



## Multiple independent origins of mitochondrial control region duplications in the order Psittaciformes

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### ABSTRACT

Mitochondrial genomes are generally thought to be under selection for compactness, due to their small size, consistent gene content, and a lack of introns or intergenic spacers. As more animal mitochondrial genomes are fully sequenced, rearrangements and partial duplications are being identified with increasing frequency, particularly in birds (Class Aves). In this study, we investigate the evolutionary history of mitochondrial control region states within the avian order Psittaciformes (parrots and cockatoos). To this aim, we reconstructed a comprehensive multi-locus phylogeny of parrots, used PCR of three diagnostic fragments to classify the mitochondrial control region state as single or duplicated, and mapped these states onto the phylogeny. We further sequenced 44 selected species to validate these inferences of control region state. Ancestral state reconstruction using a range of weighting schemes identified six independent origins of mitochondrial control region duplications within Psittaciformes. Analysis of sequence data showed that varying levels of mitochondrial gene and tRNA homology and degradation were present within a given clade exhibiting duplications. Levels of divergence between control regions within an individual varied from 0–10.9% with the differences occurring mainly between 51 and 225 nucleotides 3' of the goose hairpin in domain I. Further investigations into the fates of duplicated mitochondrial genes, the potential costs and benefits of having a second control region, and the complex relationship between evolutionary rates, selection, and time since duplication are needed to fully explain these patterns in the mitochondrial genome.

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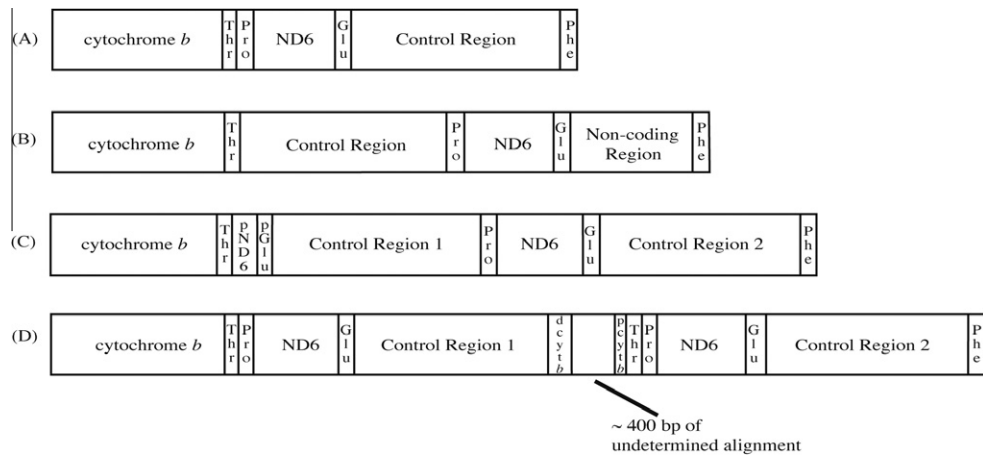
### 1. Introduction

Conservation of genome size, consistent gene content, and a lack of introns or intergenic spacers in animal mitochondria are generally interpreted as evidence that mitochondrial genomes are under selection for small size (Brown et al., 1979; Quinn and Wilson, 1993; Rand, 1993). This selection regime suggests that gene duplications in the mitochondria should be very rare or quickly eliminated because smaller genomes can replicate more quickly (Attardi, 1985; Diaz et al., 2002; Gray, 1989; Rand, 2001;

Selosse et al., 2001; Sogin, 1997). As more mitochondrial genomes are sequenced, however, duplications of mitochondrial genes have been identified with increasing frequency in diverse species such as birds, lizards, ostracods, fish, arthropods, and snakes (Abbott et al., 2005; Arndt and Smith, 1998; Bensch and Härlid, 2000; Black and Roehrdanz, 1998; Campbell and Barker, 1999; Desjardins and Morais, 1990; Eberhard et al., 2001; Gibb et al., 2007; Kumazawa et al., 1996, 1998; Lee and Kocher, 1995; Lee et al., 2001; Macey et al., 1997; Mindell et al., 1998; Moritz and Brown, 1987; Ogoh and Ohmiya, 2004, 2007; Quinn and Mindell, 1996; Shao and Barker, 2003; Shao et al., 2005). It is now clear that duplications do occur in the mitochondrial genome and are much more common than previously thought. However, understanding the underlying mechanisms, evolutionary dynamics, and fitness consequences of

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**Fig. 1.** (a) The typical avian mitochondrial gene order identified by Desjardins and Morais (1990). A tandem duplication followed by random loss of the control region and tRNAs can explain the rearrangement from the typical vertebrate gene order. (b) An alternative avian mitochondrial gene order identified by Mindell et al. (1998). A single tandem duplication followed by incomplete loss of the duplicated region may explain this rearrangement from the typical avian mitochondrial gene order. (c) The mitochondrial gene order identified by Eberhard et al. (2001) in *Amazona* parrots, in which two complete and putatively functional mitochondrial control regions appear to be maintained. (d) The mitochondrial gene order discovered by Abbott et al. (2005) in *Thalassarche* albatrosses in which cytochrome *b* to the control region is tandemly duplicated. Only the second copy of cytochrome *b* shows any degradation as depicted by the labels pcytb and dcytb, which are regions with high similarity to the full-length cytochrome *b* gene.

these duplications remains an ongoing challenge for the field of molecular evolution.

Mitochondrial duplications often occur as tandem arrays, with a gene or group of genes repeated one after the other (Campbell and Barker, 1999; Eberhard et al., 2001; Abbott et al., 2005). Several mechanisms have been proposed that would result in this type of structure. Slipped strand mispairing can frequently result in tandem duplications in the presence of repeat units or sequences that form secondary structures (Levinson and Gutman, 1987; Madsen et al., 1993; Mueller and Boore, 2005). During DNA replication, a portion of the DNA strand dissociates between two repeats forming a loop. The polymerase then reassociates at the first repeat and duplicates the looped section (Levinson and Gutman, 1987; Madsen et al., 1993; Boore, 2000). The consistent presence of repeats at either end of the junctions of mitochondrial tandem duplications in parthenogenetic lizards led Fujita et al. (2007) to conclude that slipped strand mispairing was likely the cause of duplications. Over-running the termination signal during DNA replication has also been suggested as a way to form tandem duplications (Boore, 2000; Mueller and Boore, 2005). In this case, the duplications would include the genes flanking the origin of replication, which is often seen in mitochondrial duplications (San Mauro et al., 2006). Initiation of replication at sites of secondary structure other than the origin has also been suggested to result in tandem duplications (Levinson and Gutman, 1987; Madsen et al., 1993; Stanton et al., 1994; Lunt and Hyman, 1997; Macey et al., 1997; Boore, 2000). tRNAs or other sequences that are capable of forming secondary structures often are found at the ends of duplicated regions, suggesting that these structures may cause illicit priming of mitochondrial replication (Stanton et al., 1994; San Mauro et al., 2006). Finally, unequal crossing over could potentially result in tandem duplications when two mitochondrial genomes within a single mitochondrion recombine with one genome donating its copies of a group of genes to the other genome (see Ohno, 1970; Zhang, 2003 for this mechanism in the nucleus).

Consideration of the fates of duplicated nuclear genes suggests four potential fates for duplicated mitochondrial genes: (1) non-functionalization in which one copy becomes a pseudogene and is eventually eliminated from the genome, (2) subfunctionalization, in which copies of a multifunctional gene can each become specialized for one of the different original functions, and will each be stably maintained within the genome because they are under

selection to carry out different functions, (3) neofunctionalization, in which a duplicated gene acquires a novel function due to mutations in the regulatory region or within the gene copy, and (4) redundant maintenance, in which multiple copies of a gene are maintained through gene conversion or purifying selection because the extra copies help meet high expression demands (Force et al., 1999; Lynch et al., 2001; Rastogi and Liberles, 2005; Roth et al., 2007; Zhang, 2003). However as mitochondria are believed to be under selection for compactness, it would seem that nonfunctionalization and elimination of extra gene copies would be the most likely fate of mitochondrial duplications (Rand and Harrison, 1986). Depending upon which copy of a gene is eliminated, a new gene arrangement may arise or the original gene order may be restored (Boore, 2000). This hypothesis has come to be known as the tandem duplication/random loss model of mitochondrial genome rearrangement (Boore, 2000; Macey et al., 1997; Mindell et al., 1998; Mortiz, 1991). In this model, loss of duplicated genes is thought to occur rapidly relative to evolutionary time (Mortiz, 1991; Quinn, 1997). Therefore, residual evidence of a previous duplication such as the presence of pseudogenes may be suggestive of a relatively recent event (Mortiz, 1991; Quinn, 1997).

Four different mitochondrial genome arrangements have been identified within birds (Class Aves) that differ from that of the typical vertebrate (Fig. 1). The common avian arrangement, first identified in the chicken by Desjardins and Morais (1990), can be derived from the common vertebrate arrangement (ND6/tRNA<sup>Glu</sup>/cyt *b*/tRNA<sup>Thr</sup>/tRNA<sup>Pro</sup>/control region) by one tandem duplication/random loss event (involving cyt *b*/tRNA<sup>Thr</sup>/tRNA<sup>Pro</sup>/ND6/tRNA<sup>Glu</sup>/control region). Later, Mindell et al. (1998) described a second arrangement in which a non-coding region of variable length and with some similarity to the control region was found in the typical location of the control region, while the full-length control region was located after tRNA<sup>Thr</sup>. A single tandem duplication/random loss event is necessary to derive this arrangement from the common avian mitochondrial arrangement (Mindell et al., 1998). This second gene order has been found in several diverse orders of birds, such as Piciformes (woodpeckers), Cuculiformes (cuckoos), Falconiformes (falcons), Passeriformes (oscines and suboscines), and Tinamiformes (tinamous) (Bensch and Härlid, 2000; Haddrath and Baker, 2001; Mindell et al., 1998). A third arrangement of mitochondrial genes was found in several species of *Amazona* parrots (Eberhard et al., 2001). In this case, one degenerate copy of the

duplicated ND6 and tRNA<sup>Glu</sup> was still present making the extent of the duplication more easily defined. Additionally, the second non-coding region showed high similarity with the control region and appeared to be functional. This arrangement has also been found in the osprey (*Pandion haliaetus*), ivory-billed aracari (*Pteroglossus azara*) and Philippine hornbills (*Aceros waldeni* and *Penelopides panini*) (Gibb et al., 2007; Sammler et al., 2011). The fourth arrangement was identified in *Thalassarche* albatrosses (Abbott et al., 2005). Here, the genes from cytochrome *b* to the control region were tandemly duplicated and most appeared to still be functional. However, the second copy of cytochrome *b* appeared greatly reduced in size with only portions of the 5' and 3' ends (designated as d-cyt *b* and p-cyt *b*) being alignable with the full-length copy. The two control regions were also easily alignable and appeared to be functional, but differed in sequence and length of domain III, with control region 1 lacking repeats at the 3' end (Abbott et al., 2005). A similar rearrangement has also been found in the black-faced spoonbill (*Platalea minor*) (Cho et al., 2009), the ruff (*Philomachus pugnax*) (Verkuil et al., 2010), three species of booby in the genus *Sula* (Morris-Pocock et al., 2010) and two species of Philippine hornbills (Sammler et al., 2011).

Despite the many descriptions of avian mitochondrial gene arrangements that have been published, we still lack a clear understanding of when or how often mitochondrial duplications have occurred in birds. Few orders have been systematically surveyed for gene arrangements or have been paired with a well-sampled phylogeny to allow robust conclusions about the evolutionary history of mitochondrial duplications and genome rearrangements. The order Psittaciformes (parrots and cockatoos, hereafter 'parrots'), presents an excellent opportunity to identify the frequency with which mitochondrial control region duplications occur within a clade. Eberhard et al. (2001) established that several species of Neotropical parrots contained a duplicated control region, while preliminary data from other parrots suggested that these duplications were not shared by the entire order (T.F. Wright, J.R. Eberhard, unpublished data; E.S. Tavares, C.Y. Miyaki, unpublished data).

The current study seeks to address the following two questions: (1) Does the mitochondrial control region duplication, first identified in *Amazona* parrots, exist in other parrot genera? (2) If so, was there a single origin or were there multiple independent origins of these duplications? To answer these questions, we surveyed 117 parrot species by PCR for the presence of mitochondrial control region duplications and mapped these results onto a phylogeny reconstructed from mitochondrial and nuclear intron DNA sequences.

## 2. Materials and methods

### 2.1. Taxon and character sampling

For the phylogeny and survey of mitochondrial control region duplications, we added 51 new taxa to the dataset of Wright et al. (2008) for a total of 117 parrot species representing 79 of the 82 extant genera (Tables 1S and 2S). We used a stratified sampling method to determine the number of species sampled per genus such that genera with one to four species were represented by a single species or 25–100% coverage, genera with 5–11 species had two representatives (18–40% coverage), genera with 12–16 species were represented by three species (19–25% coverage) and genera with more than 17 species had four representatives (13–24% coverage). The new species included in this study were chosen based upon the accessibility of tissue or blood samples in museum or zoo collections. Samples for three genera (*Geopsittacus*, *Ognorhynchus* and *Oreopsittacus*) were unobtainable. Taxonomic nomenclature follows Forshaw (2006) for Old World species and the 2010 AOU North American and South American checklists for

New World species (Chesser et al., 2010; Remsen et al., 2010). *Coccyzus americanus* (Cuculiformes), *Colinus colinus* (Coliiformes), *Columbina passerina* (Columbiformes), *Falco peregrinus* (Falconiformes), *Otis sunia* (Strigiformes), *Picus canus* (Piciformes), *Serinus canarius* (Passeriformes) and *Tockus flavirostris* (Coraciiformes) were included as outgroups as each has been identified as an ally or a sister group of the parrots in previous studies (Ericson et al., 2006; Fain and Houde, 2004; Hackett et al., 2008; Sibley and Ahlquist, 1990; Sorenson et al., 1999).

For the phylogenetic analyses, we sampled two mitochondrial protein-coding loci (cytochrome *c* oxidase I (COI) and nicotinamide adenosine dehydrogenase subunit 2 (ND2)) and two nuclear introns (tropomyosin intron five (TROP) and transforming growth factor beta 2 intron one (TGFB2)). These genes have proven to be informative in other phylogenetic studies of parrots (Wright et al., 2008; Joseph et al., 2012).

### 2.2. DNA extraction, PCR and sequencing

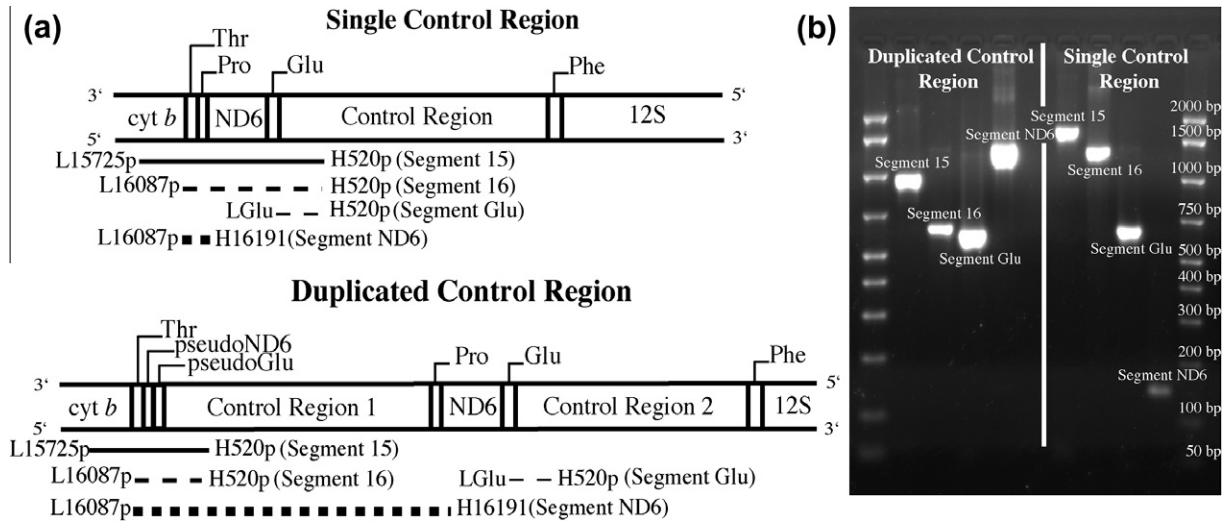
We extracted DNA from tissue or blood samples, performed polymerase chain reaction amplification (PCR), and sequenced the PCR products at laboratories in three locations due to legal restrictions on transporting specimens from endangered species. The laboratories were New Mexico State University (NMSU) in Las Cruces, New Mexico; the Instituto Nacional de Toxicología y Ciencias Forenses (Spain) in Tenerife, Canary Islands, Spain; and the University of São Paulo (Brazil) in São Paulo, Brazil.

At NMSU and in Spain we extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol for each tissue type. In Brazil, we extracted DNA from blood samples using a phenol/chloroform protocol (Bruford et al., 1992). At all locations we amplified the four gene regions by PCR using primers, reactions, and cycling conditions as described in Wright et al. (2008). PCR products were checked for correct size and the presence of multiple bands by electrophoresis on a 0.5–2% agarose gel and stained with ethidium bromide.

We cleaned PCR products using a QiaQuick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions at NMSU and in Spain, while in Brazil we used 1  $\mu$ L exonuclease I and 0.5  $\mu$ L of shrimp alkaline phosphatase per 10  $\mu$ L PCR reaction incubated at 37 °C for 30 min, then 80 °C for 15 min to clean PCR products. We sequenced each PCR product in both directions using the PCR primers and Big Dye v3.1 Terminator Cycle Sequencing chemistry (Applied Biosystems Inc, Foster City, CA). Each sequencing reaction at NMSU and in Spain consisted of 2  $\mu$ L of BigDye, 1  $\mu$ L of 5X sequencing buffer, 3.2  $\mu$ L of 1 $\mu$ M primer, 2  $\mu$ L of clean PCR product, and 11.8  $\mu$ L of water. In Brazil each sequencing reaction consisted of 2  $\mu$ L of BigDye, 2–4  $\mu$ L of clean PCR product, and 1  $\mu$ L of primer. Sequencing conditions at all locations were 25 cycles of 95 °C for 25 s, 50 °C for 5 s, and 60 °C for 4 min. We cleaned sequencing reactions at NSMU by centrifugation through Sephadex columns. Clean reactions were dried and resuspended in 20  $\mu$ L of HiDi Formamide (Applied Biosystems Inc., Foster City, CA) before sequencing on an ABI 3100 Avant automated sequencer. In Brazil sequencing reactions were cleaned by isopropanol/ethanol precipitation, dried, resuspended in 1.8  $\mu$ L of formamide, heated to 95 °C for 2 min, placed on ice until loaded on an ABI 377 automated sequencer. In Spain, we cleaned sequencing reactions by centrifugation through Centri-Sep columns in a 96 well format (Applied Biosystems Inc., Foster City, CA). The cleaned reaction was dried, resuspended in 20  $\mu$ L of formamide, and sequenced on an ABI 310 automated sequencer.

### 2.3. Phylogenetic analysis

Raw sequences were checked for ambiguous base calls in Sequencher 4.7 (Gene Codes, Ann Arbor, MI) and combined into



**Fig. 2.** Location of primer pairs and relative fragment lengths used to classify control region duplication state. (a) Schematic of fragment sizes based upon the presence of a single or duplicated mitochondrial control region. (b) A representative agarose gel of fragment sizes for a duplicated and single control region.

contigs by locus and taxon. Sequences were aligned using Clustal W with default parameters as implemented at <http://www.ebi.ac.uk/Tools/clustalw> and adjusted by eye. Gaps within introns were coded by the simple indel coding method (Simmons and Ochotereña, 2000) as implemented in IndelCoder 0.5 in the SeqState 1.40 program (Müller, 2005, 2006).

Maximum likelihood methods of phylogenetic reconstruction were implemented in GARLI v0.951 using the default settings (Zwickl, 2006). The General Time Reversible model with a gamma distribution of among site heterogeneity and a proportion of invariant sites was chosen, with parameter values estimated by the software and SPR branch swapping (Zwickl, 2006). To ensure that the tree was not located in a local optimum, 20 independent runs were conducted in GARLI and the tree with the highest likelihood was chosen for subsequent analyses (Zwickl, 2006). Nodal support was evaluated by 100 maximum likelihood bootstraps calculated in GARLI.

Bayesian methods of phylogeny reconstruction were implemented using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). A separate evolutionary model was determined for each gene region in MrModelTest 2.3 under the Akaike Information Criterion (Nylander, 2004). Gaps were coded as restriction sites using the default settings. The analysis consisted of two parallel runs, each with one cold chain and three heated chains with default parameters. The mixed model analysis of the combined dataset was run for 15,000,000 generations with trees sampled every 1000 generations and a burn-in of 25%. Convergence was assumed when the average standard deviation of split frequencies was less than or equal to 0.01 and when the effective sample size (ESS) value for parameter values was greater than 200 when viewed in Tracer v1.4 (Rambaut and Drummond, 2007).

#### 2.4. Mitochondrial control region survey

Of the 117 species included in the phylogeny 112 were surveyed at NMSU for the presence of a mitochondrial control region duplication using PCR to amplify diagnostic fragments from the regions predicted to differ in length depending on the presence or absence of a duplicated control region. We could not survey five taxa using the PCR approach (*Strigops habroptilus*, *Cyanoramphus auriceps*, *Cyanopsitta spixii*, *Enicognathus leptorhynchus* and *Triclaria malachitacea*) because tissue samples were not available at NMSU. We used the primer pairs L15725p – AAACCAGARTGATAYTTYC TMTTYGCAT (modified from Sorenson et al., 1999) and H520p –

TKGSCCTGACCKAGGAACCAG (modified from Sorenson et al., 1999), L16087p – TGGYCTTGAARCCAAARRAYGAAG (modified from Sorenson et al., 1999) and H520p and L16087p with H16191 – TCTCGDGGGGCDATTCGGC (Sorenson et al., 1999) to amplify diagnostic Segments 15, 16 and ND6 respectively. An additional primer pair, LGlu – GCCCTGAAAARCCATCGTTG (Eberhard et al., 2001) in conjunction with H520p was used to amplify Segment Glu to verify the presence of a second control region that is usually flanked by the intact tRNA<sup>Glu</sup>. The expected location of each primer and the genes contained within each segment are depicted in Fig. 2. PCR reaction conditions were the same as for the phylogeny except for the cycling conditions which were 94 °C for 4 min followed by 29 cycles of 94 °C for 25 s, 60 °C for 30 s on the initial cycle with a decrease of 0.4 °C each cycle, and 72 °C for 2 min. This was followed by 6 cycles of 94 °C for 25 s, 45 °C for 30, 72 °C for 2 min, with a final extension of 72 °C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Gels were imaged on a BioRad Gel Doc XR and band sizes for each segment were estimated using the BioRad Amplisize 50–2000 bp Molecular Ruler and the Band Analysis protocol in the Quantity One software (BioRad Life Sciences, Hercules, CA).

Expected sizes of the diagnostic segments used to score each species as a single or duplicated control region were as follows: Single Control Region, Segment 15 = 1579–1896 base pair (bp), Segment 16 = 1225–1542 bp, Segment ND6 = 132 bp; Duplicated Control Region, Segment 15 = 800–1250 bp, Segment 16 = 600–1000 bp, Segment ND6 ≥ 1200 bp. These segment sizes were derived from preliminary studies of parrot control region duplications and then further refined by analysis of the GenBank parrot mitochondrial sequences (Table 3S and T.F. Wright, J.R. Eberhard, E.E. Schirtzinger, unpublished data). Band sizes were expected to show some variation due to the variation in size of domains I and III of the control region (Baker and Marshall, 1997) as determined by alignment of primer H520p to parrot control region sequences available on GenBank. Another source of variation is the presence and size of intergenic spacers. This variation was evaluated by counting the base pairs between the 3' end of one annotated gene and the 5' end of the next annotated gene in the region of cytochrome *b* to tRNA<sup>Phe</sup> from three parrot mitochondrial genomes on GenBank (*Strigops habroptilus*, *Agapornis roseicollis* and *Melopsittacus undulatus*). These ranges were added to the sizes of the genes included in each segment to get the total estimated range of variation. A species was scored as having a single or duplicated control region based on the correspondence of its measured



segment sizes to the expected sizes for each segment (Fig. 1S). Taxa that did not amplify at least two diagnostic segments were classified as unscorable.

### 2.5. Sequencing of selected taxa

Because the expected band sizes encompass a large range, diagnostic Segment 16 and Segment Glu, from selected species were sequenced at NMSU or Brazil to confirm the status of the mitochondrial control region as classified by our PCR survey. At least one representative of each clade that contained an inferred duplicated control region was sequenced. In addition, species that were ambiguous in their classification were also sequenced. Finally, the GenBank mitochondrial sequences for *Agapornis roseicollis*, *Strigops habroptilus* (single control regions) and *Melopsittacus undulatus*, *Amazona farinosa*, *Amazona ochrocephala* and *Psittacus erithacus* (duplicated control regions) were also used as confirmation of the PCR survey results.

At NMSU, PCR products were amplified, cleaned as described above, and sent to the University of Chicago Cancer Sequencing Facility for sequencing on an ABI 3730 automated sequencer using Big Dye chemistry. In Brazil, amplifications for sequencing were performed in 10  $\mu$ L reactions with 1X buffer (GE Healthcare or Biotools), 2  $\mu$ M of dNTP, 1  $\mu$ M of each primer, 0.5 U of *Taq* polymerase, and 20–50 nanograms (ng) of template DNA, or in 25  $\mu$ L reactions with 1X buffer (Biotools), 2  $\mu$ M of dNTP, 1  $\mu$ M of each primer, 1 U of *Taq* polymerase, and 25–50 ng of DNA. PCR conditions were: initial denaturation 96 °C for 5 min, 30 cycles of 95 °C for 60 s, 50–54 °C for 25 s, and 65 °C for 40–80 s, with a final extension of 65 °C for 5 min. The size and quality of PCR products were verified, purified as described above or bands were excised from agarose gels and the product was isolated by centrifugation through filter tips (Axigen). Sequencing reactions were prepared, cleaned, and run as described above.

Raw sequences were proofread as previously described and combined into a consensus sequence by taxon and segment using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). The location of tRNA<sup>Thr</sup>, tRNA<sup>Pro</sup>, ND6, and tRNA<sup>Glu</sup> were identified by comparison with the homologous genes from the mitochondrial genome of *Melopsittacus undulatus* (NC\_009134), while the control region was identified by the presence of the goose hairpin (C<sub>7</sub>TAC<sub>7</sub>) near the 5' end. The identity of pseudogenes was based on similarity with known sequences from the *Melopsittacus undulatus* mitochondrial genome or comparison with the pseudogenes defined by Eberhard et al. (2001). Functionality of tRNAs was assessed by simulation in tRNA scan-SE (Lowe and Eddy, 1997).

For each species sequenced, the gene order from tRNA<sup>Thr</sup> through domain I of the control region was identified by similarity with previously described avian gene orders (Abbott et al., 2005; Desjardins and Morais, 1990; Eberhard et al., 2001; Mindell et al., 1998). For those species with a duplication, we measured the length of the non-coding region (calculated as the number of nucleotides from the end of tRNA<sup>Thr</sup> to the goose hairpin), and the number of nucleotide differences between the two control regions (calculated as the number of differences when the two control region fragments were aligned divided by the total length of the aligned control region segment). Because the 5' end of the control region does not have a definitive starting motif and the 3' end of the tRNA<sup>Glu</sup> could not be identified for all species, the goose hairpin was used as a proxy for the beginning of the control region.

### 2.6. Ancestral state reconstruction

Using the classifications from the PCR survey, sequences from selected taxa and GenBank sequences, the presence of a single (0) or duplicated (1) control region was coded into a matrix and

mapped onto the phylogeny. Three species (*Aprosmictus erythropterus*, *Eos reticulata*, *Pionites melanocephala*) were coded as unscorable because they either failed to amplify the diagnostic fragments by PCR or they produced ambiguous results and no confirmatory sequence was available. Ancestral state reconstructions were undertaken in Mesquite 2.01 (Maddison and Maddison, 2007) under parsimony (Fitch) and the maximum likelihood criterion. Likelihood reconstructions were undertaken using the symmetric MK1 model, which is a generalization of the Jukes–Cantor model with equal probability of changing states and the AsymmMK model, which uses different rates of change between states (Maddison and Maddison, 2007). To test the robustness of the likelihood analysis, separate reconstructions were undertaken using the AsymmMK model and rates translating to (a) gains five times as likely as losses and (b) gains 1/5 as likely as losses.

## 3. Results

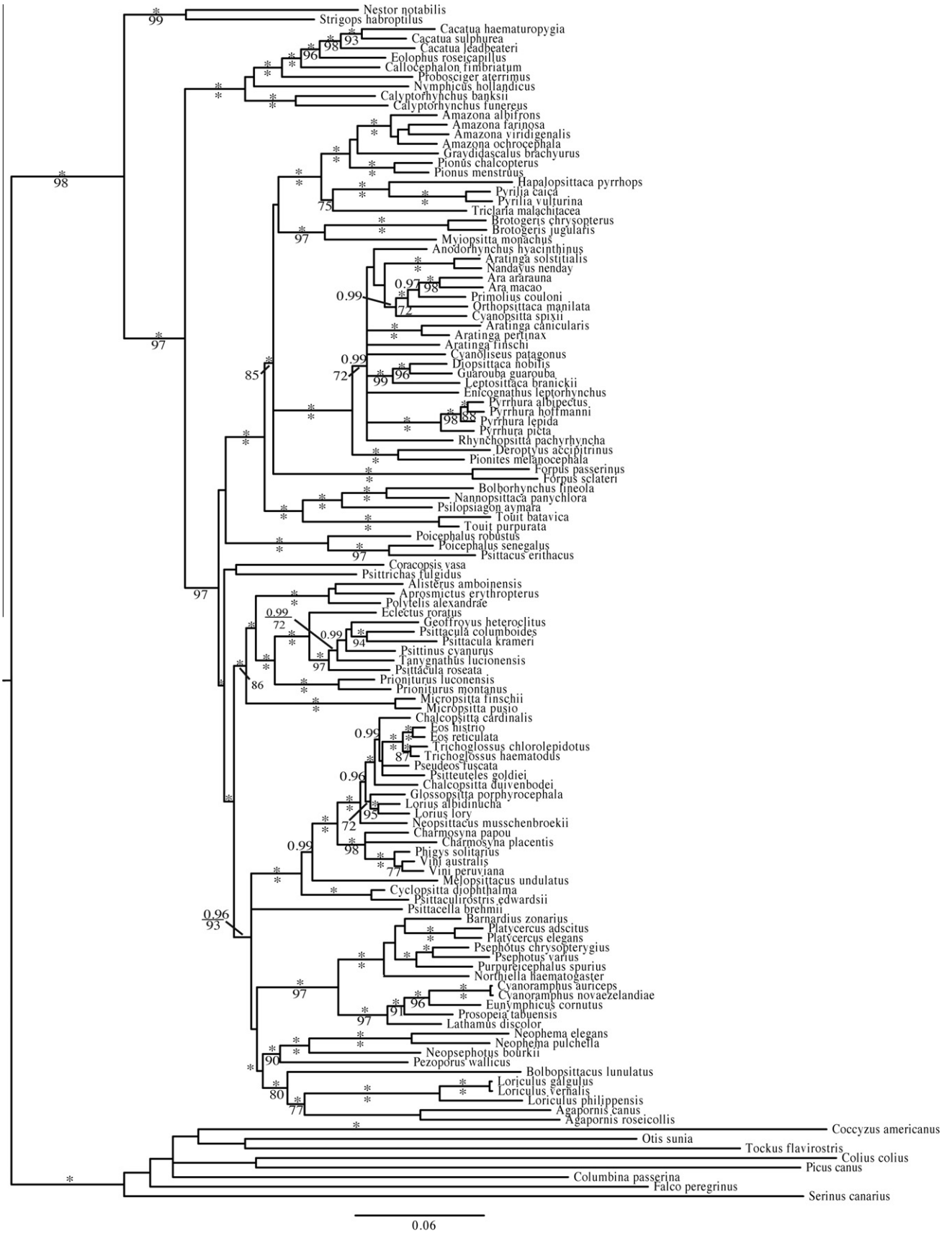
### 3.1. Phylogenetic analysis

The phylogenetic dataset consisted of 117 parrot species and eight non-parrot outgroups. The mitochondrial sequences, COI and ND2, showed no insertions/deletions (indels). The COI sequences were 570 base pairs (bp) in length while the ND2 sequences were 1041 bp. The intron sequences, TROP and TGFB2, were more variable in length due to the presence of 48 and 78 indels respectively. The TROP sequences were 498–533 bp with a total of 554 aligned bp. The TGFB2 sequences were 611–630 bp with a total of 817 aligned bp. The maximum likelihood dataset consisted of 2982 concatenated bp. The Bayesian analysis was performed on the 2982 characters included in the concatenated dataset (partitioned by gene region) and the 126 coded indels for a total of 3108 characters. Table 4S includes the genetic details for each gene region and the concatenated dataset. All new sequences have been deposited in GenBank (see Table 1S).

The most likely tree ( $-\ln L = -63846.569$ ) from 20 independent maximum likelihood (ML) analyses in GARLI of the concatenated dataset (Fig. 2S) and the consensus tree from the Bayesian analysis (Fig. 3) were broadly congruent, differing only in the placement of two genera, *Micropsitta* and *Graydidascalus*. The Bayesian analysis places *Micropsitta* with high support (posterior probability = 1) as sister to the clade consisting of *Alisterus*, *Aprosmictus*, *Polytelis*, *Eclactus*, *Geoffroyus*, *Psittacula*, *Psittinus*, *Tanygnathus* and *Prioniturus* while in the ML analysis *Micropsitta* is sister to the clade of *Alisterus*, *Aprosmictus* and *Polytelis* with poor support (ML bootstrap  $\leq 50$ ). The Bayesian analysis places *Graydidascalus* as sister to *Amazona* while in the ML analysis *Graydidascalus* is sister to the clade consisting of *Amazona* and *Pionus*. However, in both analyses the position of *Graydidascalus* is poorly supported. Nucleotide substitution models, priors for the Bayesian analysis, and final estimates of parameters for each analysis are listed in Table 5S.

### 3.2. Mitochondrial control region survey

One hundred and twelve parrot species were surveyed for the status of its mitochondrial control region by PCR of three diagnostic segments that show variation in size when a duplicated control region is present. Table 1 reports the control region status of each species surveyed and the length of each amplicon. Segment 15 was amplifiable for 96 species, while Segment 16 and Segment ND6 were amplifiable for 108 and 110 species respectively (see Table 1). *Aprosmictus erythropterus*, *Guarouba guarouba*, *Nandayus nenday* and *Rhynchopsitta pachyrhyncha* could not be scored by PCR at NMSU due to a lack of amplification. Five species (*Eos reticulata*, *Graydidascalus brachyurus*, *Hapalopsittaca pyrrhops*, *Pionites melanocephala*) were coded as unscorable because they either failed to amplify the diagnostic fragments by PCR or they produced ambiguous results and no confirmatory sequence was available.



**Fig. 3.** The partitioned Bayesian analysis of 117 parrot species reconstructed using two mitochondrial protein-coding genes (COI and ND2), two nuclear introns (TROP and TGF2) and coded gaps. Posterior probabilities >0.95 are given above the branches and maximum likelihood bootstraps >70 are given below the branches. Asterisks indicate a posterior probability of 1.0 or a maximum likelihood bootstrap value of 100.

**Table 1**  
Measured PCR fragment lengths and control region status for each parrot species in the phylogeny.

Scored by PCR	Control region status	Measured fragment size (nucleotides)			
		15	16	Glu	ND6
<i>Agapornis canus</i>	1	1781	1394	708	141
<i>Agapornis roseicollis</i>	1	1912	1399	689	144
<i>Alisterus amboinensis</i>	1	1652	1253	488	141
<i>Amazona albifrons</i>	2	1042	756	609	501
<i>Amazona farinosa</i>	2	1134	769	618	137, 1943
<i>Amazona ochrocephala</i>	2	1109	779	637	1883
<i>Amazona viridigenalis</i>	2	1182	781	626	153, 508, 2146
<i>Anodorhynchus hyacinthinus</i>	1	1672	1316	645	148
<i>Aprosmictus erythropterus</i>	UN	*	*	*	144
<i>Ara ararauna</i>	1	1742	1344	627	147
<i>Ara macao</i>	1	1738	1302	685	152
<i>Aratinga canicularis</i>	1	1833	1357	648	152
<i>Aratinga finschi</i>	1	1658	1341	630	144
<i>Aratinga pertinax</i>	1	2121	1697	640	145
<i>Aratinga solstitialis</i>	1	*	1368	627	145
<i>Barnardius zonarius</i>	1	1963	1576	847	149
<i>Bolbopsittacus lunulatus</i>	1	*	1681	901	153
<i>Bolborhynchus lineola</i>	1	1580	1255	543	149
<i>Brotogeris chrysopterus</i>	1	1809	1376	667	149
<i>Brotogeris jugularis</i>	1	1717	1343	677	145
<i>Cacatua haematurropygia</i>	1	1627	1236	640	148
<i>Cacatua leadbeateri</i>	1	1722	1245	629	149
<i>Cacatua sulphurea</i>	1	*	1227	604	144
<i>Callocephalon fimbriatum</i>	1	1505	1198	606	148
<i>Calyptorhynchus banksii</i>	1	1543	1215	549	148
<i>Calyptorhynchus funereus</i>	1	1765	1320	553	150
<i>Chalcopsitta cardinalis</i>	2	990	656	602	1346
<i>Chalcopsitta duivenbodei</i>	2	943	602	680	144, 1387
<i>Chamosyna papou</i>	2	*	1049	625	141, 315, 1164
<i>Chamosyna placentis</i>	2	998	628	625	1383
<i>Coracopsis vasa</i>	1	1572	1237	562	143
<i>Cyanoliseus patagonus</i>	1	1805	1368	671	147
<i>Cyanoramphus novaezelandiae</i>	1	2047	1626	875	144
<i>Cyclopsitta diophthalma</i>	2	*	673	636	475
<i>Deroptyus accipitrinus</i>	2	1261	952	642	147, 2244
<i>Diopsittaca nobilis</i>	1	*	1294	631	155
<i>Eclectus roratus</i>	1	1658	1253	561	153
<i>Eolophus roseicapillus</i>	1	1621	1187	*	152
<i>Eos histrio</i>	2	1010	622	573	1465
<i>Eos reticulata</i>	2?	*	627	575	145
<i>Eunymphicus cornutus</i>	1	1928	1583	861	150
<i>Forpus passerinus</i>	2	1200	810	670	138, 1813
<i>Forpus sclateri</i>	2	1234	650	672	2980
<i>Geoffroyus heteroclitus</i>	1	*	1498	781	152
<i>Glossopsitta porphyrocephala</i>	2	1088	720	687	1440
<i>Graydidascalus brachyurus</i>	2?	1410	889	700	154
<i>Guarouba guarouba</i>	UN	*	*	*	*
<i>Hapalopsittaca pyrrhops</i>	2?	1321	786	663	143
<i>Lathamus discolor</i>	1	1962	1577	840	147
<i>Leptosittaca branickii</i>	1	1726	1296	620	146
<i>Loriculus galgulus</i>	1	1571	1269	631	146
<i>Loriculus philippensis</i>	1	1598	1279	640	147
<i>Loriculus vernalis</i>	1	1636	1295	628	143
<i>Lorius albidinuchas</i>	2	998	640	618	1350
<i>Lorius lory</i>	2	984	645	580	1472
<i>Melopsittacus undulatus</i>	2	968	610	585	1612
<i>Micropsitta finschii</i>	1	1555	1180	502	149
<i>Micropsitta pusio</i>	1	1505	1142	479	142
<i>Myiopsitta monachus</i>	1	1639	1259	596	152
<i>Nandayus nenday</i>	UN	*	*	*	*
<i>Nannopsittaca panychlora</i>	1	1620	1254	556	151
<i>Neophema elegans</i>	1	*	1379	693	164
<i>Neophema pulchella</i>	1	1673	1288	640	160
<i>Neopsephotus bourkii</i>	1	1657	1313	628	154
<i>Neopsittacus musschenbroekii</i>	2	1004	640	584	1423
<i>Nestor notabilis</i>	1	1615	1264	596	156
<i>Northiella haematogaster</i>	1	1914	1322	592	147
<i>Nymphicus hollandicus</i>	1	1633	1200	591	146
<i>Orthopsittaca manilata</i>	1	1727	1363	640	152
<i>Pezoporus wallicus</i>	1	1900	1500	800	155
<i>Phigys solitarius</i>	2	1028	646	550	155, 1524
<i>Pionites melanocephala</i>	2?	*	971	626	154
<i>Pyrilia caica</i>	2?	1136	782	638	158

Table 1 (continued)

Scored by PCR	Control region status	Measured fragment size (nucleotides)			
		15	16	Glu	ND6
<i>Pyrilia vulturina</i>	2	982	615	600	166, 2223
<i>Pionus chalcopterus</i>	2	1150	781	614	157, 525, 2128
<i>Pionus menstruus</i>	2	1167	793	640	490
<i>Platycercus adscitus</i>	1	1919	1521	809	149
<i>Platycercus elegans</i>	1	1829	1449	771	148
<i>Poicephalus robustus</i>	2	1058	666	560	153, 1521, 1804
<i>Poicephalus senegalus</i>	2	978	639	531	1549
<i>Polytelis alexandrae</i>	1		1265	635	145
<i>Primolius couloni</i>	1	1685	1313	630	147
<i>Prioniturus luconensis</i>	2	1231	761	500	145, 2328
<i>Prioniturus montanus</i>	2	1130	795	492	148
<i>Probosciger aterrimus</i>	1	1601	1244	594	144
<i>Prosopaea tabuensis</i>	1	1878	1465	758	148
<i>Psephotus chrysopterygius</i>	1	1872	1494	858	138
<i>Psephotus varius</i>	1	1924	1517	837	148
<i>Pseudeos fuscata</i>	2	988	619	578	1370
<i>Psilopsiagon aymara</i>	1	1555	1210	546	151
<i>Psittacella brehmii</i>	1	1640	1476	604	150
<i>Psittacula columboides</i>	1	*	1211	550	150
<i>Psittacula krameri</i>	1	*	1343	582	136
<i>Psittacula roseata</i>	1	1503	1198	492	144
<i>Psittaculirostris edwardsii</i>	2	817	620	586	391, 680
<i>Psittacus erithacus</i>	2	984	617	545	144, 1589
<i>Psitteuteles goldiei</i>	2	1341	638	599	1455
<i>Psittinus cyanurus</i>	1	1554	1192	486	132
<i>Psittrichas fulgidus</i>	2?	1244	907	535	135
<i>Purpureicephalus spurius</i>	1	1893	1563	849	142
<i>Pyrrhura albipectus</i>	1	1712	1331	626	145
<i>Pyrrhura hoffmanni</i>	1	1563	1297	600	142
<i>Pyrrhura lepida</i>	1	1665	1306	640	136
<i>Pyrrhura picta</i>	1	1724	1310	622	154
<i>Rhynchopsitta pachyrhyncha</i>	UN	*	*	638	137
<i>Tanygnathus lucionensis</i>	1	1585	1262	550	133
<i>Touit batavica</i>	1	1700	1200	500	137
<i>Touit purpurata</i>	1	1700	1100	500	148
<i>Trichoglossus chlorolepidotus</i>	2	842	664	*	143, 175, 772, 1501
<i>Trichoglossus haematodus</i>	2	1009	643	586	1491
<i>Vini australis</i>	2	1000	650	600	142, 1348
<i>Vini peruviana</i>	2	1000	650	600	1336
	N=112	N=96	N=108	N=107	N=110
<b>Scored from Sequences (BR)</b>					
<i>Cyanopsitta spixii</i>	1				
<i>Enicognathus leptorhynchus</i>	1				
<i>Guaruba guarouba</i>	1				
<i>Nandayus nenday</i>	1				
<i>Rhynchopsitta pachyrhyncha</i>	1				
<i>Triclarina malachitacea</i>	2				
	N=6				
<b>Scored from GB Sequences or Literature</b>					
<i>Cyanoramphus auriceps</i>	1	Boon 2000			
<i>Strigops habroptilus</i>	1	AY309456			
	N=2				

\* = fragment did not amplify.

UN = unscorable by PCR.

? = ambiguous by PCR.

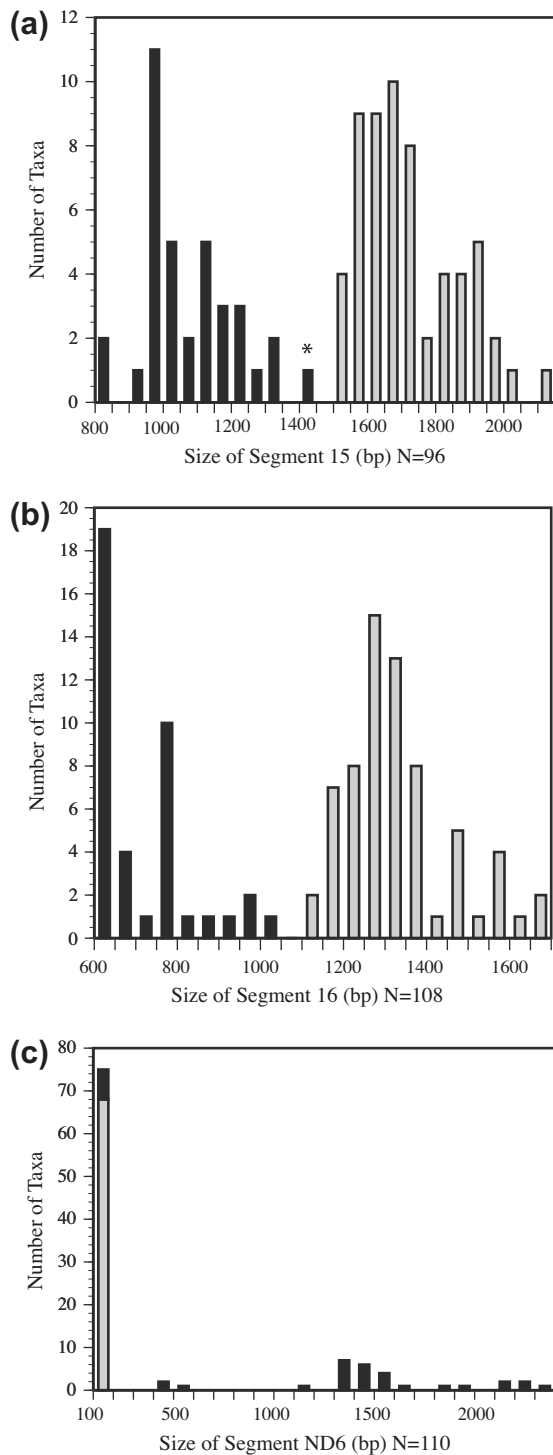
*nocephala* and *Psittrichas fulgidus*) all amplified ND6 bands indicative of a single control region while the sizes of Segment 15 and Segment 16 suggested that a duplicated control region was present. These species are listed as ambiguous in Table 1.

The band sizes for each segment are plotted in Fig. 4. Segment 15 showed a bimodal distribution, with most species clearly falling into either the single or duplicated control region size categories. However, *Gradydidascalus brachyurus* fell between the expected ranges. Segments Glu and 16 from this species were subsequently sequenced. In four taxa, Segment 15 amplicons were larger than the expected size of 1896 bp for a single control region. *Lathamus discolor* and *Barnardius zonarius* were approximately 75 bp larger than expected, while *Aratinga pertinax* and *Cyanoramphus novaezelandiae* exceeded the single control region size range by 225 bp and 150 bp.

These sizes may be stochastic effects of slightly different gel conditions that affect the distance run by the size marker used to measure the bands. The other bands for these species were also larger than expected but within the range of single control region sizes.

The size distribution for Segment 16 amplicons was also bimodal with all taxa falling within the two expected range sizes. Segment ND6 showed two different patterns: a tight cluster of species from 130 to 160 bp and a scatter of species with segment sizes from 1150 bp to over 2200 bp. *Pionus menstruus* and *Amazona albifrons* had values of 450 bp and 550 bp respectively. Based upon the other band sizes, *Amazona albifrons* and *Pionus menstruus* were classified as having a duplicated control region. The presence of a duplicated control region in *Amazona albifrons* was later confirmed by sequencing.





**Fig. 4.** Histograms of the number of species per 50 base pair bins for each amplified segment. Black bars indicate duplicate control regions. Gray bars indicate a single control region. An asterisk indicates species that fall outside of the expected fragment size ranges based on preliminary surveys of control region lengths. (a) lengths of Segment 15, (b) lengths of Segment 16, and (c) lengths of Segment ND6.

Of the 112 species surveyed by PCR, 68 were classified as having a single control region and 35 species were classified as having a duplicated control region. Four species, *Aprosmictus erythropterus*, *Guarouba guarouba*, *Nandayus nenday* and *Rhynchopsitta pachyrhyncha* were unscorable due to a lack of PCR amplification and five species (*Eos reticulata*, *Graydidascalus brachyurus*, *Hapalopsittaca pyrrhops*, *Pionites melanocephala* and *Psittichas fulgidus*) produced ambiguous results.

### 3.3. Sequencing of selected taxa

In order to validate the control region classifications from the PCR survey we sequenced 44 parrot species in the phylogeny (Table 2). The common avian gene order with a single control region was confirmed in 19 species (Desjardins and Morais, 1990), while the gene order with a duplicated control region previously described by Eberhard et al. (2001) for *Amazona* and *Pionus* species was confirmed for 25 species. Of the five species that produced ambiguous results in the PCR survey, two species (*Graydidascalus brachyurus*, *Pseudoes fuscata*) were shown to have a duplicated control region by sequencing. The aberrant size of the ND6 fragment was due to a lack of retained homology in the non-coding region. Therefore, only the functional ND6 was amplified. Sequencing of *Cyanopsitta spixii*, *Enicognathus leptorhynchus*, *Guarouba guarouba*, *Nandayus nenday*, *Rhynchopsitta pachyrhyncha* and *Triclaria malachitacea* in Brazil found that only *Triclaria malachitacea* had a duplicated control region. Three species were listed as unscorable (*Aprosmictus erythropterus*, no PCR amplification) or ambiguous (*Eos reticulata* and *Pionites melanocephala*, no sequence available to confirm status).

In the set of species with duplicated control regions, non-coding regions of various sizes were found between tRNA<sup>Thr</sup> and the first control region. These non-coding regions were examined with tRNA-Scan (Lowe and Eddy, 1997) to determine if copies of tRNA<sup>Pro</sup> or tRNA<sup>Glu</sup> retained enough homology to be identifiable. In most cases no homology could be determined due to the extent of degeneration. The control regions were identified by the presence of conserved sequences: the goose hairpin at the 5' end of domain I and the D-Box in domain II (Eberhard et al., 2001). The F-Box sequence of *Gallus gallus* was not identified in all species. An additional eight species (*Aratinga aurea*, *Aratinga leucophthalmus*, *Brotogeris chirri*, *Forpus xanthopterygius*, *Nannopsittaca dachillae*, *Pyrilia barrabandi*, *Pionites leucogaster*, and *Primolius auricollis*) were sequenced in Brazil for a different study (E. Tavares, C. Miyaki unpublished data), and served as additional confirmation of the distribution of control region duplications. Although these species were not included in the phylogeny, all are known from a broader phylogenetic survey to cluster with their congeners included in this study (E.E. Schirtzinger, unpublished data). GenBank sequences or published literature was used to confirm gene order for *Cyanoramphus auriceps*, *Strigops habroptilus*, *Amazona ochrocephala*, *Amazona farinosa*, *Pionus chalcopterus*, *Agapornis roseicollis*, *Melopsittacus undulatus*, and *Psittacus erithacus* (see Table 2 and Boon, 2000). The control region status of *Chalcopsitta duivenbodei* and *Charmosyna papou* could not be confirmed due to poor sequencing reactions.

Within the Neotropical parrots, a variation of the *Amazona* gene order was observed in *Deroptyus accipitrinus* and *Pionites leucogaster*. In contrast to all of the other species with control region duplications that we sequenced, *Deroptyus accipitrinus* and *Pionites leucogaster* retain a potentially functional copy of tRNA<sup>Pro</sup> before the non-coding region 5' to the first control region.

### 3.4. Ancestral state reconstruction

To determine if mitochondrial control region duplications in parrots originated multiple times, the character states of single (0) or duplicated control region (1) were mapped onto the Bayesian tree using parsimony and maximum likelihood methods (Fig. 5). These states were assigned using our PCR classifications (68 single control region, 35 duplicated control region, three unscorable/ambiguous), sequences from selected taxa (six single control region and three duplicated control region), GenBank sequences (one single control region), or publications (one single control region). Both methods of reconstruction identified the ancestral con-

**Table 2**

Confirmation of PCR Classified Control Region Status by Sequencing of Selected Species or by GenBank Sequences.

Scored by PCR	Control region status	Confirmed by Sequence source	GenBank #
<i>Agapornis rosiecollis</i>	1	GB	EU410486
<i>Amazona albifrons</i>	2	NMSU	JQ341164, JQ360543
<i>Amazona farinosa</i>	2	GB	AF228821
<i>Amazona ochrocephala</i>	2	NMSU/GB	AF338819, AF338820, JQ341165, JQ360544
<i>Anodorhynchus hyacinthinus</i>	1	BR	EF104124
<i>Ara ararauna</i>	1	BR	EF104127
<i>Aratinga solstitialis</i>	1	BR	EF104138
<i>Bolborhynchus lineola</i>	1	BR	EF104137
<i>Calyptorhynchus banksii</i>	1	NMSU	JQ360567
<i>Chalcopsitta cardinalis</i>	2	NMSU	JQ341170, JQ360549
<i>Chalcopsitta duivenbodei</i>	2	NMSU	JQ360545
<i>Chamosyna papou</i>	2	NMSU	JQ341166
<i>Chamosyna placensis</i>	2	NMSU	JQ341167, JQ360546
<i>Coracopsis vasa</i>	1	NMSU	JQ341168, JQ360570
<i>Cyanoliseus patagonus</i>	1	BR	EF104136
<i>Cyanopsitta spixii</i>	1	BR	EF104128
<i>Cyclopsitta diophthalma</i>	2	NMSU	JQ241169, JQ36-547
<i>Derotytus accipitrinus</i>	2	BR/NMSU	AF365437, JQ360548
<i>Diopsittaca nobilis</i>	1	BR	EF104121
<i>Enicognathus leptorhynchus</i>	1	BR	EF104139
<i>Eos histrio</i>	2	NMSU	JQ341171, JQ360550
<i>Forpus sclateri</i>	2	NMSU	JQ341172, JQ360551
<i>Glossopsitta porphyrocephala</i>	2	NMSU	JQ341173, JQ360552
<i>Graydidascalus brachyurus</i>	2	BR	EF104148
<i>Guaruba guarouba</i>	1	BR	EF104123
<i>Lorius albidinucha</i>	2	NMSU	JQ341174, JQ360553
<i>Melopsittacus undulatus</i>	2	GB	NC_009134
<i>Micropsitta pusio</i>	1	NMSU	JQ360568
<i>Myiopsitta monachus</i>	1	BR	EF104118
<i>Nandayus nenday</i>	1	BR	EF104131, EF104149
<i>Neopsittacus musschenbroekii</i>	2	NMSU	JQ341175, JQ360554
<i>Orthopsittaca manilata</i>	1	BR	EF104119
<i>Phigys solitarius</i>	2	NMSU	JQ341176, JQ360555
<i>Pyrilia caica</i>	2	NMSU	JQ341178, JQ360556
<i>Pionus chalcopterus</i>	2	NMSU/GB	AF338817, AF338818, JQ360557, JQ360558
<i>Poicephalus robustus</i>	2	NMSU	JQ360558
<i>Prioniturus montanus</i>	2	NMSU	JQ341180, JQ360566
<i>Pseudeos fuscata</i>	2	NMSU	JQ341183, JQ360561
<i>Psittacula roseata</i>	1	NMSU	JQ360569
<i>Psittaculirostris edwardsii</i>	2	NMSU	JQ341181, JQ360559
<i>Psittacus erithacus</i>	2	NMSU/GB	DQ335468, JQ341182, JQ360560
<i>Psitteuteles goldiei</i>	1	NMSU	JQ341184, JQ360562
<i>Pyrhura picta</i>	1	BR	EF104130, EF104150
<i>Rhynchopsitta pachyrhyncha</i>	1	BR	EF104135
<i>Trichoglossus haematodus</i>	2	NMSU	JQ341186, JQ360564

**Table 2 (continued)**

Scored by PCR	Control region status	Confirmed by Sequence source	GenBank #
<i>Triclaria malachitacea</i>	2	BR	EF104143, EF104146
<i>Vini australis</i>	2	NMSU	JQ341187, JQ360565
<b>Related Species Sequenced</b>			
<i>Aratinga aurea</i>	1	BR	EF104132, EF104151
<i>Aratinga leucophthalmus</i>	1	BR	EF104133
<i>Brotogeris chirri</i>	1	BR	EF104117
<i>Primolius auricollis</i>	1	BR	EF104126
<i>Pionites leucogaster</i>	2	BR	JQ749718, JQ749719
<i>Pyrilia barrabandi</i>	2	BR/NMSU	JQ341177, EF104141, EF104144
<i>Forpus xanttopterygius</i>	2	BR	EF104140, EF104147
<i>Nannopsittaca dachillae</i>	1	BR	EF104134
<i>Psittaculirostris salvadorii</i>	2	NMSU	JQ341185, JQ360563

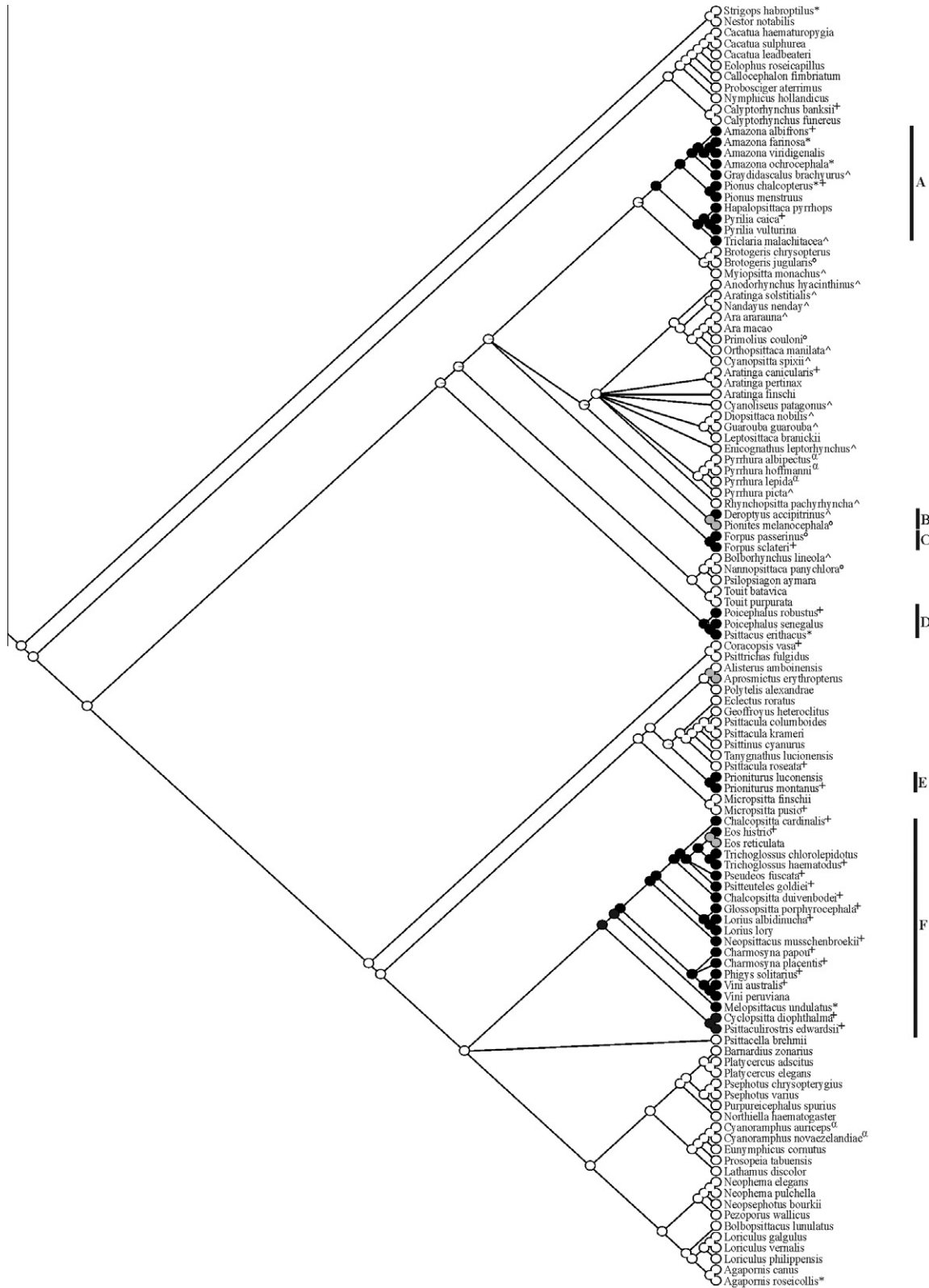
control region state in parrots as a single control region, and indicated that control region duplications have originated at least six times (Clades A–F in Fig. 5). No reversions from a duplicated control region to a single control region state were reconstructed by either method. Likelihood reconstructions using the symmetric rate (MK1) model and the asymmetric rate (AsymmMK) model did not affect the number of reconstructed independent origins or result in considerable differences in likelihoods of states at interior nodes. Similarly, changing the transition rate between states in the AsymmMK model did not affect our conclusions (See Fig. 3S and Table 6S for proportional likelihood values of each reconstructed state for interior nodes for each model analyzed).

### 3.5. Comparison of control regions

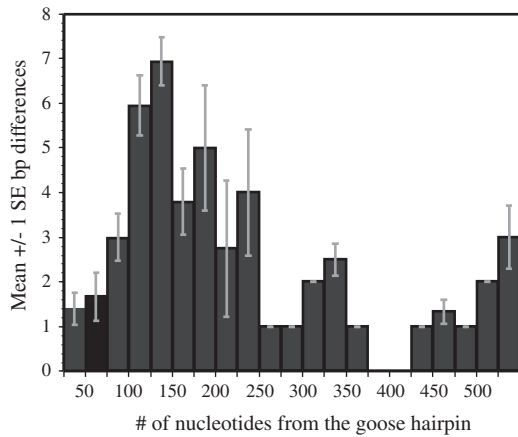
To determine if either of the two control regions had degenerated in species with a duplicated control region, the two control region fragments were aligned and nucleotide differences were calculated. These alignments found that the two control region sequences were typically highly similar, with sequence divergences ranging from 0–10.9% between the two copies within an individual. Most nucleotide differences were found between 51 and 225 nucleotides from the goose hairpin (Fig. 6). In domain I the only conserved sequence of known function is the termination-associated sequence (Baker and Marshall, 1997; Quinn, 1997; Sbisá et al., 1997). *Graydidascalus brachyurus* was not included in these calculations because neither of its control regions contained a goose hairpin.

## 4. Discussion

We reconstructed the phylogenetic relationships of 117 parrot species and classified their mitochondrial control region state from PCR fragment length analysis, DNA sequences or GenBank accessions to investigate the origins and distribution of mitochondrial control region duplications within the order Psittaciformes. A total of 76 parrot species were determined to have a single control region, while 38 parrot species were determined to have a duplicated control region. One species was unscorable and two species produced ambiguous results for which no sequence confirmation was available. Mapping the control region states onto the resulting phylogeny identified at least six independent origins of the duplicated control region state. Below we discuss the implications of



**Fig. 5.** Ancestral state reconstruction of the parrot mitochondrial control region duplications on the Bayesian tree under the maximum likelihood criterion using the MK1 model. White circles indicate species classified as having a single mitochondrial control region by the PCR fragment length analysis and/or sequencing. Black circles indicate species classified as having a duplicated mitochondrial control region. Gray circles indicate an unscorable state at the terminals and ambiguous ancestral states at interior nodes. The circles at nodes toward the interior of the tree are representative of the likelihood of each state at that node. \* = GenBank sequences, ^ = species sequenced at BR, ° = related species sequenced at BR, + = species sequenced at NMSU and α = species that have published gene orders. Letters to the right indicate clades defined by a single origin event.



**Fig. 6.** Means ( $\pm 1$  standard error) for nucleotide differences in 25 bp non-overlapping windows of aligned duplicated control regions. The figure shows that the greatest number of nucleotide differences occurs 51–225 bp from the goose hairpin in domain I of the control region.

these results for parrot evolutionary relationships and present two alternative hypotheses for the evolution of mtDNA duplications in parrots.

#### 4.1. Parrot phylogeny

The phylogeny reconstructed in this study is the most taxonomically comprehensive phylogeny to date of the Psittaciformes. The Bayesian tree and the maximum likelihood tree are generally well resolved and largely congruent in topology. Disagreement between the two analyses occurs solely on the location of the genus *Micropsitta* and *Graydidascalus brachyurus* (Fig. 2S and Fig. 3). The Bayesian phylogeny is broadly consistent with other published studies of parrot genus level relationships, in which a clade composed of the New Zealand endemics, *Strigops* and *Nestor*, was the sister group to all other parrots, and the *Cacatuoidea* (cockatoos) was the second oldest extant clade (Tavares et al., 2006; Wright et al., 2008; Schweizer et al., 2010; Joseph et al., 2012). Other well-supported parrot clades consistently recovered across various studies include the Neotropical parrots (Arinae), the African Psittacinae, the Australasian Psittaculidae and the Platycercinae from Australia, New Zealand, Oceania and Africa (de Kloet and de Kloet, 2005; Juniper and Parr, 1998; Tavares et al., 2006; Wright et al., 2008; Schweizer et al., 2010; Joseph et al., 2012). In agreement with previous studies by Tavares et al. (2006) and Wright et al. (2008), three Neotropical clades were recovered here: the parrotlets, including *Bolborhynchus*, *Nannopsittaca*, *Touit*, and *Psilopsiagon*; amazons and allies, including *Amazona*, *Pionus*, *Pyrilia*, *Triclararia* and *Graydidascalus*; and macaws and allies, including *Ara*, *Cyanopsitta*, *Aratinga*, *Orthopsittaca*, *Pyrrhura*, *Pionites* and *Anodorhynchus*.

#### 4.2. Mitochondrial gene order in parrots

Among the 114 parrot species for which data was available there is evidence for two of the four described avian mitochondrial gene orders. The typical avian mitochondrial gene order originally described by Desjardins and Morais (1990) was inferred for 76 species by PCR, sequencing and examination of GenBank sequences. In contrast, duplicate control regions and the gene order described in *Amazona* parrots by Eberhard et al. (2001) was found in 38 of the species surveyed and/or sequenced. In this genome arrangement, a non-coding region, that in some species has apparent similarity to ND6 and tRNA<sup>Glu</sup>, is located between tRNA<sup>Thr</sup> and the first control region. Two species, *Derophtus accipitrinus* and *Pionites leucogaster*, which was not in the phylogeny, were shown by sequencing

to have a variant of the *Amazona* gene order, with a functional tRNA<sup>Pro</sup> located between tRNA<sup>Thr</sup> and the first control region.

#### 4.3. Evolutionary patterns of mtDNA control region duplications

Three striking patterns are apparent in our reconstructions of the evolution of control region duplications and sequencing of duplicate control regions in parrots. First, the mapping of control region duplication states onto the phylogeny of parrots identifies at least six independent origins of the control region duplications. Second, there were no reversions to a single control region state in any of these six clades. Third, although there was considerable interspecific variation, levels of sequence similarity between duplicated control regions within an individual were typically high (89–100% similarity in the species examined), at least for the first 400–500 nucleotides of domain I that was examined. This similarity would appear to be unusual due to the fact that this segment of the control region is often very different between species with many small insertions, deletions and mutations that are thought to occur as a result of the D-loop being single-stranded and accessible to mutagenic agents, such as reactive oxygen species (Shokolenko et al., 2007).

One hypothesis to explain these patterns is that a duplication of the control region and neighboring sequences occurred in the ancestors of these six clades and was retained in all descendent species of each clade. This hypothesis begs the question of what maintains the generally high degree of sequence similarity between the duplicated control regions within each taxon. The within-individual divergences that we observed between control region copies of 0–10.9% (Table 3) fall within the range of divergences observed in other taxa with duplicated control regions such as *Amazona* parrots, albatrosses, killifish, snakes, ticks, and ostracods (Abbott et al., 2005; Campbell and Barker, 1999; Eberhard et al., 2001; Kumazawa et al., 1996, 1998; Lee et al., 2001; Ogoh and Ohmiya, 2007; Tatarenkov and Avise, 2007). A high degree of sequence similarity is often interpreted as evidence for the maintenance of function in both duplicated control regions. Portions of the control region are under selection for the ability to bind with nuclear-encoded replication factors, and for functional control of replication and transcription (Doda et al., 1981; Gensler et al., 2001; He et al., 2007; Lee and Clayton, 1998; Schultz et al., 1998; Shadel and Clayton, 1997); such functionality may provide stabilizing selection on duplicated control regions. Alternatively, several studies of organisms with a duplicated control region have explained the high degree of similarity between the two control regions as evidence of gene conversion (Eberhard et al., 2001; Kumazawa et al., 1996, 1998; Ogoh and Ohmiya, 2007; Tatarenkov and Avise, 2007; Verkuil et al., 2010), but the molecular mechanisms responsible remain unclear. In either case, the maintenance of duplicated control regions concurrent with the elimination of duplicated mitochondrial genes and tRNAs suggests an advantage to having a second control region that overrides selection for compactness. Potential advantages include faster replication (Kumazawa et al., 1996) or protection against age-related deterioration of mitochondrial function (T.F. Wright and J.R. Eberhard, unpublished data).

An alternative hypothesis for the observed patterns is that a propensity for duplications to occur such as through replication slippage due to secondary structure in the control region was present in the common ancestor of each of the six clades, leading to repeated duplications of the region from cytochrome *b* to the control region with lineage specific degradation and deletion events passing to each of the descendent taxa (Verkuil et al., 2010; Zhuang and Cheng, 2010). Zhuang and Cheng (2010) found a similar pattern within Neotrothenoid fish and suggested that within each clade with control region duplications if these mutations are neutral, each descendant

**Table 3**  
Gene order, non-coding region length and percent control region differences for parrot species with a duplicated control region.

Taxa	Source	Clade <sup>a</sup>	Gene order	Non-coding region length <sup>b</sup>	%CR differences <sup>c</sup>
<i>Amazona albifrons</i>	NMSU	A	Amazona	141	0.6
<i>Amazona farinosa</i>	AF338821	A	Amazona	158	0
<i>Amazona ochrocephala</i>	NMSU	A	Amazona	127	2.1
<i>Pyrilia caica</i>	NMSU	A	Amazona	155	0
<i>Pyrilia barrabandi</i>	BR/NMSU	A	Amazona	160	6
<i>Pionus chalcopterus</i>	NMSU/AF338817-18	A	Amazona	180	0.2
<i>Triclaria malachitacea</i>	BR	A	Amazona	112	2.5
<i>Deroytus accipitrinus</i>	BR	B	Variant A	319	4.7
<i>Pionites leucogaster</i>	BR	B	Variant A	325	10.9
<i>Forpus xanthopterygius</i>	BR	C	Amazona	129	0.2
<i>Forpus sclateri</i>	NMSU	C	Amazona	117	5
<i>Poicephalus robustus</i>	NMSU	D	Amazona	129	7.7
<i>Psittacus erithacus</i>	NMSU	D	Amazona	88	10.8
<i>Prioniturus montanus</i>	NMSU	E	Amazona	360	1.9
<i>Chalcopsitta cardinalis</i>	NMSU	F	Amazona	84	7.9
<i>Chamosyna placensis</i>	NMSU	F	Amazona	45	2.7
<i>Eos histrio</i>	NMSU	F	Amazona	84	2.3
<i>Glossopsitta porphyrocephala</i>	NMSU	F	Amazona	68	3.3
<i>Lorius albidinucha</i>	NMSU	F	Amazona	73	3.3
<i>Melopsittacus undulatus</i>	NC009134	F	Amazona	58	4.6
<i>Neopsittacus musschenbroekii</i>	NMSU	F	Amazona	88	3.3
<i>Phigys solitarius</i>	NMSU	F	Amazona	32	2.7
<i>Psittuteutes goldiei</i>	NMSU	F	Amazona	81	2.6
<i>Pseudeos fuscata</i>	NMSU	F	Amazona	71	3.5
<i>Trichoglossus haematodus</i>	NMSU	F	Amazona	86	3.3
<i>Vini australis</i>	NMSU	F	Amazona	58	3.5
<i>Cyclopsitta diophthalma</i>	NMSU	F	Amazona	62	5.1
<i>Psittaculirostris edwardsii</i>	NMSU	F	Amazona	60	5.5

<sup>a</sup> Clades labeled in Fig. 5.

<sup>b</sup> Non-coding region length was calculated as the number of nucleotides from the 3' end of tRNA<sup>THR</sup> to the 5' end of the goose hairpin.

<sup>c</sup> Percent control region differences was calculated as the number of nucleotides that differed between the 2 aligned control regions divided by the total number of overlapping nucleotides multiplied by 100. Differences were only counted if they occurred 3' of the goose hairpin.

species of a specific ancestor should have approximately the same amount of divergence between its two control regions. Table 3 shows that while species within the Australasian clade (labeled F in Fig. 5) exhibit similar levels of divergence between their duplicated control regions, the other clades show considerable variation among member species in the degree of divergence exhibited between their two control regions. Further investigation into patterns of sequence divergence over the entire length of the duplicated control region, coupled with functional studies of mitochondrial replication in species with and without control region duplications, should help distinguish between these alternative hypotheses for the evolution of duplicate control regions.

## 5. Conclusions

The presence of multiple mitochondrial gene orders within Psittaciformes supports the idea that the avian mitochondrial genome is a dynamic molecule. This study has shown that mitochondrial control region duplications have occurred many times in parrots, with ancestral state reconstructions suggesting six independent origins of the duplicated control region state and no reversions to a single control region state. Further investigations into the fates of duplicated mitochondrial genes, the potential costs and advantages of having a second control region, and the complex relationship between evolutionary rates, selection and time since duplication are needed to fully explain these patterns in the mitochondrial genome.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jymp.2012.04.009>.

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