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Temporal and spatial diversification of *Pteroglossus* araçaris (AVES: Ramphastidae) in the neotropics: Constant rate of diversification does not support an increase in radiation during the Pleistocene

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ABSTRACT

We use the small-bodied toucan genus *Pteroglossus* to test hypotheses about diversification in the lowland Neotropics. We sequenced three mitochondrial genes and one nuclear intron from all *Pteroglossus* species and used these data to reconstruct phylogenetic trees based on maximum parsimony, maximum likelihood, and Bayesian analyses. These phylogenetic trees were used to make inferences regarding both the pattern and timing of diversification for the group. We used the uplift of the Talamanca highlands of Costa Rica and western Panama as a geologic calibration for estimating divergence times on the *Pteroglossus* tree and compared these results with a standard molecular clock calibration. Then, we used likelihood methods to model the rate of diversification. Based on our analyses, the onset of the *Pteroglossus* radiation predates the Pleistocene, which has been predicted to have played a pivotal role in diversification in the Amazon rainforest biota. We found a constant rate of diversification in *Pteroglossus* evolutionary history, and thus no support that events during the Pleistocene caused an increase in diversification. We compare our data to other avian phylogenies to better understand major biogeographic events in the Neotropics. These comparisons support recurring forest connections between the Amazonian and Atlantic forests, and the splitting of cis/trans Andean species after the final uplift of the Andes. At the subspecies level, there is evidence for reciprocal monophyly and groups are often separated by major rivers, demonstrating the important role of rivers in causing or maintaining divergence. Because some of the results presented here conflict with current taxonomy of *Pteroglossus*, new taxonomic arrangements are suggested.

1. Introduction

Biologists have formulated a variety of hypotheses about the patterns and processes that may have led to the high levels of Neotropical biotic diversity, and have used a variety of analytical methods to test these hypotheses (Antonelli et al., 2010). Previous studies have focused on the effects of geological and climatic history on diversification in the lowland Neotropics. Hypotheses widely discussed in these studies include the refuge hypothesis (Haffer, 1969), the ecological gradient hypothesis (Endler, 1982), the riverine barrier hypothesis (Gascon et al., 2000), and the Miocene marine incursion hypothesis (Bates, 2001; Lovejoy et al., 2006). The refuge hypothesis, which explains diversification as a result of forest fragmentation resulting from cyclical climate oscillations, was originally based on avian distributions with emphasis placed on the Pleistocene (Haffer, 1969). However, many authors including Haffer himself (Bates et al., 1998; Haffer, 1997) have subsequently argued that much of the species-level diversification in Amazonia predates the Pleistocene. Although refugia may have played a role across a broader temporal scale, several previous studies have specifically pinpointed the Pleistocene and its dynamic paleoclimatic and geologic events as a cause of rapid diversification (Haffer, 1997; Hackett and Lehn, 1997). Additional hypotheses exist for the broader Neotropics, such as the orogeny of the Andes serving as a barrier between cis (eastern) and trans (western) Andean populations (Cracraft and Prum, 1988; Burney and Brumfield, 2009). Recent molecular studies indicate that Neotropical diversification is very complex and one hypothesis cannot be generalized to explain all present-day species distributions and diversity (Bermingham and Avise, 1986; Prum, 1988; Bates et al., 1998; Marks et al., 2002; Miller et al., 2008). Thus, additional biogeographic analyses using calibrated phylogenies for different sets of taxa are needed to further understand diversification patterns in the Neotropics.

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Aleixo and Rossetti (2007) encouraged testing hypotheses of diversification by studying groups with "high species richness, high ecological diversity, and widespread distribution." The *Pteroglossus* aracaris exhibit many of these characteristics and have previously served as a model genus to evaluate the Pleistocene refuge and other hypotheses (Haffer, 1974) of Neotropical diversification (Hackett and Lehn, 1997; Eberhard and Bermingham, 2005). Araçaris are medium-sized frugivorous birds of the toucan clade Ramphastidae. Currently 11 species are recognized, but many are polytypic with multiple named subspecies (AOU, 1998; Remsen et al., 2010). *Pteroglossus* has a widespread distribution in lowland tropical forests from Mexico south through Central America, the Chocó lowlands west of the Andes and from Amazonia to the Atlantic forest of Brazil and northeastern Argentina.

Hackett and Lehn (1997) analyzed allozyme data to reconstruct evolutionary relationships between *Pteroglossus* species and reported low genetic differentiation consistent with diversification events in the Pleistocene, which was concordant with Haffer's original refuge hypothesis. Their study also indicated that the Atlantic forest endemic *Pteroglossus bailloni*, previously thought to constitute a monotypic genus (*Baillonius*), was actually nested within *Pteroglossus*. Subsequently, Eberhard and Bermingham (2005) used DNA sequence data (ATPase 6 and 8, COI, and cyt *b*) to reconstruct a species-level phylogeny and infer historical relationships and timing of speciation within *Pteroglossus*, using the standard mitochondrial evolutionary rate of 2% per million years (Shields and Wilson, 1987), which may or may not be appropriate for *Pteroglossus* (Weir and Schluter, 2008). They concluded that the onset of *Pteroglossus* diversification was during the Late Pliocene. As with many avian lineages, calibrating the *Pteroglossus* tree is difficult due to a lack of fossil data for ramphastids, and involves making a number of assumptions (Moyle et al., 2009). Using Eberhard and Bermingham's (2005) data, Pereira and Wajntal (2008) employed additional calibration methods, including distantly related fossils and geologic dates to test hypotheses about the timing of *Pteroglossus* diversification. They found that the *Pteroglossus* diversification began during the Late Miocene, well before the time Eberhard and Bermingham (2005) had suggested (Pereira and Wajntal, 2008). Most of the calibration points Pereira and Wajntal (2008) used were much older than 12 Mya, which can be problematic due to saturation of mtDNA sequences (Arbogast et al., 2002; Weir and Schluter, 2008). For example, Pereira and Wajntal (2008) used the fossil record to date the minimum split age between Picidae and Ramphastidae to around 30 Mya. They also used the uplift of the northern end of the Cordillera of the Andes between 2 and 13 Mya to date divergences between sister taxa with cis- and trans-Andean distributions. However, lowland taxa on either side of the Andes could have dispersed through the South American Caribbean lowlands or through low passes in the Andes (Nores, 2004; Miller et al., 2008; Burney and Brumfield, 2009). We argue that for *Pteroglossus* and other lowland taxa with presumably high dispersal capabilities, the use of the Andean uplift as a maximum divergence calibration point is not appropriate. Pereira and Wajntal's (2008) use of this calibration point may have resulted in skewed calibrations and unrealistic divergence times.

Our study is based on more thorough taxonomic sampling and a larger molecular dataset than those previously published (Hackett and Lehn, 1997; Eberhard and Bermingham, 2005; Pereira and Wajntal, 2008). We include DNA sequence data for multiple mitochondrial genes and one nuclear locus from all known species and multiple subspecies. Furthermore, we also calibrated diversification times on the phylogeny using points that differ from those used by Pereira and Wajntal (2008) and believe that these are more appropriate for dating divergences for the genus. Lastly, we model the rate of diversification to explicitly examine the role of the Pleistocene events on diversification in this group. A diversification rate increase would be consistent with events during the Pleistocene causing increased diversification. On the other hand, a constant rate of diversification indicates that the rate during the Pleistocene is no different than the background level of speciation and thus that Pleistocene events did not play a special role in the diversification of this group. Using these data and time calibrated phylogenies, we address the following questions: (a) what are the evolutionary relationships among the species (and subspecies) of *Pteroglossus*? (b) are the recovered relationships among *Pteroglossus* taxa consistent with current taxonomy and recognized species limits? and (c) how does the timing and rate of diversification in *Pteroglossus* correlate with the timing of known paleoclimatic and geologic events in the Neotropics?

2. Materials and methods

2.1. DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from 34 frozen tissue samples using the DNeasy tissue extraction kit (Qiagen, Valencia, California). We analyzed samples of all *Pteroglossus* species including much of the described subspecific variation (Table 1). The mitochondrial cytochrome oxidase I (COI), cytochrome *b* (cyt *b*), and NADH dehydrogenase subunit 2 (ND2) genes, and the nuclear β-fibrinogen intron 7 (βFib7), were amplified via the polymerase chain reaction using primers previously designed from other studies of toucans. In total, 3215 bp were amplified and sequenced. Primers L6625 and H7005 (Hafner et al., 1994) were used to amplify COI. Cyt *b* was amplified either in its entirety using external primers L14841 (Kocher et al., 1989) and H16065 (Helm-Bychowski and Cracraft, 1993) or alternatively in two pieces in combination with internal primers ToucCBH and ToucCBL (Weckstein, 2005). Similarly, ND2 was either amplified in its entirety using external primers L5215 (Hackett, 1996) and H6313 (Sorenson et al., 1999) or in two pieces with internals L5758Touc and H5776Touc (Weckstein, 2005) or H5776Touc22 (this study). βFib7 was amplified either as one piece using only external primers Fib-B17L and Fib-B17Uc (Moyle, 2004) or in two pieces with internal primers FibL2 and FibU2 (Prychitko and Moore, 1997). All primers and their sequences are listed in Table 2. The thermocycling regime for the mitochondrial genes was as follows: denaturation at 94 °C, annealing at 46 °C for COI and 50 °C for cyt *b* and ND2, extension at 72 °C. This cycle was repeated 35 times and then held at 4 °C. For βFib7, the annealing temperature was incrementally decreased from 58 °C for five cycles to 54 °C for five cycles and 50 °C for 30 cycles. A small aliquot from each amplification was run on an agarose gel to check whether there was a single amplified fragment of sufficient quantity for sequencing. Amplifications of COI were cleaned using the Exonuclease and Shrimp Alkaline Phosphatase enzymatic reactions (United States Biochemical), whereas cyt *b* and ND2 were cleaned by cutting a single band of PCR product from a low melt agarose gel and cleaning with GELase (Epicentre Technologies, Madison, WI) following the manufacturer's recommended protocol. Amplifications were cycle-sequenced using a BigDye 3.1 Terminator kit (BigDye, Applied Biosystems, Foster City, CA) and the same primers used for amplification. Cycle sequencing reactions were cleaned with ethanol EDTA precipitation, and resuspended in Hi-Di formamide. Sequences were then visualized through an ABI 3730 automated sequencer and aligned and reconciled using the computer program Sequencher 3.1.1 (Gene Codes Corp, Ann Arbor, MI). All new sequences and associated voucher data were deposited in GenBank (HQ424015-HQ424126). A few sequences were from previously published studies GenBank (AY959799-AY959802, AY959810, AY959826-AY959829,

Table 1
Specimens used in this study.

#	Species	Tissue number	Voucher deposition	Locality	Common name
Ingroup					
1	*Pteroglossus azara azara*	FMNH 456642/MPEG 62554	MPEG	Brazil: Amazonas	Ivory-billed Araçari
2	*P. azara azara*	FMNH 456643/MPEG 62555	FMNH	Brazil: Amazonas	Ivory-billed Araçari
3	*P. azara flavirostris*	FMNH 456647/MPEG 62550	MPEG	Brazil: Amazonas	Ivory-billed Araçari
4	*P. azara flavirostris*	FMNH 456648/MPEG 62551	FMNH	Brazil: Amazonas	Ivory-billed Araçari
5	*P. azara mariae*	LSU B8991	LSU	Bolivia: Pando	Ivory-billed Araçari
6	*P. azara mariae*	LSU B40614	LSU	Peru: Loreto Department	Ivory-billed Araçari
7	*P. viridis*	LSU B20231	LSU	Brazil: Amazonas	Green Araçari
8	*P. inscriptus inscriptus*	LSU B35444	MPEG	Brazil: Mato Grosso	Lettered Araçari
9	*P. inscriptus humboldtii*	LSU B8819	LSU	Bolivia: Pando	Lettered Araçari
10	*P. inscriptus humboldtii*	LSU B39983	PERU	Peru: Loreto Department	Lettered Araçari
11	*P. inscriptus humboldtii*	FMNH 456652/MPEG 62560	MPEG	Brazil: Amazonas	Lettered Araçari
12	*P. bitorquatus sturmi*	LSU B35533	MPEG	Brazil: Mato Grosso	Red-necked Araçari
13	*P. bitorquatus reichenowi*	FMNH 456656/MPEG 61671	MPEG	Brazil: Pará	Red-necked Araçari
14	*P. aracari atricollis*	USNM B09755	KU	Guyana: Northwest district	Black-necked Araçari
15	*P. aracari aracari*	LSU B35547	MPEG	Brazil: Pará	Black-necked Araçari
16	*P. aracari aracari*	FMNH 456655/MPEG 61670	MPEG	Brazil: Pará	Black-necked Araçari
17	*P. castanotis castanotis*	LSU B27624	LSU	Peru: Loreto Department	Chestnut-eared Araçari
18	*P. castanotis castanotis*	FMNH 456651/MPEG 62559	MPEG	Brazil: Amazonas	Chestnut-eared Araçari
19	*P. castanotis australis*	LSU B37611	LSU	Bolivia: Santa Cruz	Chestnut-eared Araçari
20	*P. castanotis australis*	LSU B35266	MPEG	Brazil: Mato Grosso	Chestnut-eared Araçari
21	*P. pluricinctus*	LSU B7112	LSU	Peru: Loreto Department	Many-banded Araçari
22	*P. torquatus torquatus*	LSU B28584	LSU	Panama: Colón	Collared Araçari
23	*P. torquatus sanguineus*	LSU B11783	ANSP	Ecuador: Esmeraldas	Stripe-billed Araçari
24	*P. torquatus erythropygius*	LSU B100021	LSU	Zoo/Captive	Pale-mandibled Araçari
25	*P. frantzii*	LSU B16075	LSU	Costa Rica: Puntarenas	Fiery-billed Araçari
26	*P. beauharnaesii*	LSU B35532	MPEG	Brazil: Mato Grosso	Curl-crested Araçari
27	*P. bailloni*	LSU B25891	LSU	Paraguay: Caazapa	Saffron Toucanet
Outgroups					
28	*Ramphastos vitellinus ariel*	LSU B35555	LSU	Brazil: São Paulo	Channel-billed Toucan
29	*Semnornis frantzii*	LSU B16019	LSU	Costa Rica: Heredia	Prong-billed Barbet
30	*S. ramphastinus*	LSU B7771	ANSP	Ecuador: Pichincha	Toucan Barbet
31	*Aulacorhynchus prasinus atrogularis*	LSU B21201	LSU	Peru: Madre de Dios	Emerald Toucanet
32	*A. prasinus caeruleogularis*	LSU B26403	LSU	Panama: Chiriqui	Emerald Toucanet
33	*Selenidera reinwardtii*	LSU B27756	LSU	Peru: Loreto Department	Golden-collared Toucanet
34	*Andigena cucullata*	LSU B1273	LSU	Bolivia: La Paz	Hooded Mountain Toucan

Notes: FMNH = Field Museum of Natural History, LSU = Louisiana State University Museum of Natural Science, MPEG = Museu Paraense Emílio Goeldi, ANSP = Academy of Natural Sciences Philadelphia.

Table 2
Primers used for PCR amplification and sequencing in the study.

Gene	Primer	Sequence
cyt *b*	L14841[a]	5′-GCTTCCATCCAACATCTCAGCATGATG-3′
	TOUCCBH[b]	5′-GAGAARTARGGGTGRAATGG-3′
	TOUCCBL[b]	5′-CTTCCTNCTNCCATTCCTAATYRCAGG-3′
	H16065[c]	5′-GGAGTCTTCAGTCTCTGGTTTACAAGAC-3′
COI	L6625[d]	5′-CCGGATCCTTYTGRTTYTTYGGNCAYCC-3′
	H7005[d]	5′-CCGGATCCACNACRTARTANGTRTCRTG-3′
ND2	L5215[e]	5′-TATCGGGCCCATACCCCGAAAAT-3′
	H5776TOUC22	5′-GGTGGGAGATGGARGAGAAGGC-3′
	L5758TOUC[b]	5′-GGCTGARYAGGCMTCAACCARAC-3′
	H6313[h]	5′-CTCTTATTTAAGGCTTTGAAGGC-3′
βFib7	FIB-B17L[f]	5′-TCCCCAGTAGTATCTGCCATTAGGGTT-3′
	FIB U2[g]	5′-GTAACCCATAATGGGTCCTGAG-3′
	FIB L2[g]	5′-CTTCTGAGTAGGCAGAACTT-3′
	FIB-B17Uc[f]	5′-GGAGAAAACAGGACAATGACAATTCAC-3′

[a] Kocher et al. (1989).
[b] Weckstein (2005).
[c] Helm-Bychowski and Cracraft (1993).
[d] Hafner et al. (1994).
[e] Hackett (1996).
[f] Moyle (2004).
[g] Prychitko and Moore (1997).
[h] Sorenson et al. (1999).

AY959837, AY959853, AY959855, AY959856, AY959864, GQ457981-GQ457984, GQ458000, GQ458001, GQ458014, GQ458015; Weckstein, 2005; Patané et al., 2009).

2.2. Phylogenetic analyses

We aligned sequences of the 34 individuals for each gene in Sequencher and concatenated them into one dataset. For outgroups, we used representatives of four Ramphastidae genera, which are known to be the closest relatives of *Pteroglossus* (Barker and Lanyon, 2000), including *Ramphastos vitellinus ariel*, *Aulacorhynchus prasinus atrogularis*, *Aulacorhynchus prasinus caeruleogularis*, *Selenidera reinwardtii*, *Andigena cucullata* (Table 1). We also used *Semnornis frantzii* and *Semnornis ramphastinus* because they were critical outgroups for calibrating the *Pteroglossus* phylogeny. Phylogenies were reconstructed using maximum parsimony (MP) and maximum likelihood (ML) methods as implemented in PAUP* and Bayesian Inference (BI) as implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Genetic distances also were calculated in PAUP*. We used the incongruence length difference test (ILD) as implemented in PAUP* (Swofford, 2002) to test for phylogenetic congruence between the three mtDNA partitions (COI, cyt *b* and ND2), between mtDNA and βFib7, and between all four gene partitions, and then used the suggested partitioning for parsimony and likelihood analyses.

We ran a MP analysis using a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 100 random addition replicates. Support for each node was assessed by 1000 bootstrap replicates using a heuristic search, TBR branch swapping and 10 random additions per replicate.

For ML analyses, we used a general-time-reversible model of sequence evolution with invariable sites and gamma rate heteroge-

neity (GTR + Γ + I), which according to Modeltest v3.7 (Posada and Crandall, 1998) was the best model of sequence evolution based on the Akaike Information Criterion (AIC). Nodal support in ML analyses was assessed by 100 bootstrap replicates with TBR branch swapping and one random addition per replicate.

For BI analyses, we used MrModeltest v2.3 (Nylander, 2004) to find the best model of evolution for each partitioning scheme and then tested different partitioning schemes with Bayes Factor Analysis (Kass and Raftery, 1995): (1) all data combined; (2) two partitions (mtDNA and βFib7); (3) four partitions (mtDNA 1st codon position, 2nd codon position, 3rd codon position, and βFib7); and (4) 10 partitions (COI 1st codon position, 2nd codon position, 3rd codon position, cyt *b* 1st codon position, 2nd codon position, 3rd codon position, ND2 1st codon position, 2nd codon position, 3rd codon position, and βFib7). A Bayes Factor Analysis (Kass and Raftery, 1995) selected four partitions (three mtDNA codon positions and βFib7) as the best partitioning scheme. Each partition was assigned its own likelihood model based on the MrModeltest results. For the four-partition scheme, likelihood model settings were as follows: mtDNA 1st codon positions with GTR + Γ + I and equal state frequencies; mtDNA 2nd codon position with HKY + Γ + I and a flat Dirichlet prior for state frequencies; mtDNA 3rd codon positions with GTR + Γ + I and a flat Dirichlet prior for state frequencies; and βFib7 with HKY + Γ with a flat Direchlet prior for state frequencies. All parameters were unlinked between partitions (except for the topology and branch length parameters) and were estimated as part of the analysis. We used two parallel runs, each with four Markov chains and for 5 million generations, to insure that our analyses were not stuck at local optima (Huelsenbeck and Bollback, 2001). We sampled the Markov chains every 500 generations and used the resulting 10,000 parameter point-estimates minus the burn-in generations (500) to create a 50% majority-rule consensus tree and to calculate Bayesian Posterior Probabilities (PP) to assess nodal support.

2.3. Geologic and timing analyses

We used a Markov Chain Monte Carlo (MCMC) algorithm as implemented in Beast v1.4.8 (Drummond and Rambaut, 2007) to make Bayesian estimates on the timing of speciation events within *Pteroglossus*. We pruned the data to include only one individual of each subspecies. We used calibrated age splits between two *Semnornis* species and two *Aulacorhynchus* subspecies to estimate divergence events on the tree. One taxon in each of these outgroups is endemic to the Central American highlands, whereas the other is only found in the South American highlands. The uplift of the Talamanca highlands of Costa Rica and western Panama, which connected these two regions, took place approximately 4.5 Mya (Abratis and Worner, 2001; Grafe et al., 2002) and we used this as the maximum age for the split of both *Semnornis* and *Aulacorhynchus* taxa. For the non-calibrated nodes we set a birth–death prior (Gernhard, 2008) with default parameters. We conducted the Bayesian dating analysis using three data partitions (COI, cyt *b*, ND2), each with individual models chosen by MrModeltest (Nylander, 2004), with priors following a uniform [0.0, 4.5] distribution for the two calibration nodes, and individual uncorrelated lognormal rate priors with mean (parameter ucld.mean) following a normal distribution [$\mu = 0.01105$; $\sigma = 0.0034$] of substitutions/site/branch/Mya (s/s/b/Mya), (Weir and Schluter, 2008), and standard deviation (parameter ucld.stdev) following a uniform distribution [0, 10] of s/s/b/Mya for each partition. We ran the analysis for 10,000,000 generations and sampled the chain every 1000th generation. Stationarity of the MCMC chain, parameter effective sample sizes (ESSs), and posterior intervals spanning the 95% highest posterior density (HPD) were assessed using Tracer v1.4.1 (Rambaut and Drummond, 2008). Intervals of divergence were associated with their respective geologic periods according to Gradstein et al. (2004).

Using the branching time information obtained from the Beast analysis, we assessed the rate of diversification in this group with the LASER package (Rabosky, 2007) as implemented in R (R Development Core Team, 2009). If geologic and paleoclimatic events during the Pleistocene did cause an increase in diversification then we would expect to see an increased rate of speciation during or following this time period. First, we plotted the lineages through time (LTT) and calculated the γ-statistic (Pybus and Harvey, 2000), which tests for decreasing rates of diversification, as may be common for other lowland groups (Weir, 2006). Then, we used maximum likelihood to explore and estimate parameters of various models of speciation, including rate constant models, such as pure birth (Yule, 1924) and birth–death (Kendall, 1948), and rate variable models (density dependent exponential, density dependent logarithmic and a two-rate model; Rabosky, 2006). After examining our LTT plot, we also tested a model with a constant extinction and an exponentially decreasing rate of speciation (using fitSPVAR in LASER) because simulations of this model have previously shown patterns similar to our LTT (Rabosky and Lovette, 2008). Through these analyses, we examined whether a change in the diversification rate during the Pleistocene is more likely than a constant rate of diversification. We compared these models to each other using AIC and the likelihood ratio test (where applicable).

3. Results

3.1. Molecular results

For nearly all taxa in the study, we obtained 379 base pairs (bp) for COI, 1048 bp for cyt *b*, 1041 bp for ND2, and 747 bp for βFib7 for a combined total data set of 3215 bp of DNA sequence for 34 taxa (27 ingroup, 7 outgroup). Due to the poor quality of the tissue sample available for *Pteroglossus inscriptus humboldtii* B39983, we were unable to obtain sequences of βFib7 for this individual. Thus, in all phylogenetic estimates, βFib7 sequences were missing for this sample. Also, a short portion of the βFib7 sequences was missing for four of the outgroups (*A. cucullata*, *S. reinwardtii*, *S. frantzii*, and *S. ramphastinus*). There was a total of 737 potentially parsimony informative sites for the combined dataset (84 for COI, 282 for cyt *b*, 327 for ND2, and 44 for βFib7). No individuals were identical across all four genes and therefore all were used in the phylogenetic analyses. The ILD test indicated no significant conflict in underlying phylogenetic signal between the partitions in any of the partition schemes employed ($P = 0.10$ [COI, cyt *b*, ND2], 0.465 [mtDNA and βFib7], 0.20 [all four genes]). Therefore, we combined these sequences into one data set for tree reconstruction. Among ingroup taxa, the average uncorrected sequence divergence was 4.16% (0.08–8.04%) for the combined data set, 4.23% (0–7.12%) for COI, 5.28% (0–8.68%) for cyt *b*, 5.37% (0.09–9.22%) for ND2, and 0.58% (0.00–2.71%) for βFib7.

There was a single codon missing in six individuals between position 1040–1042 of the cyt *b* gene, which is the third to last amino acid in the protein. The six samples are *Pteroglossus frantzii* B16075, *Pteroglossus torquatus torquatus* B28584, *Pteroglossus torquatus sanguineus* B11783, *Pteroglossus torquatus erythropigius* B13479, *Pteroglossus castanotis australis* B37611, and *Pteroglossus pluricinctus* 7112. Of these six samples, *P. frantzii* B16075, *P. t. torquatus* B28584, *P. t. sanguineus* B11783, and *P. t. erythropygius* B13479 form a monophyletic group. In MacClade (version 4.05; Maddison and Maddison, 2002) a parsimony reconstruction (not shown) of the presence/absence of the indel on the MP, ML, and BI trees suggests that it involved three independent losses of the codon on the *Pteroglossus* phylogeny. We translated the cyt *b* sequences and

found that when present, this codon codes for asparagine, except in the *Pteroglossus inscriptus* clade, where it codes for serine. Both of these amino acids are similarly hydrophilic polar amino acids (Nelson and Cox, 2004).

3.2. Phylogenetic results

Maximum parsimony analysis resulted in three most parsimonious trees (TL = 2252, CI = 0.563, CI excluding uninformative characters = 0.492, RI = 0.718), which were the same except for the relationships among individuals in the subspecies *P. i. humboldtii*. These were unresolved in the MP bootstrap 50% consensus tree (Fig. 1). The topology of the ML 50% majority rule bootstrap consensus tree was identical to the tree that resulted from Bayesian analyses (Fig. 2). The Bayesian tree is consistent with the ML tree presented by Eberhard and Bermingham (2005) but includes more subspecific resolution due to our increased taxon sampling (Fig. 2); however, their MP tree was slightly different. Our MP trees have *P. bailloni* sister to a monophyletic *Pteroglossus*, whereas Eberhard and Bermingham (2005) found that it nested with moderate support within *Pteroglossus* as sister to the *Pteroglossus viridis*/*Pteroglossus inscriptus* clade. Our ML and Bayesian analyses are consistent with those in Eberhard and Bermingham (2005) and indicate strong support (ML = 96%, PP = 1.0) for *P. bailloni* being imbedded within *Pteroglossus* and sister to the *P. viridis*/*P. inscriptus* clade (Fig. 2). MP bootstrap support for the relationship of *P. bailloni* within the *P. viridis*/*P. inscriptus* clade was only 37.6%.

3.3. Biogeographic and timing results

In the BEAST analysis, a burn-in of 1% was sufficient for parameters to reach stationarity, and ESSs were all above 200. We obtained a chronogram showing 95% confidence intervals for the divergence times at each node (Fig. 3). Root height had a 95% posterior distribution of 10.38–15.61 Mya. Here we report posterior rates in s/s/Mya, instead of s/s/*b*/Mya (which is roughly half the value of a rate in s/s/Mya). Rates (parameter meanRate) were 1.72–2.99% s/s/Mya for COI, 2.47–3.73% for cyt *b*, and 2.74–4.11% for ND2. Therefore, only the range of rate values for COI encompassed the commonly reported 2% rate in avian studies (García-Moreno, 2004; Lovette, 2004; Weir and Schluter, 2008).

The LTT plot (Fig. 4) was linear, with calculated γ-statistic 0.09729179, which can be the result of either a constant diversification rate or constant extinction rate with decreasing speciation (Rabosky and Lovette, 2008). When fitting the model of constant extinction rate with decreasing speciation, the rate of exponential decrease in speciation was close to zero and yielded a likelihood value similar to the pure birth model, which was the best rate constant model. According to AIC, the best fit model overall was pure birth, indicating that extinction probably did not play a role in the diversification of this group and that the rate of speciation was constant throughout (Table 3). The likelihood ratio test was consistent with this result by indicating that the other more complex models (birth death, density dependent exponential, density dependent logarithmic, two rate, and constant extinction/

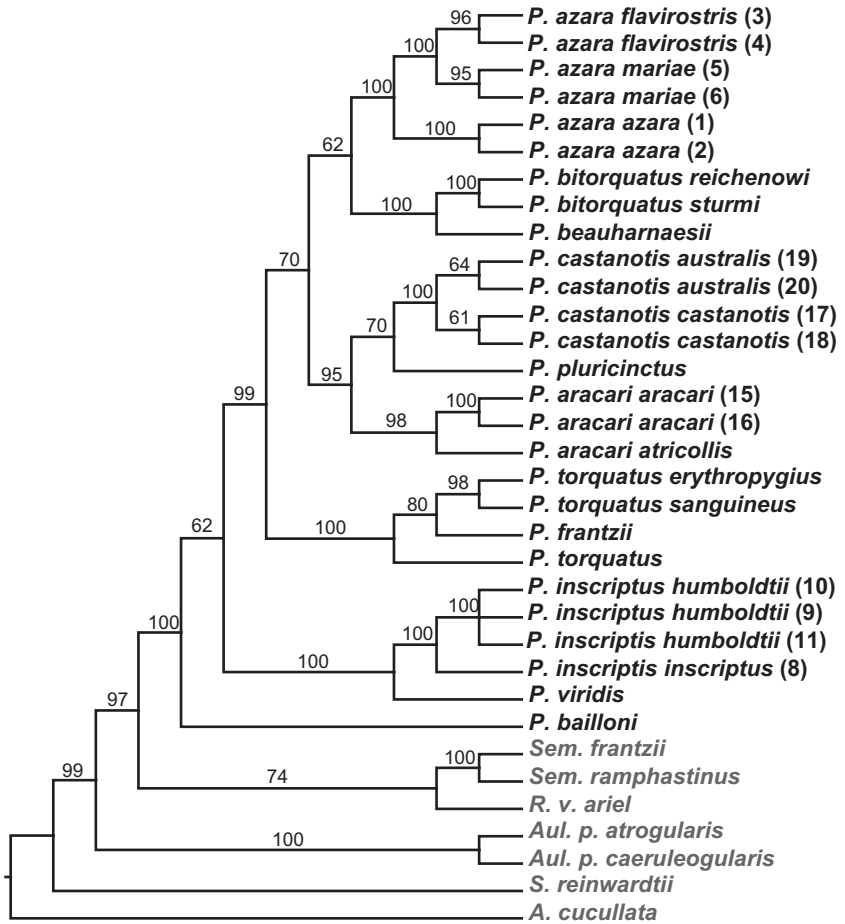

Fig. 1. Maximum parsimony 50% majority rule bootstrap consensus of *Pteroglossus* from the combined analysis of three mtDNA genes (COI, cyt *b*, ND2) and one nuclear intron (βFib7). MP bootstrap values are shown above nodes for support. Numbers next to individuals correspond to numbers in Table 1. Abbreviations are as follows: *P.* = *Pteroglossus*, *Sem.* = *Semnornis*, *R.* = *Ramphastos*, *Aul.* = *Aulacorhnychus*, *S.* = *Selenidera*, *A.* = *Andigena*.

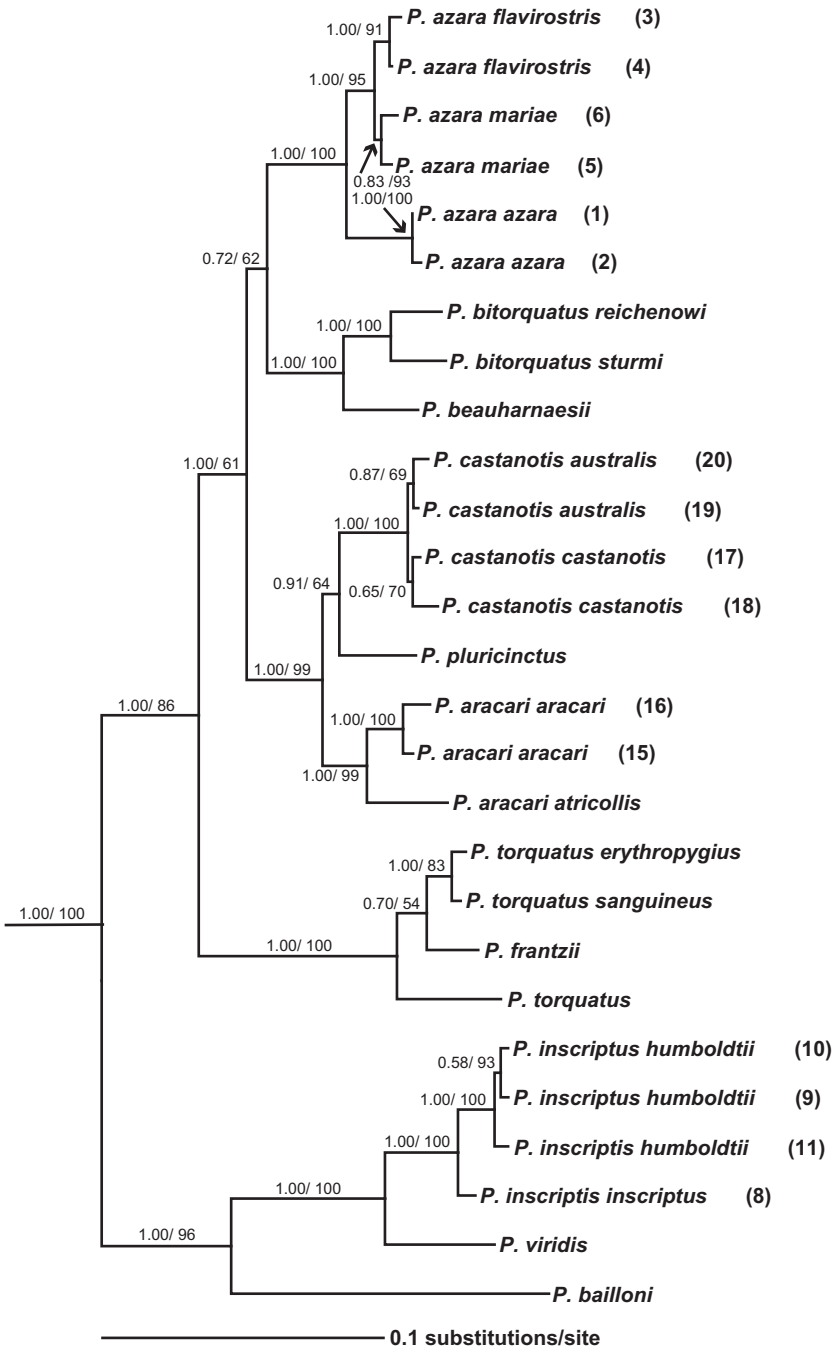

Fig. 2. Phylogram of the Bayesian consensus tree of *Pteroglossus* from the four partition analysis (1st, 2nd, 3rd codons of the mtDNA genes [COI, cyt *b*, and ND2] and βFib7 intron) with branch lengths proportional to substitutions per site. Support values correspond to Bayesian posterior probabilities and ML bootstrap values, respectively. Numbers next to individuals correspond to numbers in Table 1. Abbreviations are the same as in Fig. 1. The outgroup taxa are pruned from this tree.

decreasing speciation) did not significantly fit the data better than pure birth. If we force a two-rate model, the most likely breakpoint is at 0.5 Mya, at which point the diversification rate slightly decreases.

4. Discussion

4.1. Phylogenetic relationships and taxonomy

The phylogenetic relationships reconstructed here are consistent with the phylogeny presented by Eberhard and Bermingham (2005) but include more subspecific resolution. The *P.* (*viridis*) superspecies, which includes *P.* (*viridis*) *inscriptus*, is monophyletic, as Haffer (1974) predicted based on morphology. However, the *P.* (*araçari*) superspecies, in which Haffer (1974) groups *P. castanotis*, *Pteroglossus aracari*, *P. pluricinctus*, and *Pteroglossus torquatus*, is not. In our results, *P. castanotis*, *P. aracari*, and *P. pluricinctus* are more closely related to *Pteroglossus bitorquatus*, *Pteroglossus beauharnaesii*, and *Pteroglossus azara* than to *P. torquatus*, a result also found by Eberhard and Bermingham (2005). Haffer (1974) thought that the *P.* (*bitorquatus*) superspecies had no close relatives and was thus a comparatively "old" taxon. However, our results strongly support *P. beauharnaesii* as sister to *P. bitorquatus*, to the

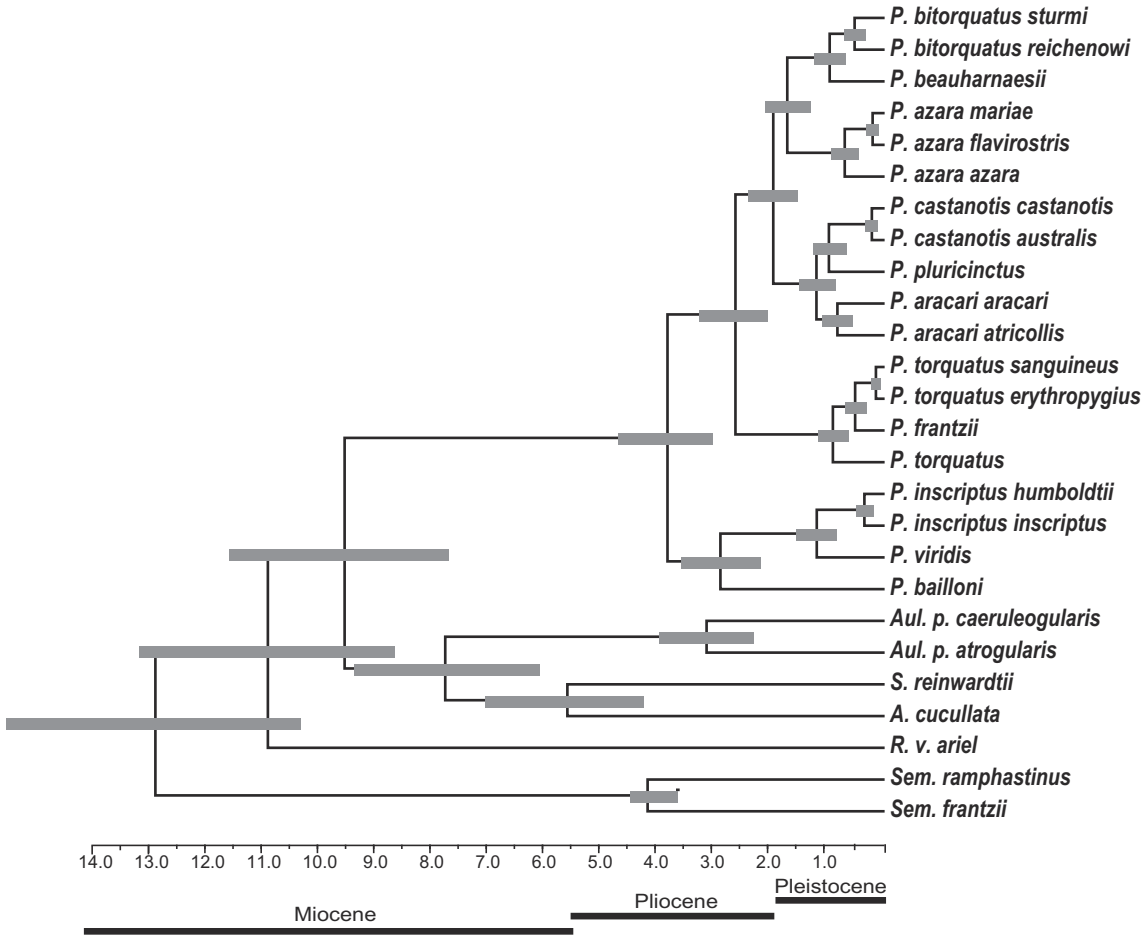

Fig. 3. Chronogram of *Pteroglossus* indicating divergence time estimates based on Bayesian relaxed clock analysis. Gray bars on nodes correspond to the 95% confidence intervals of the time estimates. Abbreviations are the same as in Fig. 1. Scale numbers correspond to millions of years before present.

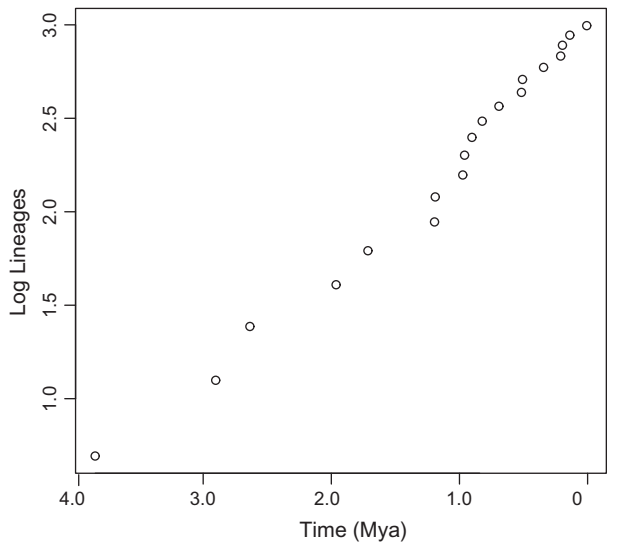

Fig. 4. Log lineages through time plot of *Pteroglossus* based on mean divergence time estimates.

exclusion of *P. azara* (Fig. 2), rendering Haffer's (1974) *P.* (*bitorquatus*) superspecies paraphyletic. Therefore, based on our results and those of Eberhard and Bermingham (2005), four main natural groups can be recognized in *Pteroglossus*: (1) *P.* (*viridis*) including *P. bailloni*, *P. viridis*, and *P. inscriptus*; (2) *P.* (*torquatus*) including *P. torquatus* and *P. frantzii*; (3) *P.* (*aracari*) including *P. aracari*, *P. pluricinctus*, and *P. castanotis*; and (4) *P.* (*bitorquatus*) including *P. bitorquatus*, *P. beauharnaesii*, and *P. azara*.

Previously, *P. bailloni* was placed in a monotypic genus (*Baillonius*), thought to be closely related to *Pteroglossus* (Cracraft and Prum, 1988; Hackett and Lehn, 1997; Haffer, 1974). Eberhard and Bermingham (2005) found moderate support for the placement of *P. bailloni* within *Pteroglossus*, but indicated that they could not reject the taxonomic placement of this species as sister to *Pteroglossus*. Kimura et al. (2004) found strong support for *P. bailloni* being nested within *Pteroglossus*, based on a fragment of cyt *b*. The placement of this species within and not as sister to *Pteroglossus*, although weakly supported by our MP-bootstrap analysis (38%), was strongly supported by our ML bootstrap and Bayesian posterior probabilities (PP; 96% and 1.0 respectively) analyses. The MP-bootstrap results may be affected by saturation and homoplasy, which are mitigated by the use of molecular models under ML and BI. Therefore, we favor nesting *P. bailloni* inside *Pteroglossus* and sister to the *viridis* superspecies.

The improved taxon sampling in our study provides additional information about subspecific relationships at the tips of this evolutionary tree. In all cases where we sampled multiple individuals of a subspecies, those individuals formed a monophyletic group, with moderate to strong support (61–100% MP-bootstrap from Fig. 1, 69–100% ML bootstrap, 0.59–1.0 PP from Fig. 2). In some cases, the reciprocal monophyly of these subspecies, relatively high divergences, and diagnostic morphological features suggest that

Table 3
AIC and log likelihood values for fitting models to *Pteroglossus* chronogram.

Model	Parameters	AIC	Log likelihood
Pure Birth[a]	r	−23.12342	12.56171
Birth Death[b]	r, a	−21.15471	12.57736
Two rate[c]	r_1, t, r_2	−19.84406	12.92203
Density Dependent Exponential[c]	r_o, x	−21.12451	12.56226
Density Dependent Logarithmic[c]	r_o, k	−21.22804	12.61402
High Extinction, Decreasing Speciation[d]	k, λ_o, μ	−19.15202	12.57601

[a] Yule (1924).
[b] Kendall (1948).
[c] Rabosky (2006).
[d] Rabosky and Lovette (2008).

taxa such as *Pteroglossus azara flavirostris* and *Pteroglossus azara mariae* might best be considered valid species under lineage based species concepts (i.e., de Queiroz, 2005), as already recognized by the Brazilian Checklist Committee for *P. azara mariae/flavirostris* (CBRO, 2009). Additional population level sampling is needed to test the robustness of patterns of reciprocal monophyly recovered in this study for taxa grouped under the other polytypic *Pteroglossus* taxa, such as *P. inscriptus*, *P. aracari*, *P. castanotis* and *P. bitorquatus*.

Our results and those of Eberhard and Bermingham (2005) indicate that *P. torquatus* was paraphyletic with respect to the Central American endemic *P. frantzii*, which is sister to the South American subspecies *P. t. erythropygius* and *P. t. sanguineus*. Nominate *torquatus* was sister to the *frantzii–erythropygius–sanguineus* clade (Fig. 2). Support for the node uniting *erythropygius* and *sanguineus* to *P. frantzii* was moderate according to our results (Figs. 1 and 2), but high in Eberhard and Bermingham (2005). Previously, *frantzii*, *torquatus*, *sanguineus*, and *erythropygius* were each considered separate species (Meyer de Schauensee, 1970), but Haffer (1974) treated them as subspecies of *P. torquatus* based on purported hybrids between *sanguineus* and *torquatus* from northwestern Colombia, and that *erythropygius* and *frantzii* looked no more different from each other than the hybridizing taxa, although evidence of intergradation involving either *erythropygius* and *frantzii* was lacking. Subsequently, authors differed significantly in their treatment of the *P. torquatus* superspecies, ranging from considering all taxa as separate species (Hilty and Brown, 1986; Ridgely and Greenfield, 2001) to lumping them into two or three biological species (Short and Horne, 2001, 2002). The AOU (1998), and South American Classification Committee (Remsen et al., 2010) treat *P. frantzii* as a separate species, lumping all remaining taxa under *P. torquatus*. When viewed together, our study and that of Eberhard and Bermingham (2005) sampled a total of 11 individuals belonging to the *P. torquatus* superspecies (3 *torquatus*, 3 *frantzii*, 3 *sanguineus*, and 2 *erythropygius*), and consistently recovered the same topology in which these taxa appear as reciprocally monophyletic. Therefore, even if hybridization is present among members of this superspecies, it may be so restricted (as documented by Haffer, 1974) that it has not led to the loss of reciprocal genetic and morphological diagnoses among the taxa involved. Hence, using the increased sampling in this group, we infer that all members of the *P. torquatus* superspecies are better treated as separate evolutionary and even biological species. No evidence is available to date showing that *P. frantzii* hybridizes with *P. torquatus* where both overlap on the Pacific coast of Costa Rica and Panama (Short and Horne, 2001).

4.2. Timing of diversification and biogeography

Peterson (2007) documented wide variation in approaches to molecular clock calibration, and *Pteroglossus* provides a good example of this for a single lineage. Our dating estimate of the first diversification event within extant *Pteroglossus* lineages (3.05–4.76 Mya, Middle to Early Pliocene) differs greatly from that of Pereira and Wajntal (2008), who estimated this event to have taken place in the Late to Middle Miocene (9.8–13.0 Mya). We believe that our estimates are more accurate because Pereira and Wajntal (2008) calibrated their divergences using the Andean uplift and many older calibration points in the avian tree. These calibration points are problematic for two reasons. First, the Andes may not serve as a strong barrier even to the dispersal of small passerines, such as *Mionectes* flycatchers (Miller et al., 2008), inhabiting the forest understory and therefore would be an even more permeable barrier to canopy birds such as *Pteroglossus* (Burney and Brumfield, 2009). Second, the old calibrations used by Pereira and Wajntal (2008; many older than 12 Mya) may be biased due to mtDNA saturation, which may lead to overestimates of divergence times (Weir and Schluter, 2008).

We estimate that *P. bailloni* diverged from the *viridis* superspecies group between 2.25 and 3.68 Mya, which spans the latter half of the Pliocene. *P. bailloni* is found only in the Atlantic forest, whereas the *viridis* superspecies is an endemic Amazonian group. Six previously published studies have comparable Amazonia/Atlantic forest avian splits (*Pionopsitta* and *Pteroglossus*: Eberhard and Bermingham, 2005; *Pionus*: Ribas et al., 2007; *Xiphorhynchus*: Cabanne et al., 2008; *Mionectes*: Miller et al., 2008; *Ramphastos*: Patané et al., 2009; and *Brotogeris*: Ribas et al., 2009; Table 4). In comparing these taxa, one can see that the patterns and timing of diversification are quite variable. Because of the inconsistency of dating methods across different studies (Peterson, 2007), we use the most conservative estimates (widest time interval) to draw comparisons. The Amazonia/Atlantic Forest splits for *Ramphastos vitellinus* (0.77–1.40 Mya; Patané et al., 2009), *Pionus menstruus/reichenowi* (0.15–1.41 Mya; Ribas et al., 2007), and *Mionectes oleagineus* (ca. 0.9 Mya; Miller et al., 2008) are more recent, whereas *Xiphorhynchus fuscus*, *Mionectes rufiventris*, and *Brotogeris tirica*, all Atlantic forest endemics, split from their Amazonian sister lineages around the same time (ca. 3.0 Mya: Cabanne et al., 2008; ca. 2.9 Mya: Miller et al., 2008; 3.0–3.6 Mya: Ribas et al., 2009) as the *P. bailloni* split estimate from this study as well as from

Table 4
Comparative timing of Amazonia/Atlantic Forest and cis/trans-Andean splitting events in some Neotropical avian lineages.[a]

	Amazonia/Atlantic Forest	Cis/trans Andean
Pteroglossus[b] (Ramphastidae)	2.18–3.59	2.05–3.28
Pteroglossus[c] (Ramphastidae)	3.1	2.6
Pteroglossus[d] (Ramphastidae)	10.0	9.9
Ramphastos[e] (Ramphastidae)	0.77–1.40	2.60–4.15
		0.99–1.75
		0.33–0.81
		0.20–1.40
Brotogeris[f] (Psittacidae)	3.0–3.6	~3
Pionus[g] (Psittacidae)	0.15–1.41	0.15–1.41
Pionopsitta[c] (Psittacidae)	6.6	4.2
Xiphorhynchus[h] (Dendrocolaptidae)	~3.0	–
Mionectes[i] (Tyrannidae)	~2.3	~1.9
		~1.5
	~0.9	~0.3

[a] Dates reported in million years before present.
[b] This study.
[c] Eberhard and Bermingham (2005).
[d] Pereira and Wajntal (2008).
[e] Patané et al. (2009).
[f] Ribas et al. (2009).
[g] Ribas et al. (2007).
[h] Cabanne et al. (2008).
[i] Miller et al. (2008).

Eberhard and Bermingham (2005; Table 4). These differences in timing suggest that distinct geological and climatic events have led to multiple dispersal and vicariance events between Amazonian and Atlantic Forest regions or different responses to the same barrier. Our estimates, in conjunction with these previous studies, do not match the timing of marine transgression and subsequent formation of savanna between the Atlantic and Amazon forests in the Miocene that Pereira and Wajntal (2008) suggested as the vicariance event leading to this divergence. Willis (1992) and DeOliveira et al. (1999) suggested that forested links existed between the Amazon and Atlantic forests periodically during the Pleistocene; however, as the *P. bailloni* split and the other datasets discussed above illustrate, the historical exchange between the Atlantic forest and Amazonia biotas predates the Pleistocene, and therefore was likely the result of multiple events.

In four of five of these Neotropical groups (*Pteroglossus*, *Ramphastos*, *Brotogeris* and *Mionectes*), a cis/trans Andean split followed the Atlantic forest divergence. We estimate that the *P. torquatus* species complex, a monophyletic group found west of the Andes (Chocó and Central America), split from its cis-Andean sister lineage around 2.05–3.28 Mya, which is within the range of estimates for the final uplift of the Andes (~2–5 Mya; Gregory-Wodzicki, 2000). Eberhard and Bermingham (2005) had a similar estimate of ~2.6 Mya. Therefore, our estimates are consistent with orogeny as a vicariance event that drove diversification of *Pteroglossus* on either side of the Andes. The alternative is that there was a dispersal event around the Andes through the Caribbean lowlands (Nores, 2004) at this time. Studies of some avian lineages have identified discordance between the estimated time of speciation and uplift of the Andes, and thus suggest that cis/trans splits were due to dispersal around the Andes (Miller et al., 2008; Patané et al., 2009; Ribas et al., 2009). *Ramphastos* alone shows four independent cis/trans Andean speciation events (Table 3; Patané et al., 2009), whereas *Mionectes* shows three (Miller et al., 2008). Patané et al. (2009) hypothesized that the earliest of these four events in *Ramphastos* (2.60–4.15 Mya) was concordant with the uplift of the Andes and that the three more recent events were due to dispersal. Ribas et al. (2009) suggested dispersal around the Andes in *Brotogeris* for a speciation event ~3.0 Mya. Differentiating between vicariance due to uplift and dispersal around the Andes is not always straightforward and further information is needed on the temporal relationship between the vicariance and speciation events. This is likely to be dependent on the dispersal abilities of the group being considered, as is the case for vicariance due to rivers (Burney and Brumfield, 2009).

The topology within the *torquatus* group suggests that dispersal events subsequent to the rise of the Andes had to occur to lead to present-day distributions. We estimate that at ca. 0.31–0.7 Mya, the Chocó endemic taxa (*P. t. sanguineus* and *P. t. erythropygius*) split off from the other Central American members of the *torquatus* group (*P. torquatus* and *P. frantzii*). One possibility is that the common ancestor of the *torquatus* group dispersed to a widespread trans-Andean distribution (Central America and Chocó) and then differentiated *in situ* to present-day patterns, possibly as the result of sea level rises. However, the timing of the sea level rises remains controversial and may have occurred during the Late Pliocene, well before this split (Nores, 2004). Another possibility is that the ancestor to *torquatus* dispersed from Western Amazonia (cis-Andean) to Central America, and then the populations dispersed back to the Chocó to form the present-day *sanguineus* and *erythropygius* groups. Future phylogeographic and population genetics studies focusing on the *torquatus* group should test those two alternative hypotheses.

Among Amazonian lineages of *Pteroglossus*, many reciprocally monophyletic and morphologically diagnosable taxa are separated by wide rivers such as the Amazon (e.g., *P. viridis* and *P. inscriptus*; *P. aracari aracari* and *Pteroglossus aracari atricollis*), Solimões/Amazon (*P. azara mariae* and *Pteroglossus azara azara*/*Pteroglossus azara flavirostris*), Madeira (*P. inscriptus inscriptus* and *P. inscriptus humboldtii*; *P. castanotis castanotis* and *P. c. australis*), and Tapajós (*Pteroglossus bitorquatus reichenowi* and *Pteroglossus bitorquatus sturmi*) rivers. The majority of lineages separated by major rivers involve intra-specific taxa, which have not previously been included in any *Pteroglossus* phylogeny (Hackett and Lehn, 1997; Eberhard and Bermingham, 2005). In fact, all subspecies of *Pteroglossus* in the Amazon are delimited by a major Amazonian river (Short and Horne, 2002). This demonstrates the important role that these rivers have played on more recent *Pteroglossus* diversification, beginning in the early/middle Pleistocene. Sister lineages at the species level often have overlapping distributions that span these rivers (e.g. *P. beauharnaesii*/*P. bitorquatus*, and *P. pluricinctus*/*P. castanotis*). One possible explanation for this pattern is that species diverged, then expanded their ranges to become secondarily sympatric, and are now further diverging into subspecies due to the vicariance effect of the Amazonian tributaries (Haffer, 1997). According to some estimates, the modern Amazon drainage was already in place about 2.4 Mya (Figueiredo et al., 2009), but other studies support younger dates stretching well into the Pleistocene (Rossetti et al., 2005; Campbell et al., 2006). Our results are more consistent with a "young" age for the Amazon drainage, but it is also conceivable that the possible delay in cladogenesis seen in *Pteroglossus* under the "old" Amazon drainage hypothesis may be due to the fairly good dispersal ability of these toucans. This might have led to greater coalescent times and a delayed response to vicariance barriers, as predicted for other avian lineages with similar natural history attributes (Hackett and Lehn, 1997; Burney and Brumfield, 2009). Our subspecific phylogenetic resolution provides further insight into the influence of the Amazon river basin on diversification patterns of this group, but broad generalizations will be possible only when densely sampled phylogenies become available for a diverse array of lineages and organisms.

4.3. Diversification rates

According to our estimates, *Pteroglossus* diversification began between 3.04 and 4.71 Mya (95% confidence interval), well before the start of the Pleistocene. Although the onset of the *Pteroglossus* diversification predates the Pleistocene, most of the speciation within *Pteroglossus* took place during this epoch, which may make it seem as if events during the Pleistocene did cause more diversification (Fig. 3). However, one cannot simply count speciation events in a given time period to determine the rate of diversification. Instead, one has to use methods of analyzing rates of diversification to test whether there was an actual rate increase or if diversification is occurring at a background level. In other words, if Pleistocene events did cause an increase in diversification, we would expect to see an increase in the rate some time after the onset of the Pleistocene. Statistical analysis on rates of diversification indicated a constant rate of speciation across the entire *Pteroglossus* tree. Thus, although most splits in *Pteroglossus* occurred during the Pleistocene, the results of our lineage through time analyses do not support an increase in diversification during this time as previously hypothesized for this group (Haffer, 1974; Hackett and Lehn, 1997). In a study of multiple avian groups, including the Eberhard and Bermingham (2005) *Pteroglossus* data, Weir (2006) also showed that diversification in lowland groups did not increase during the Pleistocene. On the contrary, he showed a density dependent rate for lowland taxa, in which speciation decreases as the number of existing lineages increases (Weir, 2006). Our results do not show this decrease. One possibility for this discrepancy is our inclusion of all major *Pteroglossus* phylogroups (i.e., all recognized species and most subspecies), which prevented a taxonomic

bias towards not recognizing relatively young independent evolutionary lineages in a phylogeny, and thus leading to an overall underestimation of rates of diversification (Tobias et al., 2008; Cusimano and Renner, 2010). From another perspective, although our results (constant diversification rate) differ from Weir's (2006) decreased diversification rate, both studies agree with the conclusion that speciation rates did not increase significantly during the Pleistocene, thus undermining the hypothesis that events during the Pleistocene led to a rapid radiation.

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