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Molecular systematics in *Aratinga* parakeets: species limits and historical biogeography in the '*solstitialis*' group, and the systematic position of *Nandayus nenday*

Camila C. Ribas and Cristina Y. Miyaki*

Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, R. do Matão 277, 05508-090 São Paulo, SP, Brazil

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Abstract

The parrot genus *Aratinga* comprises 20 species that can be separated, based on morphological characters, in at least three distinct groups. We performed a phylogenetic analysis based on mtDNA sequences of individuals belonging to the *solstitialis* group with the objectives of: (1) assessing the genetic differences among individuals in order to clarify their specific status; (2) testing the monophyly of the group and establishing its phylogenetic position relative to other *Aratinga* species, (3) making inferences about temporal and geographical patterns of diversification in the Neotropics. As a result of the analysis, the three taxa belonging to the *Aratinga solstitialis* complex were found to be diagnosable phylogenetic species, the monotypic genus *Nandayus* was found to be included in the *solstitialis* group and the non-monophyly of the genus *Aratinga* was confirmed. Most of the speciation events occurred during the Pliocene and Pleistocene and may be related to habitat shifts associated to climate oscillation during these periods.

1. Introduction

The study of the distribution of organisms in South America has revealed areas of endemism that are common to different groups of animals (Brown, 1977; Cracraft, 1985; Haffer, 1969; Vanzolini and Williams, 1970). The origin of these areas as well as the origin of the high species diversity found in some of them has been the subject of many investigations, and different hypotheses have been proposed to account for these observations. Some of them postulate that the high diversity is due to the tropics being old and stable and having had low extinction rates, so that endemic species accumulated over time (Fjeldså, 1994). Other hypotheses are based on factors that promote speciation in tropical faunas and focus on the geographic context of speciation and the physical causes of isolation. These hypotheses try to deduce historical biogeographic events that promoted speciation based on distributions and phylogenetic relationships of extant taxa, and different models have been proposed (see Bush, 1994; Haffer, 1997; Moritz et al., 2000 for reviews). Among them, the most discussed has been the Pleistocene refugia model (Haffer, 1969), which proposes that climatic changes during the Pleistocene generated contractions and expansions of rainforests and savannas, promoting speciation. Alternatively, an emerging view of diversification in the Neotropics is that no single model can explain it, but instead several of the current models might be integrated to give a better picture of the complex processes producing biological diversity (Bush, 1994; Marroig and Cerqueira, 1997).

Despite all the proposed hypotheses, the origin and history of Neotropical patterns of endemism and diversity are still poorly understood (Bates et al., 1998; Cracraft and Prum, 1988) and comparisons among patterns of distribution and phylogenetic relationships of closely related, allopatric groups of taxa can provide important information to address this issue (Cracraft, 1985; Cracraft and Prum, 1988; Crisci, 2001).

There are 148 recognized species of parrots (Psittacidae) in South America (Forshaw and Cooper, 1989). Most of these species contain subspecies and many of

* Corresponding author. Fax: +55-11-30917553.
E-mail address: cymiyaki@usp.br (C.Y. Miyaki).

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them are endemic. This high degree of geographical differentiation complicates the systematic arrangement of the group, mainly at the specific and subspecific levels, but at the same time makes it a very informative group for biogeographical studies. Detailed systematic studies of the various complexes of subspecies existing today are needed, as the current taxonomy may be hiding a great amount of the diversity that exists in the group.

The genus *Aratinga* comprises 20 species distributed throughout South America, Central America, and the West Indies (Forshaw and Cooper, 1989). Molecular systematic studies of Neotropical genera of psitacids fail to give good support to the monophyly of this genus (Tavares, 2001). Based on plumage and osteological characters several groups of taxa within the genus can be defined. One such group, from now on referred to as the '*solstitialis* group,' comprises *Aratinga weddelli* and three allopatric taxa whose taxonomic status is uncertain: *A. solstitialis* (Linnaeus) 1758, *A. jandaya* (Gmelin) 1788, and *A. auricapilla* (Kuhl) 1820. Peters (1937) listed these as separate species but noted without qualification that they were 'probably conspecific'; this treatment is followed by Forshaw and Cooper (1989). Sick (1997) considers the three taxa 'geographic races (semispecies)' of *A. solstitialis*. Recent treatments recognize the three species but note that they are 'allospecies' (Juniper and Parr, 1998) forming a 'superspecies' (Collar, 1999). The plumage characters that unite *A. solstitialis*, *A. jandaya*, *A.auricapilla*, and *A. weddelli* include: dark blue on the apex of the primaries, dark blue in the primary coverts, ventral side of the rectrices dark, and apex of the dorsal face of the rectrices dark blue. These characters are found only in these taxa among all *Aratinga* species (Silveira, L.F., Lima, F.T., Höfling, E., in preparation. A new species of *Aratinga Spix*, 1824 (Psittacidae) from Brazil, with a revision of the *Aratinga Solstitialis* complex).

Aratinga weddelli occurs in 'terra firme' forest in southwestern Amazonia, *A. solstitialis* in open savannas and savanna woodland, várzea forest and secondary vegetation in eastern Amazonia, *A. jandaya* in northeastern Brazil, where it is restricted to wooded cover, including humid forest edge and adjacent open areas and avoids more arid vegetation, such as caatinga (Fig. 1). *A. auricapilla* occurs in forest edges and cerrado regions in southeastern Brazil (Fig. 1), and two subspecies have been described: *A. a. auricapilla* in the northern part of its range, and *A. a. aurifrons* in the southern part of its range, but the validity of the morphological differences that led to this distinction is being questioned (Silveira et al. in prep.). Individuals of the three taxa are known to hybridize in captivity, but hybridization has never been confirmed in nature (Joseph, 1992).

The taxonomic status of *A. solstitialis*, *A. jandaya*, and *A. auricapilla* as subspecies or distinct species remains uncertain. This has led to uncertainties about

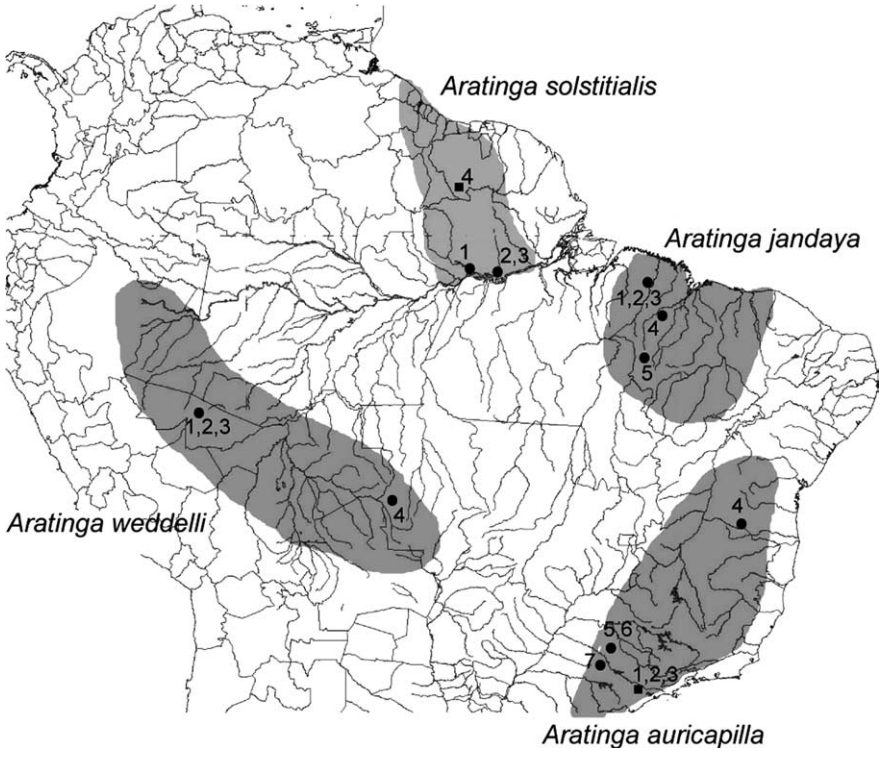

Fig. 1. Distribution of *Aratinga solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* (modified from Forshaw and Cooper, 1989; Sick, 1997; Collar, 1999). Numbers show approximate locations for all samples used in this study. Circles: collection locality; squares: region from which sample originated, exact location not available.

the degree of vulnerability of these taxa and to errors when referring to individuals of the three taxa simply as *A. solstitialis*. Defining the limits of the geographical patterns of variation is important for both taxonomy and conservation, as it allows the recognition of discrete evolutionary and taxonomic units in nature (Cracraft, 1987). Also, the determination of the phylogenetic relationships among these taxa, which present allopatric patterns of distribution that roughly correspond to different areas of endemism described for South America (Cracraft, 1985; Haffer, 1985), will contribute, together with other such studies, to test hypotheses of area relationships (Bates et al., 1998; Cracraft and Prum, 1988; Marks et al., 2002) and to clarify the history of the diversification in the continent.

The aim of this study was to perform a molecular systematic investigation of the relationships and genetic differences among individuals belonging to each of the taxa included in the *solstitialis* group in order to clarify their specific status. Also, by comparing these taxa with other *Aratinga* species and with other psittacine genera we intend to test the monophyly of the *solstitialis* group and establish its phylogenetic position relative to some other *Aratinga* species. Finally, by determining the phylogenetic relationships and the degree of differentiation among taxa in the *solstitialis* group, we try to understand the historical biogeography of the group and to make inferences about the historical processes that originated the present patterns of geographical variation and diversity.

Here we present partial sequences of the mitochondrial 12S and 16S ribosomal subunits, cytochrome *b* gene, as well as the control region for individuals of the four *Aratinga* species mentioned above. The data produced were analyzed in conjunction with a broader data set including 12 species of psitacids. Sequence divergence analysis and molecular clock dating were performed and the divergence dates are related to biogeographical models.

2. Materials and methods

2.1. Taxon sampling

In order to assess patterns of evolution and species limits, sequences were obtained from *Aratinga solstitialis* (four individuals), *A. jandaya* (5), *A. auricapilla* (7), and *A. weddelli* (4) (Table 1, GenBank accession numbers for 12S rDNA, 16S rDNA, cytochrome *b*, and control region, respectively). Blood samples were collected in the field or in captivity, whenever reliable information about geographical origin was available (Fig. 1, Table 1). All the birds were identified by plumage characters and their geographical origins agreed with known species distributions.

In order to test for the monophyly of the *solstitialis* group and to establish how it relates to other psitacid genera, the sequences obtained in the preset study were analyzed in conjunction with a broader data set that included sequences obtained from the GenBank. The potential outgroup sequences used, followed by their GenBank accession numbers for 12S rDNA, 16S rDNA, cytochrome *b*, and control region, respectively, are presented in Table 1. Sequences from two individuals per species were used for *Aratinga cactorum*, *A. aurea*, *A. leucophthalmus*, and *Nandayus nenday*. Nomenclature follows Forshaw and Cooper (1989) and Penhallurick (2001).

2.2. DNA extraction, amplification, and sequencing

Total DNA was isolated from blood samples stored in 95% ethanol using proteinase K, and the phenol-chloroform procedure according to Bruford et al. (1992).

Fragments of four mitochondrial regions were amplified via the polymerase chain reaction (PCR) using the following primers: rDNA12S: L1735 (GGATTAGA TACCCCACTATGC); H2170 (AGGGTGACGGGCG GTATGTACG), rDNA16S: L2702 (CCTACCGAGC TGGGTGATAGCTGGTT), H3309 (TGCGCTACC TTCGCACGGT) (Miyaki et al., 1998); cytochrome *b* L15298 (TGAGGCCAAATATCATTCTGAGGGGC) (Cheng et al., 1994), H15764 (CCTCCTAGTTTGTTG GGGATTGA) (Miyaki et al., 1998); control region: Lglu 16737 (GCCCTGAAAARCCATCGTTG); H522 (TGG CCCTGACYTAGGAACCAG) (Eberhard et al., 2001); and L420 (CACGAGAGATCAYCAACCCGGTGT), H1032 (AAGTGTAAAACAAAGTGCATCAGGGT) (Tavares, 2001).

The amplifications were performed in 10 μl reactions that contained 0.8 mM dNTP, 1 μM of each primer, 0.5 U *Taq* polymerase (Pharmacia), buffer 1× (Pharmacia), and 1 μl of template DNA. Thermal cycling conditions were: an initial denaturation step of 95 °C for 5 min followed by 30 cycles of 95 °C (1 min), 53 °C (40 s), 72 °C (40 s), and a final incubation of 72 °C for 5 min. PCR products were visualized by electrophoresis on agarose gels and purified using Shrimp alkaline phosphatase (0.5 U, USB) and Exonuclease I (5 U, USB). Purified PCR products were used as templates for Big Dye (Perkin–Elmer) terminator cycle sequencing reactions following the manufacturer's recommendations. The same primers used for the first amplification were used in the sequencing reaction. Sequences were read with an ABI 377 automated sequencer. Both strands were sequenced for each region studied.

2.3. Sequence alignment and statistics

The sequences obtained were determined by comparing the heavy and light strands using the programs

Table 1
Individuals studied, their origin and their sequences' GenBank accession numbers (12S, 16S, cytochrome *b*, and control region, respectively)

Taxon	#[a]	Locality	GenBank Accession No.
Ingroup (sequences obtained in the present study)			
Aratinga solstitialis 1	3460	Alenquer, Pará, Brazil	AY208190, AY208210, AY208230, AY208250
Aratinga solstitialis 2	0941	Monte Alegre, Pará, Brazil	AY208193, AY208213, AY208233, AY208253
Aratinga solstitialis 3	0940	Monte Alegre, Pará, Brazil	AY208191, AY208211, AY208231, AY208251
Aratinga solstitialis 4	2807	Pará, Brazil	AY208192, AY208212, AY208232, AY208252
Aratinga jandaya 1	1312	Rio Gurupi, Pará, Brazil	AY208204, AY208224, AY208244, AY208264
Aratinga jandaya 2	0877	Rio Gurupi, Para, Brazil	AY208205, AY208225, AY208245, AY208265
Aratinga jandaya 3	1314	Rio Gurupi, Pará, Brazil	AY208202, AY208222, AY208242, AY208262
Aratinga jandaya 4	4027	Santa Ines, Maranhão, Brazil	AY208203, AY208223, AY208243, AY208263
Aratinga jandaya 5	4026	Imperatriz, Maranhão, Brazil	AY208201, AY208221, AY208241, AY208261
Aratinga auricapilla 1	0662	São Paulo, Brazil	AY208199, AY208219, AY208239, AY208259
Aratinga auricapilla 2	0659	São Paulo, Brazil	AY208196, AY208216, AY208236, AY208256
Aratinga auricapilla 3	0145	São Paulo, Brazil	AY208200, AY208220, AY208240, AY208260
Aratinga auricapilla 4	0036	Rio Pardo, Bahia, Brazil	AY208197, AY208217, AY208237, AY208257
Aratinga auricapilla 5	1144	Adamantina, São Paulo, Brazil	AY208194, AY208214, AY208234, AY208254
Aratinga auricapilla 6	1142	Adamantina, São Paulo, Brazil	AY208195, AY208215, AY208235, AY208255
Aratinga auricapilla 7	4249	Aracatuba, São Paulo, Brazil	AY208198, AY208218, AY208238, AY208258
Aratinga weddellii 1	2065	Acre, Brazil	AY208208, AY208228, AY208248, AY208268
Aratinga weddellii 2	2085	Acre, Brazil	AY208206, AY208226, AY208246, AY208266
Aratinga weddellii 3	2086	Acre, Brazil	AY208207, AY208227, AY208247, AY208267
Aratinga weddellii 4	1318	Pontes e Lacerda, Mato Grosso, Brazil	AY208209, AY208229, AY208249, AY208269
Outgroup (sequences obtained from previous studies[b])			
Anodorhychus hyacinthinus	0114	Mato Grosso do Sul, Brazil	U70741, U70752, U70763, AF430809
Anodorhynchus leari	0410	Raso da Catarina, Brazil	AF362914, AF365407, AF370763, AF430808
Orthopsittaca manilata	2385	Mato Grosso, Brazil	AF362924, AF365417, AF370770, AF430828
Nandayus nenday 1	0143	Mato Grosso do Sul, Brazil	U70746, U70757, AY219914, AF430833
Nandayus nenday 2	0377	Mato Grosso do Sul, Brazil	AF 362937, AF365428, AY219915, AF430834
Aratinga cactorum 1	0837	Tocantins, Brazil	AF362920, AF365413, AF370768, AF430823
Aratinga cactorum 2	0992	Tocantins, Brazil	AF362921, AF365414, AY281254, AF430824
Aratinga aurea 1	0346	Goias, Brazil	U70740, U70751, U70762, AF438818
Aratinga aurea 2	0461	Mato Grosso, Brazil	AY286200, AY286217, AY286208, AF438819
Aratinga leucophthalmus 1	1625	Ilha do Marajo, Para, Brazil	AF362918, AF365411, AF370767, AF430822
Aratinga leucophthalmus 2	2090	Acre, Brazil	AF362919, AF365412, AY281253, AF365434
Ara ararauna	0013	Goias, Brazil	U70739, U70750, U70761, AF430811
Primolius couloni	3142	Acre, Brazil	AF362940, AF365431, AF370780, AF430835
Primolius auricolis	1742	Mato Grosso, Brazil	AF362926, AF365419, AF370772, AF430829
Diopsittaca nobilis	1126	Para, Brazil	AF362923, AF365416, AF370769, AF430826
Guarouba guarouba	0004	Maranhao, Brazil	U70744, U70755, U70766, AF430813

[a] Catalog number at the genetic resources collection, LGEMA, Universidade de São Paulo.
[b] Miyaki et al. (1998), Tavares (2001), and Tavares, E.S., Yamashita, C.Y. (submitted). Phylogenetic relationships among some Neotropical parrot genera (Psittacidae; Aves) based on mitochondrial sequences.

Sequence Navigator and Sequence Analysis (Applied Biosystems). The alignments were performed in Clustal X (Thompson et al., 1997) and manually corrected using MacClade 4.0 (Maddison and Maddison, 1999). The alignments were used to construct character matrices for each studied gene region as well as for combined data. Aligned sequences were examined in order to verify if there were fixed molecular markers characteristic of each of the taxa studied.

Base composition, T_s/T_v ratios, percent sequence divergence (*p*-distance; Nei, 1987) and Kimura's two-parameter distances (K2-P; Kimura, 1980) among sequences were calculated using MEGA (Kumar et al., 2001) and PAUP* (version 4.0b10; Swofford, 1998). Saturation was evaluated for each region by plotting the absolute numbers of transitions against *p*-distance and against K2-P distances for all pairwise comparisons. *p*-Distances were also plotted against K2-P distances for all pairwise comparisons. In both analyses, deviations from linearity would indicate saturation effects.

2.4. Phylogenetic analyses

All phylogenetic analyses were conducted in PAUP* (version 4.0b10; Swofford, 1998). Sequences from the different regions were analyzed both independently and combined using the methods of maximum parsimony (MP) and maximum likelihood (ML). MP analyses were performed with heuristic tree searches, using unordered and equally weighted characters, tree bisection–reconnection (TBR) branch swapping, and 10 random-addition sequence replications. Bootstrap

(1000 replications) and decay indices (Bremer, 1994) were used to determine the relative support for inferred monophyletic groupings.

The likelihood-ratio test as implemented in MODELTEST (Posada and Crandall, 1998) was used to select the simplest model of molecular evolution yielding a significantly higher likelihood than others. The model selected was used for the ML analyses, which were performed using heuristic tree search, TBR branch swapping and 10 random addition replicates. The robustness of the trees found was determined by 100 bootstrap replications.

2.5. Molecular clock and divergence dates estimates

Due to the absence of a reliable psitacid fossil record it was not possible to calibrate a local substitution rate for our data. For this reason, rates of substitution that have been calculated for other avian orders were used. Two different substitution rates were applied to the data: one derived from geological dating of island formation (1.6%/Ma; Fleischer et al., 1998) and the other estimated from fossil records (2.0%/Ma; Randi, 1996; Shields and Wilson, 1987). As both these rates were calculated for cytochrome *b* sequences and the dataset obtained in the present work includes other mitochondrial regions as well, we applied these rates to estimate the date of the most basal split in the ingroup (*A. weddelli* × all other taxa) using the branch lengths of the ML tree constructed from a matrix containing only cytochrome *b* sequences. We then used this date to calculate an internal rate for the ingroup based on the ML tree constructed from the combined dataset. This rate was then applied to the branch lengths of the linearized ML tree constructed using the combined dataset to estimate the divergence dates. For these analyses, matrices containing only the ingroup (22 terminals, see Results) and one outgroup (*Aratinga aurea*) were used. A likelihood ratio test assuming a X^2 distribution with number of taxa minus two degrees of freedom (Huelsenbeck and Rannala, 1997) was applied to these two matrices (cytochrome *b* only and combined) in order to compare the log-likelihood values from ML trees constructed with and without a molecular clock constraint.

3. Results

3.1. Sequence characteristics

Nuclear copies of mitochondrial genes in birds have been previously reported (Quinn, 1997). In the present study, no contaminants were detected in the PCR products; the base composition found was typical of avian mitochondrial DNA; the primers used were designed to amplify avian mitochondrial regions; and all the sequences obtained aligned easily to other psittacine mitochondrial sequences. These facts strongly suggest that the sequences obtained are of mitochondrial origins.

Eberhard et al. (2001) found that the control region is duplicated in the mitochondrial genome of parrots belonging to the genera *Amazona* and *Pionus*. Tavares et al. (Tavares, E.S., Lourenço, R., Miyaki, C.Y., in preparation. Mitochondrial gene arrangements around the control region in some Neotropical Psittacidae genera) have found, through PCR and sequencing analyses of the control region and its flanking genes, that this duplication does not exist in *Anodorhynchus hyacinthinus*, *Ara ararauna*, *Diopsittaca nobilis*, *Guarouba guarouba*, and *Cyanopsitta spixii*, so that these species have the same mitochondrial gene order found in *Gallus gallus*. Through the analysis of the size of PCR amplified fragments (data not shown) we found that the *Aratinga* species studied here also do not present this duplication, so that the control region sequences could be used without concern about wrong hypotheses of homology.

A total of 2010 base pairs (bp) from four different mitochondrial regions (Table 2), were obtained from individuals of *Aratinga solstitialis* ($n = 4$), *A. jandaya* ($n = 5$), *A. auricapilla* ($n = 7$), and *A. weddelli* ($n = 4$). When these sequences were aligned with sequences from

Table 2
Variable sites, informative sites, nucleotide composition, and T_s/T_v ratios of the mitochondrial regions studied

	12S rDNA	16S rDNA	Cytochrome *b*	Control region
Number of base pairs	295	461	340	914
Number variable (%)	14 (4.7)	45 (9.8)	45 (13.2)	137 (15.0)
Number informative (%)	13 (4.4)	42 (9.1)	40 (11.7)	123 (13.5)
%A	31.4	35.2	28.2	22.9
%C	29.2	31.6	34.4	25.5
%G	22.0	16.4	10.7	19.3
%T	17.3	16.7	26.7	32.2
T_s/T_v ratio	7.36 (SE[a] = 3.26)	10.41 (SE = 4.49)	10.21 (SE = 5.80)	5.59 (SE = 1.47)

All 20 *Aratinga* individuals were included in this analysis.
[a] SE, standard error.

the other psittacine genera, a matrix containing 2029 characters was obtained. This length difference is due to the indels that were introduced in the alignment when the more divergent sequences were added. The statistics shown in Table 2 refer only to the sequences obtained in the present work. The alignments are available upon request to the authors.

The alignment of the *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* sequences (ingroup) showed that a small number of indels were present in the 12S and 16S rDNAs and in the control region. There were no indels in the cytochrome *b* region. Base composition analysis of the ingroup sequences showed a higher frequency of cytosine and adenine in all regions with the exception of the control region in which a higher frequency of thymine was found (Table 2). No base composition bias was detected in the dataset, including variable sites only for all the sequences ($X^2 = 10.6734$, $df = 105$, and $P = 1.000$). The number of transitions observed was higher than the number of transversions in all regions (Table 2).

Among the mitochondrial regions sequenced, the rDNAs were the least variable and had the lowest phylogenetic signal (Table 2). Cytochrome *b* had higher variability, mainly in third positions. Translation of the cytochrome *b* sequences showed that there were no differences in amino acid sequence among all *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals. The control region was the most variable region and also had the highest phylogenetic information content for the taxa analyzed (Table 2). This confirms the utility of this region for the study of closely related taxa.

Saturation plots for each studied region did not indicate saturation when comparing the number of transitions with the uncorrected distances for each pairwise comparison (data not shown). A linear relationship between *p*-distances and K2-P distances for all pairwise comparisons was also found for all regions (data not shown). Transition/transversion ratios were high among *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals, which accumulated a small number of transversions.

3.2. Phylogenetic analyses

Phylogenetic analyses included all the sequences obtained in the present work (20 individuals) and data from 16 other psittacines obtained from GenBank (Table 1). This matrix contained 2029 characters and 36 terminals. The inclusion of 16 sequences as putative outgroups had the objective of testing for the monophyly of the ingroup, analyzing the relationship of the ingroup with the other *Aratinga* species included, and placing the new taxa in a phylogenetic context among the other Neotropical psittacine for which sequences were available. As the relationships among Neotropical psittacine genera are not well established and the alignment of the control region among 'long' and 'short' tailed genera is problematic because of a duplication of this region in some short tailed genera (Eberhard et al., 2001), we decided to root the trees obtained with the most distantly related outgroup taxa, the two species belonging the genus *Anodorhynchus*. This procedure did not affect the relationships inside the ingroup, which are the main focus of the present analysis.

In the MP analysis there were 527 parsimony-informative characters, and six most parsimonious trees were generated having 1465 steps, a consistency index of 0.566, and a retention index of 0.786. The strict consensus tree is shown in Fig. 2, along with the values of bootstrap support (1000 replications) and decay indices (Bremer, 1994) (heuristic search, random addition of taxa with 100 replications, TBR). The independent analysis of each region (data not shown) yielded very poor resolution among the outgroup sequences. All four isolated regions datasets recovered the clade composed of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *N. nenday*. Control region and 16S rDNA analysis also included *A. weddelli* in this clade.

For the ML analysis, the evolutionary model chosen by MODELTEST was Tamura–Nei with a proportion of invariable sites of 0.3969 and the gamma distribution shape parameter of 0.6395, the tree obtained had log likelihood of 9895.80761 (Fig. 3). One hundred bootstrap replications were performed, and bootstrap values are shown in Fig. 3. Independent analysis of the different regions (data not shown) generated many polytomies, with the exception of the control region, which did recover all the main clades found in the total evidence analysis.

MP and ML analyses of the combined dataset resulted in similar topologies (Figs. 2 and 3), with some small differences at the tips. In all trees, individuals of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, and *A. weddelli* formed distinct, reciprocally monophyletic clades with high bootstrap and Bremer supports. *A. jandaya* and *A. auricapilla* appeared as sister groups with *A. sosltitialis* external to them. *Nandayus nenday* always appeared as being the sister taxon to the (*A. solstitialis*, *A. auricapilla*, and *A. jandaya*) clade with high bootstrap support, and *A. weddelli* was the next most closely related species to this group. The branch lengths inside this group were very short in both analyses. These results show that *N. nenday* is included in the ingroup. The node uniting *Aratinga cactorum* and *Aratinga aurea* to the ingroup had low bootstrap and Bremer supports in the MP analysis.

3.3. Genetic distances and diagnostic characters

Measures of uncorrected pairwise sequence divergence (*p*-distances) among individuals of *A. solstitialis*, *A. jandaya*, *A. auricapilla*, *N. nenday*, and *A. weddelli* were obtained for each region independently. Individuals

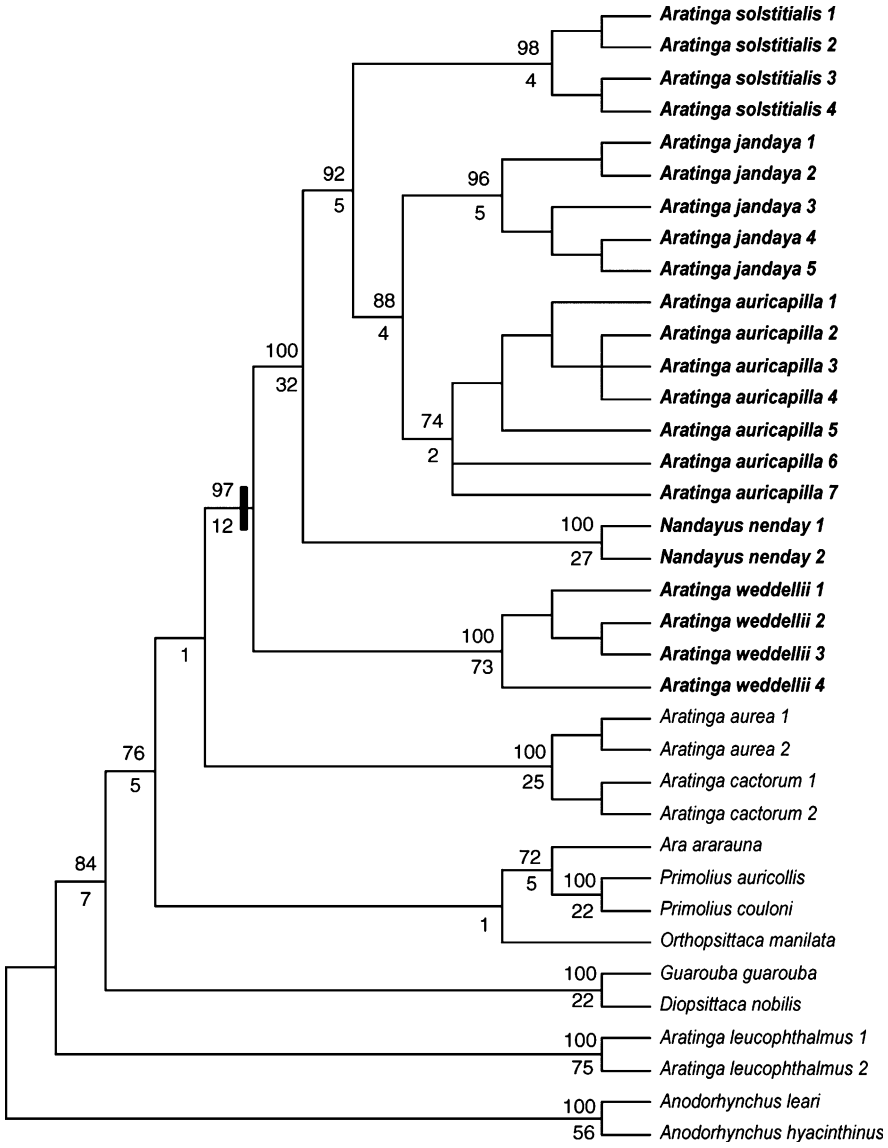

Fig. 2. Phylogenetic reconstruction based on 2029 bp of mitochondrial sequences using MP assuming equal weighting of characters (strict consensus of six trees, $L = 1465$ steps). Bootstrap values (1000 replications) greater than 50% are shown above branches and Bremer decay indices are shown below branches. Bar is located at the base of the ingroup.

of each taxon were grouped and the distances and standard errors for each region were measured within and between groups (Tables 3 and 4). Distances among individuals of the same species ranged from 0.0 to 1.4%, while distances among individuals belonging to different species ranged from 0.0 to 10.7%. The greatest distances were found when comparing control region sequences.

There were 70 variable characters among *A. solstitialis*, *A. jandaya*, and *A. auricapilla*. Forty eight of them were phylogenetically informative for parsimony (three in the 12S rDNA sequence, four in 16S rDNA, seven in cytochrome *b*, and 34 in the control region). Among these, 17 were common to all *A. solstitialis* and differentiate them from *A. jandaya* and *A. auricapilla*, five of these are shared with the outgroup and thus are primitive retentions, and the other 12 are diagnostic for the *A. solstitialis* haplotypes, being ten transitions and two transversions. Eight characters are diagnostic for *A. jandaya* haplotypes, one of them (1147) has a reversion in one *A. auricapilla* sequence (individual 1142) and another (1279) is shared with the outgroup and may be interpretable as a primitive retention depending on the optimization used. This also happens with two (1236 and 1833) of the four diagnostic characters for *A. auricapilla*. The remaining phylogenetically informative characters were common to some individuals belonging to the same species. All the differences involving *A. auricapilla* and *A. jandaya* were transitions.

A MP analysis of the 16 ingroup haplotypes using *N. nenday* as outgroup was performed with the branch and bound option in PAUP*, resulting in four equally

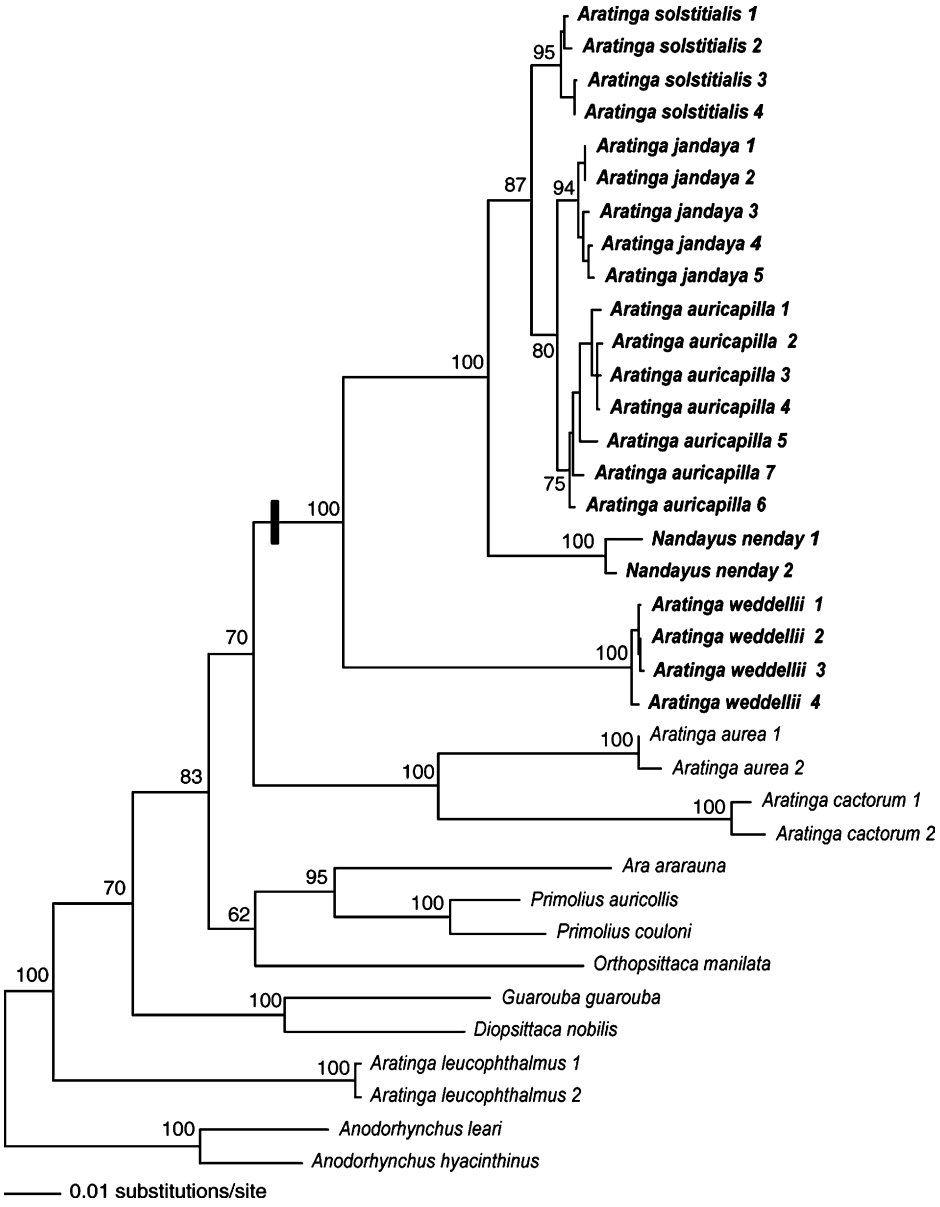

Fig. 3. Phylogenetic reconstruction based on 2029 bp of mitochondrial sequences using Maximum Likelihood (Tr$N + I + \Gamma$; $I = 0.3841$, $\alpha = 0.6459$), bootstrap values (100 replications) shown on branches. Bar is located at the base of the ingroup.

Table 3
Average *p*-distances (%) and standard errors (within brackets) for 12S (295 bp, below diagonal) and 16S (461 bp, above diagonal) between groups of individuals belonging to each taxon. Average *p*-distances within groups (%) shown in the diagonal for 12S and 16S, respectively

	N[a]	1	2	3	4	5
1. *A. auricapilla*	7	**0.0/0.1**	0.7 (0.4)	0.4 (0.3)	2.7 (0.7)	4.5 (0.8)
2. *A. jandaya*	5	0.0 (0.0)	**0.0/0.0**	0.6 (0.3)	2.9 (0.8)	4.7 (0.9)
3. *A. solstitialis*	4	0.7 (0.4)	0.7 (0.4)	**0.5/0.2**	2.6 (0.7)	4.3 (0.8)
4. *N. nenday*	2	0.9 (0.5)	0.9 (0.5)	1.6 (0.6)	**0.3/0.2**	5.8 (1.0)
5. *A. weddelli*	4	3.4 (1.0)	3.4 (1.0)	3.1 (0.9)	3.6 (1.0)	**0.0/0.1**

[a] Number of analyzed individuals from each species.

parsimonious trees of 37 steps (data not shown). The support values were obtained from 200 bootstrap replicates. Individuals belonging to each species cluster together with high bootstrap support (100% for *A. solstitialis* individuals, 97% for *A. jandaya*, and 92% for *A. auricapilla*).

Table 4
Average *p*-distances (%) and standard errors (within brackets) for cytochrome *b* (340 bp, below diagonal) and control region (914 bp, above diagonal) between groups of individuals belonging to each taxon. Average *p*-distances within groups (%) shown in the diagonal for cyt b and CR, respectively

	N^a	1	2	3	4	5
1. *A. auricapilla*	7	**0.6/0.8**	1.9 (0.4)	2.3 (0.4)	4.7 (0.7)	10.5 (0.9)
2. *A. jandaya*	5	0.4 (0.2)	**0.0/0.6**	2.5 (0.5)	5.0 (0.7)	10.1 (0.9)
3. *A. solstitialis*	4	1.7 (0.7)	1.3 (0.6)	**0.2/0.3**	5.1 (0.7)	10.5 (0.9)
4. *N. nenday*	2	3.5 (0.9)	3.1 (0.9)	3.2 (0.9)	**0.3/1.4**	10.7 (0.9)
5. *A. weddelli*	4	9.4 (1.5)	9.2 (1.5)	8.5 (1.5)	10.5 (1.6)	**0.1/0.4**

[a] Number of analyzed individuals from each species.

3.4. Molecular clock

Two different approaches may be used to estimate divergence times from sequence data: a fixed rate of sequence evolution found for other groups of organisms may be applied to the data, or the calibration used may be derived from the data itself, when there is a node that can be dated by the fossil record or some other means. It was not possible to apply this second approach to our data due to the absence of good fossil calibration, and only the rates of sequence evolution were used. Two matrices were constructed containing only the ingroup (*Aratinga solstitialis*, *A. jandaya*, *A. auricapilla*, *Nandayus nenday*, and *A. weddelli*) and one outgroup (*Aratinga aurea*). One matrix contained only the cytochrome *b* sequence (340 bp), whereas the other contained the combined dataset (2012 bp). ML trees were constructed using the models selected by MODELTEST for each matrix: HKY, $T_i/T_v = 7.54$, $I = 0$, equal rates for all sites for the cytochrome *b* data, and $\mathrm{Tr}N + \Gamma$ ($\alpha = 0.153$) for the combined dataset. ML scores for both trees with or without a molecular clock enforced did not show significant differences (LRT: $X^2 = 31.84$, $df = 21$, and $P > 0.05$ for cytochrome *b*, and $X^2 = 28.52$, $df = 21$, and $P > 0.10$ for the combined dataset).

Two different rates were applied to the ML tree derived from the cytochrome *b* data: 1.6%/Ma (Fleischer et al., 1998) and 2.0%/Ma (Randi, 1996; Shields and Wilson, 1987), and two time estimates were obtained for the split between *A. weddelli* and all other taxa (Fig. 4). Using these two time estimates, two new rates were calculated on the ML tree constructed with the combined dataset (1.97% and 2.46%). These new rates refer only to the ingroup sequences and were used to estimate the divergence dates for the other three nodes (Fig. 4).

4. Discussion

4.1. Systematic relationships within Aratinga

The analysis of the 36 psittacine sequences, comprising 16 taxa, showed that *Aratinga solstitialis*, *A.*

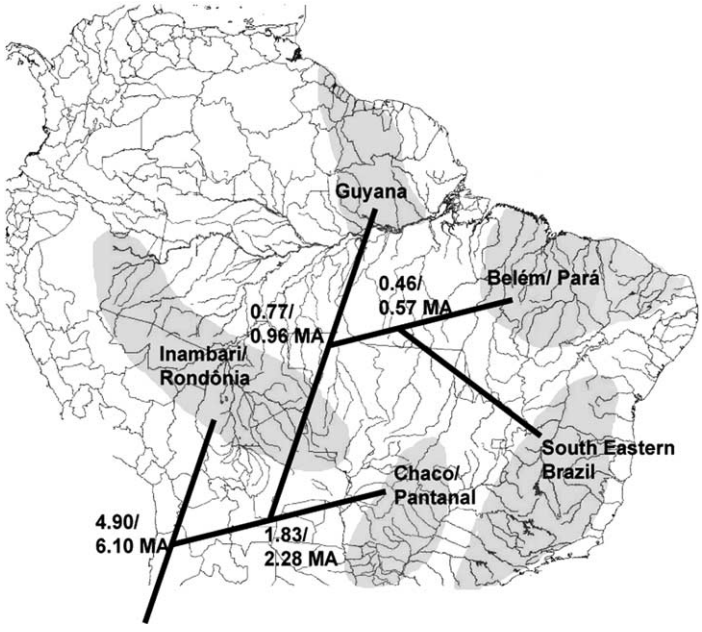

Fig. 4. Area cladogram showing the distribution of the ingroup taxa studied, the areas of endemism where each one occurs and the divergence times estimated for each node using the rates of sequence divergence of 2.0 and 1.6% per million years, respectively.

jandaya, and *A. auricapilla* are closely related, and that individuals belonging to the same taxon cluster together into reciprocally monophyletic groups. Both MP and ML analyses showed that *Nandayus nenday*, initially included as one of the outgroup taxa, is more closely related to (*A. solstitialis*, *A. jandaya*, and *A. auricapilla*) than *A. weddelli*. This close relationship between these taxa has been suggested before only by Miranda-Ribeiro (1920), who proposed the inclusion of *Aratinga solstitialis*, *A. jandaya*, and *A. auricapilla* in the genus *Nandayus*. Our results show that *N. nenday*, presently classified in a monotypic genus, is imbedded in a closely related group of species. *Aratinga cactorum* and *Aratinga aurea* form the sister group to the ingroup in both analyses, but this is not well supported by the MP analysis. *Aratinga leucophthalmus* is distantly related to the other *Aratinga* species in all analyses. Its phylogenetic position among the other psitacines is not well defined with the current taxon sampling, but it is clear that it does not form a monophyletic group with the other *Aratinga* species.

As *A. solstitialis* is the type species of the genus *Aratinga*, the results suggest that *N. nenday* should be included in the genus *Aratinga* and that *A. leucophthalmus* should be assigned to another genus. The analyses presented here also suggest that *A. aurea* and *A. cactorum* may group with the other *Aratinga*, but a better taxon sampling will be necessary to test this issue. Further analyses will also be necessary to determine which other species, presently included in the genus *Aratinga*, group with *A. leucophthalmus*.

4.2. Sequence divergence and diagnosable units

The divergence between *A. jandaya* and *A. auricapilla* individuals is very small and the control region sequence is the only one to show a significantly larger distance between these taxa ($1.9\% \pm 0.4$) when compared to the intra-taxon distances ($0.6\% \pm 0.2$ for *A. jandaya* individuals and $0.8\% \pm 0.2$ for *A. auricapilla* individuals). The divergences found when comparing control region sequences of *A. jandaya* and *A. auricapilla* with *A. solstitialis* are similar ($2.5\% \pm 0.5$ for *A. jandaya* and $2.3\% \pm 0.4$ for *A. auricapilla*) and still quite small when compared to the divergences found between other congeneric pairs of psittacine species, such as *Anodorhynchus leari* and *A. hyacinthinus*, which have a divergence of 5.2% in this same region of the mtDNA. The small amount of genetic divergence found among *A. solstitialis*, *A. jandaya*, and *A. auricapilla* individuals indicates a recent origin for these three forms, but the results presented here suggest that they are distinct and give no indication of interbreeding in nature.

Despite having diagnostic plumage characters, *A. solstitialis*, *A. jandaya*, and *A. auricapilla* are still considered by some authors as subspecies within *A. solstitialis* (e.g., Sick, 1997). Our analyses show that these three taxa have diagnostic characters and form reciprocally monophyletic groupings in the phylogenetic analyses, meeting the two criteria—diagnosability (Cracraft, 1983; Eldredge and Cracraft, 1980) and monophyly (de Queiroz and Donoghue, 1988; Donoghue, 1985; Moritz, 1994)—required to be considered distinct species under the Phylogenetic Species Concept (Cracraft, 1987). This concept recognizes basal and diagnosably distinct taxa as species, avoiding further subdivision of species in subspecies, and thus is appropriate, in this case, to define the evolutionary units.

The sequences analyzed here provide evidence that *A. solstitialis*, *A. jandaya*, and *A. auricapilla* can be distinguished by exclusive nucleotide differences and cannot be further subdivided. Under the Phylogenetic Species Concept they are three separate and well-defined species. It is relevant to note that despite the great similarity among their DNA sequences and osteology, differences in plumage are very distinctive. This is evidence that changes in plumage patterns can occur very rapidly after isolation when compared to genetic divergence, and thus plumage variation may be an important factor driving speciation in these birds. This lack of correspondence between genetic and plumage divergence has been observed in other groups of birds (Driskell, 2002; Greenberg et al., 1998; Zink and Dittmann, 1993), showing that plumage differentiation may sometimes occur so rapidly that there is no time for corresponding genetic differentiation to arise.

4.3. Tempo and mode of diversification

The adoption of a molecular clock to estimate divergence dates based on sequence data is subject to many sources of errors, including the possibility that the molecular data may not evolve in a clock-like manner (Mindell et al., 1996) and that the calibration may be poor. On the other hand, the possibility of adding a temporal framework to phylogenetic analyses allows inferences about the past history of taxa. In this way, despite the many errors that time estimates may incorporate, they have been widely used in phylogenetic analyses (Dimcheff et al., 2002; Groombridge et al., 2002), and different tests and methodologies have been developed to estimate, correct, and reduce errors (Huelsenbeck and Crandall, 1997).

Many authors have estimated rates for mitochondrial sequence divergence and most of them range around 2% sequence divergence per million years (Fleischer and McIntosh, 2001). These rates seem to be good predictors of time for divergences up to 5 Ma. Simulations have shown that predictions of older dates are generally underestimated (Moore et al., 1999). According to our estimates, *A. solstitialis*, *A. jandaya*, and *A.auricapilla* diverged during the Pleistocene, their split from *N.*

nenday occurred during the end of the Pliocene, and *A. weddelli* separated from this group in the end of the Miocene.

The end of the Pliocene and the Pleistocene in South America are marked by the climatic cycles of temperature and humidity change (Hooghiemstra and Ran, 1994). The responses of the vegetation to these changes from hot and humid to dry and cold climate are still poorly understood, and the extent to which these changes influenced the distribution of forests and savannas in the continent is the subject of continuous debate (Bush et al., 1990; Colinvaux and De Oliveira, 2001; van der Hammen, 1974; van der Hammen and Hooghiemstra, 2000). One of the most common critics to the Pleistocene refugia hypothesis is that most species studied so far seem to be older than the Pleistocene, and thus their diversification could not have been caused by the refuges.

The pattern of diversification described here, with at least two speciation events dating to the Pleistocene and another one to the end of the Pliocene (Fig. 4) shows that there are speciation events that may have been related to Pleistocene climatic changes and is one example of species that originated recently enough to have been affected by the Pleistocenic ice ages and the changes in vegetation and climate caused by them. Many other examples of this kind may be found if sufficiently young divergences are investigated. Perhaps in other groups of birds plumage differentiation has been slower, and thus diversification is still not reflected in external characters (and these taxa are still considered conspecific). Detailed population genetic studies may reveal a diversity that is still not known in Neotropical species.

The pattern of phylogenetic relationships found also indicates a shift in habitat preference corresponding to the divergence between the forestal species *A. weddelli* and its sister group. The diversification of *A. solstitialis*, *A. jandaya*, and *A. auricapilla* occurred in open areas associated with forest edges and was probably driven by isolation of the open area habitats of eastern Brazil during more humid periods. These species are still more associated with forests than *N. nenday*, which occurs in the habitats of the open vegetation Chaco area. These habitat shifts may have been important mechanisms of speciation during the climate oscillations that occurred in South America before and during the Pleistocene.

4.4. Historical biogeography

The species studied here have distributions that roughly correspond to one or more areas of endemism delimited by Cracraft (1985), Haffer (1985), and Ridgely and Tudor (1989). *A weddelli* is the only one strictly associated to forests and occurs in the Inambari and Rondônia areas of endemism. Among the other four species, *N. nenday* is strictly associated with open areas, occurring in the Chaco area of endemism. The other three species occur in dry vegetation areas, but are associated with forest edges. *A. solstitialis* occurs in the Guianas area of endemism, but is found only in the Amazonian savannas. *A. jandaya* occurs in the Cerrado and Belém/Pará areas, occupying the edges of the south eastern Amazonian forest. *A. auricapilla* occurs mainly in regions of transition between the Cerrado and the Atlantic Forest areas (Fig. 1).

Some hypotheses, based on the phylogenetic relationships within different groups of birds (Cracraft and Prum, 1988; Prum, 1988; Marks et al., 2002) or on analysis of distributional data of bird species (Bates et al., 1998), have been proposed to explain the relationships among South American areas of endemism. The Atlantic Forest appears in some hypotheses as basal to the Amazonian areas (Bates et al., 1998) and in others it appears as closely related to the southeastern Amazon Belém/Pará area of endemism (Marks et al., 2002). Cracraft and Prum (1988) have suggested that this region is a composite, or biogeographic 'hybrid' area having component taxa with different biogeographic histories.

The sister relationship between *A. jandaya* and *A. auricapilla* is another example of the close association between southeastern Amazonian and southeastern Brazilian faunas (Fig. 4). The barriers isolating these taxa may be related to the arid vegetation that occurs in northeastern Brazil. According to the molecular clock estimations, their separation occurred around 500,000 years ago. There's much debate about the extent to which the climatic changes that occurred during the Pleistocene affected the distribution of forests and open areas, but many authors agree that the climate became significantly cooler during glacial periods (van der Hammen and Hooghiemstra, 2000). As these two species are associated with lowland forest edges and open areas, their differentiation may be related to the development and expansion, in glacial periods, of the very dry vegetation of the caatinga.

Aratinga solstitialis, distributed in the Guiana area, appears as a basal sister-group to the (*A. jandaya* and *A. auricapilla*) clade, thus showing an older split between northern and southern Amazonia than between northeast and southeast Brazil. The history of the Amazonian arid lands is closely related to the interplay between forest and savanna vegetation during the Pleistocene ice ages. If corridors of open vegetation were created in the Amazon forest, the fauna currently associated to the savannas could have been isolated by the re-expansion of the forest and by the marine introgressions postulated to have occurred during the interglacial periods (Haffer, 1969; Haffer, 1997; Marroig and Cerqueira, 1997). The origin of *A. solstitialis* dates to 770,000 to 960,000 years ago and may be related to the 'Amazonian lagoon' postulated to have been formed around 750,000 years ago (Klammer, 1984; Marroig and Cerqueira, 1997).

The split between *N. nenday* and the (*A. solstitialis*, *A. jandaya*, and *A. auricapilla*) clade occurred at the end of the Pliocene. *N. nenday* is part of the Chaco avifauna. This avifauna has low endemism and its components are derived from other habitats, including forest edges (Short, 1975). The lineage that gave origin to *N. nenday* may have originated from a forest ancestor, inasmuch as *A. weddelli* is found in forested habitats (Fig. 4).

Aratinga weddelli from the 'terra firme' forest in western Amazonia is basal to all four species above, indicating that the earliest speciation event separated the western Amazonian species from species associated with more arid vegetation in central and eastern South America and a posterior diversification separated the open area species *N. nenday* from the three *Aratinga* species, associated to forest edges (Fig. 4). This pattern suggests a closer relationship of the Amazonian aridlands with the eastern Brazilian open areas than with western Amazonia. This group of taxa is also an example of a close relationship between forest and arid land species, contradicting the hypothesis of an old split between biotas associated with these two habitats (Haffer, 1985).

The pattern of diversification described here for the *Aratinga* species included in the *solstitialis* group is one more piece of information about the systematic of this complex genus and about the processes of diversification that originated the diversity of species found in South America. Other studies, sampling more species presently included in the genus *Aratinga* will be needed in order to understand the evolutionary relationships among them, and define the monophyletic groupings. The factors that affected speciation in the *Aratinga* species studied here may have been important for many other groups as well and, as phylogenetic studies of South American groups accumulate, the patterns will become clearer making it easier to understand the processes of diversification in the continent.

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