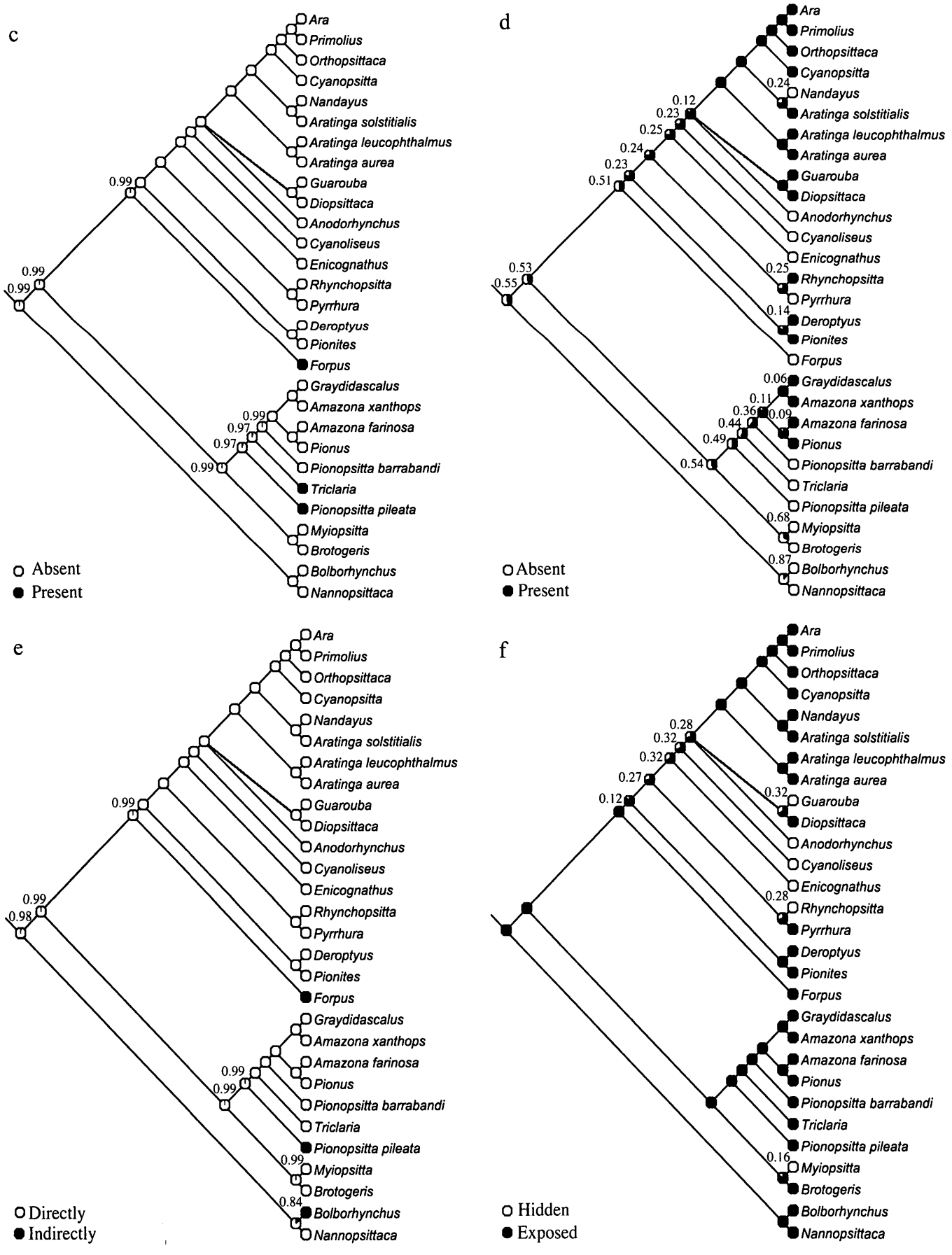

FIGURE 3. (Continued)

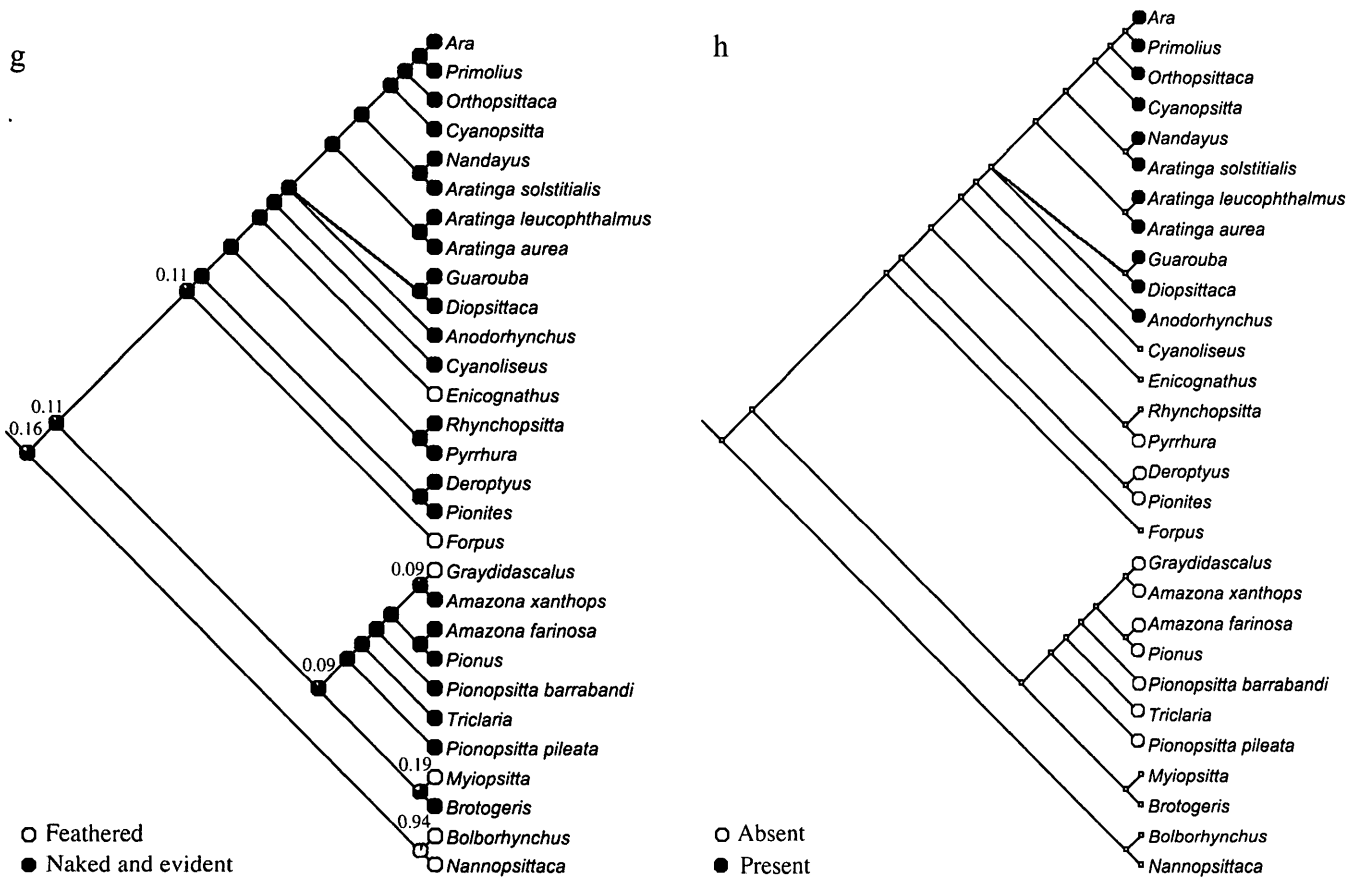

FIGURE 3. (Continued)

(i.e., the separation of New Zealand from the rest of the Gondwanaland) allowed rates to be estimated from the data set, instead of assuming rates estimated for other avian groups (Lovette, 2004).

Based on 95% credibility intervals, the estimated time of divergence of the ancestral Neotropical lineage from the Australian parrots between 66 and 51 Mya implies that at this time the Psittaciformes were widely distributed across the southern continents including Antarctica and South America. This transantarctic pattern of relationship between the fauna of South America and that of Australia and New Zealand has been suggested before for many avian groups, including Psittaciformes (Cracraft, 2001). The separation of Australia from the Antarctica/South America landmass around 55 to 45 Mya, as well as a rise in sea level due to global increase in temperatures (Woodbourne and Case, 1996), is a candidate vicariant event for the split of Neotropical and Australian parrots. Between 60 and 50 Mya, Antarctica was covered with lush, green cool-temperate forests similar to those found today in New Zealand and Tasmania (Poole et al., 2001, 2005) and could have supported a limited diversity of parrots.

The diversification of Neotropical parrots into three major lineages occurred between 57 and 41 Mya, which corresponds to the Late Paleocene and most of the Eocene. At this time, Antarctica was ice-free, wetter, and warmer than today and still connected to South America (Lawver and Gahagan, 2003). Sea level was on average 100 to 200 m above current level (Haq et al., 1987), fragmenting the continent into East Antarctica and West Antarctica by a seaway flowing from the Ross Sea towards the Weddell Sea (Lawver and Gahagan, 2003). Similarly, the southern cone of South America was isolated from the rest of South America by a transcontinental seaway throughout the Eocene and Early Oligocene until at least 30 Mya (Smith et al., 1994). We hypothesize that these isolated areas could have harbored the common ancestors of the three main lineages of extant Neotropical parrots (clades A, B, and C in Fig. 4).

Although it is not clear from our data or from the fossil record which clades were present in those areas, the two contrasting temporal patterns of genus diversification found among clades of Neotropical parrots may give us some clues (Fig. 4). The pattern observed within clade B (amazons and allies) is one of continuous increase in genus diversity throughout the Eocene, Oligocene, and Miocene after the clade originated in the Eocene between 54 and 39 Mya. Therefore, we hypothesize that the ancestor of this clade may already have been present in the southern region of South America or was able to cross the Patagonian transcontinental sea. Because the

TABLE 3. Estimated divergence times of parrot lineages (million of years ago, Mya) using the PL method fixing the node that splits *Strigops* from the other parrots at 85 and 82 Mya and associated 95% confidence intervals (95% CI); and with a Bayesian method and the 95% credibility intervals (95% CrI). Nodes are numbered as in Figure 4.

	PL method				Bayesian Method	
Node	Date	95% CI	Date	95% CI	Date	95% CrI
1	85	Fix	82	Fix	82.9	(82.0; 84.6)
2	72.3	(69.3; 75.0)	69.8	(66.9; 72.3)	67.6	(60.4; 74.8)
3	64.3	(60.8; 68.5)	62.1	(59.0; 65.1)	59.0	(51.5; 66.8)
4	54.5	(51.0; 57.8)	52.6	(49.3; 59.0)	49.7	(42.5; 57.3)
5	21.0	(18.1; 24.0)	20.3	(17.7; 23.2)	18.4	(13.4; 24.2)
6	52.7	(49.3; 56.0)	50.9	(47.7; 54.1)	48.3	(41.2; 55.9)
7	51.2	(47.7; 54.6)	49.4	(16.2; 52.7)	46.1	(39.0; 53.8)
8	37.9	(34.5; 41.4)	36.7	(33.3; 40.0)	32.5	(25.5; 40.2)
9	41.5	(36.8; 45.1)	40.1	(36.7; 43.6)	39.2	(32.2; 46.6)
10	38.7	(35.2; 42.3)	37.4	(34.0; 40.9)	36.2	(29.7; 43.4)
11	37.0	(33.4; 40.6)	35.7	(32.4; 39.2)	33.2	(26.8; 40.2)
12	29.9	(26.3; 33.6)	28.9	(25.4; 32.4)	24.9	(19.4; 31.1)
13	25.8	(22.4; 29.4)	24.9	(21.6; 28.4)	22.4	(17.0; 28.7)
14	20.4	(17.4; 24.2)	19.7	(16.8; 22.9)	16.6	(11.7; 22.1)
15	50.0	(46.6; 53.4)	48.3	(45.1; 51.6)	46.2	(39.0; 53.8)
16	31.4	(28.6; 34.5)	30.4	(27.6; 33.3)	28.2	(22.5; 34.8)
17	23.9	(20.0; 27.0)	23.1	(20.1; 26.1)	22.3	(16.6; 28.7)
18	26.3	(23.5; 29.3)	25.4	(22.7; 28.3)	25.2	(20.0; 31.2)
19	26.0	(23.1; 29.1)	25.1	(22.4; 28.1)	23.4	(18.1; 29.6)
20	24.1	(21.7; 26.9)	23.3	(20.9; 26.7)	23.4	(19.0; 29.2)
21	22.8	(21.0; 25.6)	22.1	(19.8; 24.6)	22.1	(17.3; 27.5)
22	20.9	(18.7; 23.3)	20.2	(17.9; 22.6)	20.6	(16.1; 25.7)
23	9.7	(7.9; 11.8)	9.4	(7.7; 11.3)	8.9	(6.1; 12.3)
24	19.4	(17.3; 21.8)	18.7	(16.7; 21.1)	19.9	(15.5; 24.9)
25	16.7	(14.7; 19.0)	16.2	(14.3; 18.4)	18.3	(13.9; 23.2)
26	18.4	(16.3; 20.7)	17.8	(15.9; 20.1)	19.0	(14.8; 24.0)
27	5.1	(4.3; 6.0)	4.9	(4.2; 5.8)	5.5	(3.6; 7.7)
28	16.7	(14.9; 19.0)	16.3	(14.4; 18.4)	17.2	(13.0; 22.0)
29	14.4	(12.5; 18.6)	13.9	(12.1; 15.9)	15.0	(11.2; 19.5)
30	13.3	(11.4; 15.3)	12.8	(11.1; 14.8)	13.6	(9.9; 18,0)

pattern of cladogenesis corresponds to times of major paleoenvironmental changes in South America, we infer that this diversification could well have occurred exclusively in South America. Conversely, ancestors of parrotlets and of macaws, conures, and allies may have been isolated in Antarctica and/or the southern cone of South America, and only dispersed out of these southern regions when climate cooled and Antarctica became ice-encrusted about 35 Mya (Dingle and Lavelle, 1998; Shevenall et al., 2004). This 'ice house' climate caused a significant drop in sea level during the Early Oligocene (Haq et al., 1987) and caused considerable changes in oceanic and atmospheric circulation (Flynn and Wyss, 1998). A similar pattern of cladogenesis showing a gap between the origin of the group and the radiation of genera has been detected in other Neotropical birds, such as cracids (Pereira et al., 2002) and toucans (Nahum et al., 2003).

The upheaval of the Andes, especially in the last 30 to 8 Mya, changed river systems, atmospheric circulation and rainfall throughout the continent and altered the geographic distribution of the Neotropical biota to a large extent (Lundberg et al., 1998; Nores, 2004). The Andes blocked humid Pacific winds from reaching the interior of the continent (Hooghiemstra and van der Hammer, 1998), leading to the formation of open habitats and grasslands, as inferred by the estimates for the time of diversification of plants of dry environments (Pennington et al., 2004) and by the observation that the mammalian fauna was dominated by hypsodont herbivores, which are usually inferred to be grazers (Flynn and Wyss, 1998). The origin of Neotropical parrots that are today found in open areas, grasslands and savannah, especially in clade C, coincides with the formation of these habitats in South America. The rapid radiation of genera in clade C (macaws, conures, and allies) points to an important role for ecological speciation (Schluter, 2000) via niche diversification as these open-dry areas were colonized from forests.

Although we did not include African parrots in our study, we do not expect their phylogenetic placement to change our biogeographic interpretation of the evolution of Neotropical parrots. Previous work has suggested that African parrots are not monophyletic and may have invaded Africa twice, via Southeastern Asia, and separately via South America (Barrowclough et al., 2004; de Kloet and de Kloet, 2005). The putative colonists from South America are the sister group to the monophyletic Neotropical parrots and therefore their divergence time cannot be younger than 57 to 41 Mya, which is the estimated age for the beginning of the diversification of Neotropical parrots based on our nuclear and

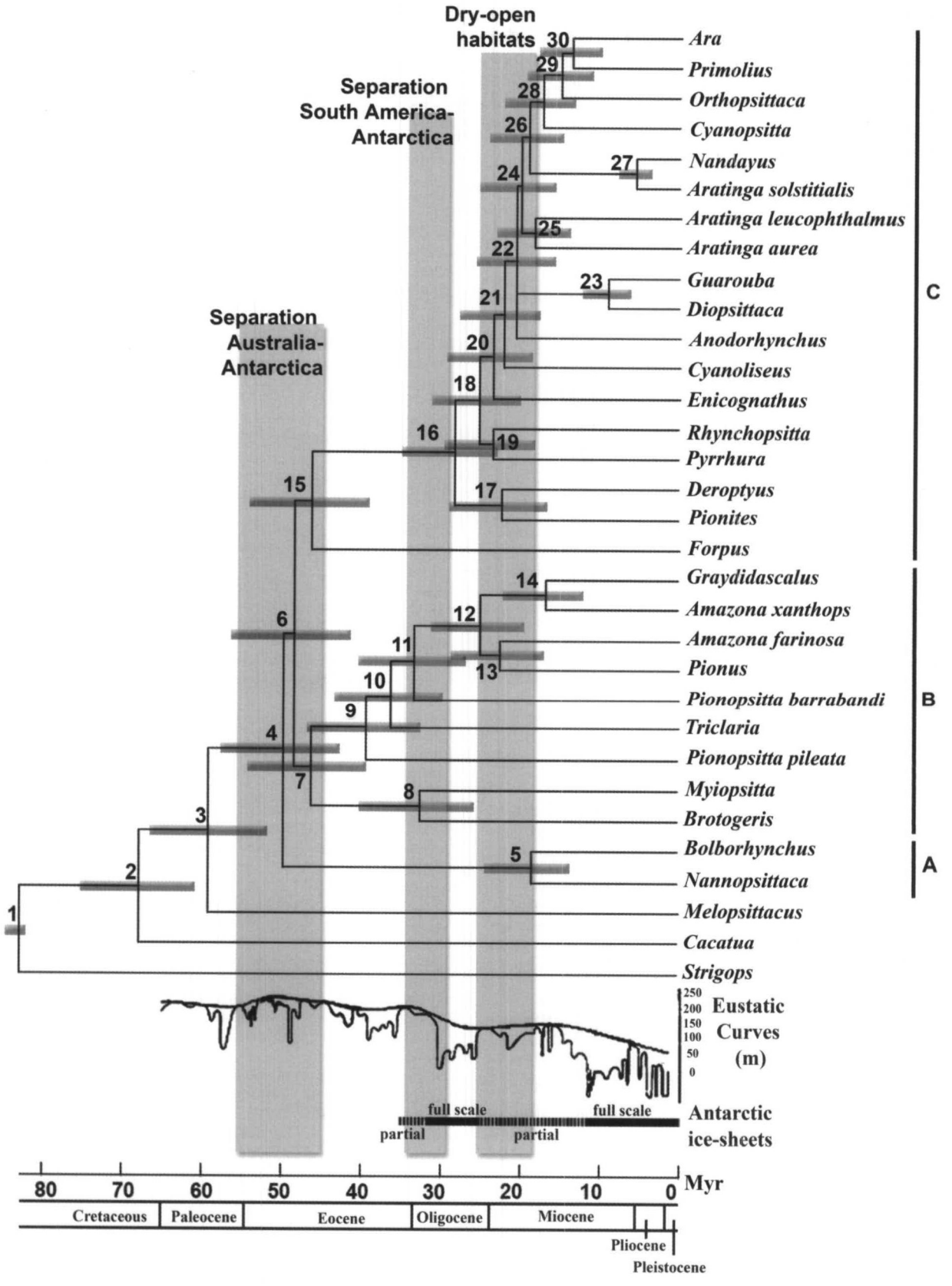

FIGURE 4. Chronogram showing divergence times among the parrot genera and paleoevents possibly related to the Neotropical diversification. Numbers on nodes correspond to the estimated divergence times of lineages as given in Table 3. Horizontal bars at nodes are 95% credibility intervals of divergence times. Clades are defined as A, parrotlets; B, amazons and allies; and C, macaws, conures, and allies. Eustatic curves of sea level are from Haq et al. (1987), and Antarctic ice sheet scale is from Zachos et al. (2001).

mitochondrial DNA sequences. This implies that the invasion of Africa from South America occurred at a time when Africa was already isolated from a supercontinent that would later be fragmented into Antarctica, Australia, and South America and reinforces our hypothesis that the ancestor of parrots and allies were widespread in Gondwanaland.

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Cyanopsitta spixii—monotypic species of its genus, extinct in the wild and last seen in Curaçá (Bahia, Brazil).