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Pliocene diversification within the South American Forest falcons (Falconidae: *Micrastur*)

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ABSTRACT

The Neotropics are one of the most species rich regions on Earth, with over 3150 species of birds. This unrivaled biodiversity has been attributed to higher proportions of mountain ranges, tropical rain forest or rain fall in the forest than in any other major biogeographic regions. Five primary hypotheses aim to explain processes of diversification within the Neotropics; (1) the Pleistocene refuge hypothesis, (2) the riverine barrier hypothesis, (3) the Miocene marine incursions hypothesis, (4) the ecological gradient hypothesis, and (5) the impact of the last Andean uplift serving as a barrier between eastern and western population Andean populations. We assessed these hypotheses to see which best explained the species richness of the forest-falcons (*Micrastur*), a poorly known lineage of birds that inhabit lowland and mid-elevation humid forest. Our analyses suggest all speciation events within the genus *Micrastur* probably occurred in the last 2.5–3.6 myrs, at or before the Pliocene/Pleistocene boundary, with the basal split within the genus being 7 myrs old. Hence our data allow us to formerly reject the classical Pleistocene refuge for *Micrastur*, Our divergence time estimates are younger that dates for the Miocene marine incursions, the riverine barrier and the Andean uplift hypotheses.

1. Introduction

The Neotropics are one of the most species-rich regions on Earth, with over 3150 species of birds (Fjeldså, 1994; Gentry, 1988). This unrivaled species richness has been attributed to a higher proportion of mountain ranges, tropical rain forest or rain-fall than in any other major biogeographic regions (e.g. Afrotropics, Indo-Malaya). Because of its high diversity, the Neotropics has been a major focus for studies of endemism and processes of biodiversity formation (Wallace, 1852; Haffer, 1969, 1997; Cracraft, 1985; Fjeldså, 1994; Bates et al., 1998). There are five primary hypotheses that aim to explain processes of diversification within the Neotropics (Haffer, 1997), (1) the classical Pleistocene refuge hypothesis (Haffer, 1969, 1997 but see Bush and Oliveira, 2006), (2) the riverine barrier hypothesis (Wallace, 1852; Bates et al., 2004 but see Aleixo, 2004), (3) the Miocene marine incursions hypothesis (16–21 mya and 11 mya; Lovejoy et al., 2006), (4) the ecological gradient hypothesis (Endler, 1982), and (5) the impact of the Andean uplift serving as a barrier between eastern and western population Andean populations (e.g. Burney and Brumfield, 2009). Previous studies report strikingly different patterns and timings of Neotropical species origins, suggesting that no single hypothesis is sufficient (e.g. Bates et al., 1998; Marks et al., 2002; Costa, 2003; Miller et al., 2008; Santos et al., 2009), and that multiple factors have, on multiple occasions, impacted the genetic structure within species.

The genus *Micrastur* (forest falcons) includes seven species that are distributed in the lowland and mid-elevation humid forest of Central and South America. The biology of most species is poorly known due to their secretive behavior, and confusion exists about species limits. This situation is exemplified by the *Micrastur ruficollis* species group. Three primary taxa were traditionally recognized within that lineage (*M. ruficollis*, *Micrastur gilvicollis* and *Micrastur plumbeus*). *Micrastur gilvicollis* was considered to be conspecific with *M. ruficollis* by some (e.g. Amadon, 1964) until Schwartz (1972), using morphology and vocalizations data, indicated they were distinct species. Depending how *M. gilvicollis* was treated taxonomically, *M. plumbeus* was either considered a subspecies of *M. gilvicollis* (e.g. Stresemann and Amadon, 1979) or a distinct species if *M. gilvicollis* was considered a subspecies of *M. ruficollis* (Bierregaard, 1994a). The latter treatment was justified by evidence of sympatry with *M. ruficollis interstes* in Colombia. A new species was added recently to the *M. ruficollis* species group with the name Cryptic Forest-Falcon (*Micrastur mintoni*) (Whittaker, 2002). Because poor knowledge about species limits and genetic diversity prevents any efficient conservation planning (e.g. Sinclair et al., 2005; Johnson et al., 2007), a robust phylogenetic hypothesis

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may help to identify taxa that are in critical need of conservation. With one species (*M. plumbeus*) listed as Vulnerable by the IUCN and two others only poorly known (*M. mintoni* was described in 2002, *Micrastur buckleyi* is only known from 11 specimens, Whittaker, 2001), the genus *Micrastur* is in clear need of study. The forest falcons are often classified in a separate subfamily, the Herpetotherinae, together with their closest relative, the Laughing Falcon (*Herpetotheres cachinnans*). The latter clade appears to be the first clade to branch off in the Falconidae phylogeny (Griffiths et al., 2004). This phylogenetic placement as an early off-shoot and the fact that this clade is species-poor, relative to the other two subfamilies, contributes to their distinctiveness and conservation value.

Here, we address the phylogenetic relationships and biogeographic history of the forest-falcons using over 7400 bp of DNA sequence data gathered from all *Micrastur* species.

2. Material and methods

2.1. Sampling

We sampled 1–7 individuals for all species of *Micrastur* and two individuals of *H. cachinnans*, the sister taxon to *Micratur* (Table 1). Representatives of all primary lineages within Falconidae (i.e. Falconinae and Polyborinae) were included as proximate outgroups to allow the use of a fossil calibration point. Sequences from representatives of several avian orders (Table 1) were used as distant outgroups.

DNA was extracted from fresh tissues (muscles, liver, kidney) using the Qiagen extraction kit (Valencia, CA) following the manufacturer's protocol. We extracted DNA from the *M. buckleyi* toe-pad sample in a room dedicated to work with ancient DNA and used a phenol–chloroform extraction protocol and 20 l of dithiothreitol (DTT, 0.1 M).

We analyzed DNA sequences from eight independent loci, including a mitochondrial fragment of ca 2.4 kb (encompassing the tRNA-Leu, ND1, trNA-Ile, tRNA-Gln, tRNA-Met and ND2 region) and seven autosomal loci (Myoglobin intron-2 -MB, β-fibrinogen intron-5 -FGB, transforming growth factor beta2 intron-5-TGFB2, phosphoenol pyruvate carboxykinase intron-9 -PEPCK, vimentin intron-8 -VIM, period homolog 2 intron-9, -PER and recombination activating gene 1 – RAG1). The primer sequences used for PCR-amplification and sequencing are detailed in Supplementary material Tables S1a and S1b. We amplified the mitochondrial region in one fragment using the primers L3827 and H613 using the TaKaRa LA Taq (TaKaRa Co. Ltd., Tokyo, Japan). The thermocycling conditions for the mitochondrial fragment included a hotstart at 94 °C,

Table 1
List of taxa studied (following Dickinson, 2003), and tissue or voucher number informations. All *Micrastur* species but *M. ruficollis* and *M. semitorquatus* are monotypic. Abbreviations: AMNH, American Museum of Natural History, New York, USA; CAS, California Academy of Sciences, San Francisco, USA; FMNH, Field Museum of Natural History, Chicago, USA; KUNHM, University of Kansas, Natural History Museum, Lawrence, USA; LSUMNS, Louisiana State University, Baton-Rouge, USA; PF, Peregrine Fund; NRM, Swedish Museum of Natural History, Stockholn, Sweden, USNM, National Museum of Natural History, Smithsonian Institution, Washington UWBM, University of Washington, Burke Museum, Seattle, USA. Asterisks indicate tissue with voucher specimens.

Subfamily	Genus	Species	Subspecies	Voucher/tissue number	Country
Polyborinae	*Caracara*	*cheriway*		LSUMNS B-8513*	USA
Polyborinae	*Ibycter*	*americanus*		LSUMNS B-1019*	Bolivia
Falconinae	*Falco*	*columbarius*		CAS 91441*	USA
Falconinae	*Falco*	*peregrinus*		CAS 90669*	USA
Herpetotherinae	*Herpetotheres*	*cachinnans*	*queribundus*	KUNHM 90147*	Paraguay
Herpetotherinae	*Herpetotheres*	*cachinnans*	*cachinnans*	USNM612262*	Panama
Herpetotherinae	*Micrastur*	*buckleyi*		MVZ165101*	Peru
Herpetotherinae	*Micrastur*	*gilvicollis*		LSUMNS B-20376*	Brazil
Herpetotherinae	*Micrastur*	*gilvicollis*		LSUMNS B-55277*	Surinam
Herpetotherinae	*Micrastur*	*gilvicollis*		LSUMNS B-5502*	Peru
Herpetotherinae	*Micrastur*	*mintoni*		FMNH 389678*	Brazil
Herpetotherinae	*Micrastur*	*mintoni*		FMNH 389679*	Brazil
Herpetotherinae	*Micrastur*	*mintoni*		FMNH 456434*	Brazil
Herpetotherinae	*Micrastur*	*mirandollei*		LSUMNS B-4991*	Peru
Herpetotherinae	*Micrastur*	*mirandollei*		LSUMNS B-4998*	Peru
Herpetotherinae	*Micrastur*	*plumbeus*		LSUMNS B-11798*	Ecuador
Herpetotherinae	*Micrastur*	*plumbeus*		LSUMNS B-11846*	Ecuador
Herpetotherinae	*Micrastur*	*ruficollis*	*guerilla*	FMNH 393898*	Mexico
Herpetotherinae	*Micrastur*	*ruficollis*	*guerilla*	PF-Falco24	Guatemala
Herpetotherinae	*Micrastur*	*ruficollis*	*pelzelni*	FMNH 433039*	Peru
Herpetotherinae	*Micrastur*	*ruficollis*	*pelzelni*	LSUMNS B-9716*	Bolivia
Herpetotherinae	*Micrastur*	*ruficollis*	*pelzelni*	LSUMNS B-27771*	Peru
Herpetotherinae	*Micrastur*	*ruficollis*	*ruficollis*	NRM 937326*	Paraguay
Herpetotherinae	*Micrastur*	*semitorquatus*	*naso*	PF-Falco22	Guatemala
Herpetotherinae	*Micrastur*	*semitorquatus*	*naso*	PF-Falco23	Guatemala
Herpetotherinae	*Micrastur*	*semitorquatus*	*naso*	UWBM 77034*	Panama
Herpetotherinae	*Micrastur*	*semitorquatus*	*semitorquatus*	LSUMNS B-11298*	Peru
Falconinae	*Microhierax*	*caerulescens*		AMNH DOT10891	Unknown
Polyborinae	*Milvago*	*chimango*		USNM 614585*/USNM 635931*	Argentina/Uruguay
Polyborinae	*Phalcoboenus*	*megalopterus*		LSUMNS B-22907*	Bolivia
Falconinae	*Polihierax*	*semitorquatus*		FMNH 391014*	Captive
Polyborinae	*Spiziapteryx*	*circumcincta*		LSUMNS B-18584*	Bolivia
Outgroup					
	Sphyrapicus	*varius*		CAS89230*	USA
	Otus	*kennicottii*		CAS90394*	USA
	Buteo	*jamaicensis*		CAS89962*	USA
	Vultur	*gryphus*		CAS86013*	Captive
	Pandion	*haliaetus*		CAS95638*	USA
	Corvus	*corax*		CAS90612*	USA

an initial denaturation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 40 s, 56 °C for 40 s, and 72 °C for 3 min, and was completed by a final extension at 72 °C for 15 min. The thermocycling conditions for the nuclear introns included a hotstart at 94 °C, an initial denaturation at 94 °C for 3 min, followed by 35–40 cycles at 94 °C for 40 s, 55–60 °C for 30–45 s, and 72 °C for 30–45 s, and was completed by a final extension at 72 °C for 10 min. Nuclear introns were amplified using the Recombinant Invitrogen Taq (Invitrogen Co., Carlsbad, CA). Purified PCR products were cycle-sequenced using the Big Dye terminator chemistry (ABI, Applied Biosystems) in both directions with the same primers used for PCR amplification, and run on an automated ABI 3100 DNA sequencer. We used an additional set of primers to sequence the mitochondrial fragment (see Supplementary material Table S1b). Heterozygous sites in the nuclear loci (double peaks) were coded using the appropriate IUPAC code. Apparent length polymorphisms were cloned using the TOPO TA cloning kit with pCR2.1 vector and Mach1 cells (Invitrogen Co., Carlsbad, CA), following the manufacturer's protocol. Between four and ten clones were sequenced per individual. All sequences have been deposited in Genbank (Accession Numbers JF899877–JF899917 and JF909604–JF909802).

2.1.1. Phylogenetic analyses

Phylogenetic analyses including individual gene trees and a concatenated approach were conducted using maximum likelihood and Bayesian inference, as implemented in RAxML V7.0.4 (Stamatakis, 2006; Stamatakis et al., 2008), MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003) and BEAST 1.5.4 (Drummond et al., 2002, 2006; Drummond and Rambaut, 2007). The most appropriate models of nucleotide substitution were determined with TOPALi v2.5 (Milne et al., 2009) and the Bayesian Information Criterion (BIC). Maximum likelihood and Bayesian analyses under the concatenated approach were performed allowing the different parameters of the substitution model to vary among loci (i.e. partitioned analyses, Nylander et al., 2004). The relevance of partitioning the protein coding genes by codon position was assessed using Bayes Factors (B_F; Nylander et al., 2004). A value greater than 4.6 for $\ln B_F$ was considered as very strong evidence against the simpler model (Jeffreys, 1961); this value is very similar to the threshold proposed by Kass and Raftery (1995) for very strong support for the more complex model ($2 \ln B_F = 10$). For MRBAYES 3.1.2, we used default priors for the base frequency and substitutions models. We ran several preliminary analyses by changing the branch length prior, from *unconstrained: exp (10)* to *unconstrained: exp (50),* and the temperature from 0.2 to 0.1. We observed better mixing of the chains using the *unconstrained: exp (50)* prior. In all MRBAYES analyses, four Metropolis-coupled Markov Chains Monte Carlo, one cold and three heated, were run for fifty million iterations with trees sampled every 1000 iterations. Two independent Bayesian runs initiated from random starting trees were performed for each data set, and the log-likelihood values and posterior probabilities were checked to ascertain that the chains had reached the posterior distribution. We ensured that the potential scale reduction factor (PSRF) approached 1.0 for all parameters and that the average standard deviation of split frequencies converged towards zero. For BEAST 1.5.4, we assigned each locus its own substitution model and uncorrelated lognormal clock model and used uniform priors for the rate matrix (0, 5) and for the gamma parameter (0, 15). We ran the analyses for fifty millions iterations with trees sampled every 1000 iterations.

As an alternative to the traditional concatenated approach (see Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007 for caveats regarding concatenation), we estimated the species tree using the coalescent method *BEAST (Heled and Drummond, 2010) implemented in BEAST 1.5.4 (Drummond et al., 2002, 2006; Drummond and Rambaut, 2007). We use the traditional species delineations as 'species' in the analyses. We assumed an uncorrelated lognormal molecular clock model for all loci and used the best fit model for each locus. Each locus had its own specific substitution and clock model. We assumed a Yule speciation process and ran the chains for 150 million iterations.

We used TRACER v1.5 (Rambaut and Drummond, 2007) to ensure that our sampling of the posterior distribution had reached a sufficient effective sample size (ESS > 200) for meaningful parameter estimation. The species tree was summarized as a Maximum Clade Credibility tree using TREEANNOTATOR; the first 25 millions generations were discarded as burn-in.

2.1.2. Divergence time estimates

We used BEAST 1.5.4, assuming an uncorrelated lognormal clock model (Drummond et al., 2006) and the best fit substitution models for each loci, to estimate the divergence time within the Falconidae. We used a Yule speciation process for the tree prior. As a calibration point we used the split between the Falconidae and the Polyborinae, estimated to have occurred at least 16.3 myrs ago. This estimate is based on the fossil *Pediohierax ramenta* (Wetmore, 1936) which has been suggested to be the earliest Falconinae (Late Hemingfordian–Early Barstovian) (Becker, 1987). We used a lognormal distribution (zero set off 16.3, lognormal mean: 0.8, lognormal standard deviation: 0.61); the 95% credibility interval of the prior distribution was 17–23.7 myrs ago. The latter value corresponds to the beginning of the Miocene epoch. We compared the divergence time estimates obtained using the fossil calibration point with estimates obtained using a molecular clock rate as recently estimated in Passeriformes for ND2 (6.1%/Myr, Arbogast et al., 2006) by specifying the *meanRate* parameter to be 0.0305 substitutions/site/Myr/lineage. Mitochondrial protein-coding genes evolve about 50% faster in Passeriformes than Falconidae (Pacheco et al., in press). Thus, if the fossil calibration point is representative, we expect that the mitochondrial rate will give younger estimates than the fossil calibration point. We used uniform priors (0, 5) for the substitutions matrices for the three mitochondrial partitions, uniform priors for each rate heterogeneity parameter (Γ) and default priors for the other parameters. MCMC chains were run for $10 * 10^7$ steps and were sampled every 1000 steps. TRACER v1.5 was used to visualize the posterior distributions for every parameter.

3. Results

Our final DNA alignment consisted of 7428 bp of DNA sequence data obtained for all *Micrastur* species. This includes partial sequences for PER, MB, RAG1 and VIM (minimum 67% of the total alignment length of the locus) for *M. buckleyi*. All substitution models selected for the phylogenetic analyses under the BIC are listed in Table 2.

3.1. mtDNA

The gene order was the same for all species we studied and is similar to the typical avian gene order for that region (Mindell et al., 1998); only differences in the presence/absence or length of intergenic regions were detected (range: 2320 bp for *P. haliaetus* versus 2353 bp for *Daptrius americanus* – 2364 bp if we consider the *Micrastur semitorquatus* LSUMNS B-11298 pseudogene – see below).

For ND1, an insertion of one codon was observed in *Spiziapteryx* and a change in the specific stop codon was recovered in the Polyborinae and Falconinae: TAA was the stop codon found in the Polyborinae, Falconinae as well as in one of the outgroup (*Pandion*) whereas AGA/AGG was the stop codon in the Herpetotherinae

Table 2
List of substitutions models selected under the Bayesian Information Criterion for each data set and number of variable and informative sites for each locus (Falconidae only). Stop codon and non-coding regions were excluded from the mtDNA alignments.

Loci	Alignment (bp)	Variable/ informative	Substitution model
mtDNA	2308	1014/847	TVM + Γ + I
tRNA	292	75/62	TrN + Γ
ND1-ND2 1st pos	672	244/187	TVM + Γ + I
ND1-ND2 2nd pos	672	98/69	TVM + Γ + I
ND1-ND2 3rd pos	672	597/529	TVM + Γ + I
ND1	978	413/358	TVM + Γ + I
ND2	1038	526/427	TIM + Γ + I
ND1 1st pos	326	90/72	TVMef + Γ + I
ND1 2nd pos	326	32/21	HKY + I
ND1 3rd pos	326	291/265	TrN + Γ
ND2 1st pos	346	154/115	GTR + Γ + I
ND2 2nd pos	346	66/48	TVM + Γ + I
ND2 3rd pos	346	306/264	HKY + Γ
MB	730	100/50	TVMef + Γ
VIM	581	110/64	HKY
PER	802	140/88	TVM + Γ
TGFb2	602	113/81	TrNef + Γ
FGB	633	122/89	HKY + Γ
PEPCK	702	123/83	K81uf + Γ
RAG1	1070	122/83	TVM + Γ
RAG1 1st pos	357	28/20	HKY
RAG1 2nd pos	356	33/22	TVM + Γ
RAG1 3rd pos	357	61/41	K81 + Γ

and all outgroups but *Pandion*, Within the Herpetotherinae the last two G nucleotides of the ND1 stop codon overlapped with the neighboring $tRNA_{Ile}$ for Herpetotheres and the four species of the *M. ruficollis* species group where the three species. There was no overlap for the stop codon (AGA) between between ND1 and the $tRNA_{Ile}$ for the three species of the *M. semitorquatus* group; an insertion of 5–6 bp of non-coding DNA was also found. The complete ND1 sequence was 975 bp long for the majority of ingroup taxa, while it was 978 bp for *Spiziapteryx* and the Herpetotherinae. We did not detect any evidence of double peaks in the sequences and no stop codons were present in the ND1 and ND2 coding regions. The only exception was *M. semitorquatus* LSUMNS B-11298 for which we detected evidence of length polymorphism in the ND1 region, suggesting the presence of a pseudogene (numt) or heteroplasmy. Cloning of the whole fragment for LSUMNS B-11298 indicates that a 20 bp insertion occurred in ND1 in one of the two recovered haplotypes. We assumed that the haplotype with the 20 bp insertion was non-functional as several stop codons were found downstream. The haplotype with the insertion was only found in LSUMNS B-11298 and also differs from the inferred functional sequence by two further substitutions. Both the functional and non-functional sequences were placed together in the mtDNA tree with high support in the ML and BI analyses. We conclude that this case (numt or heteroplasmy) is restricted to LSUMNS B-11298 and is likely to be more recent than any diversification within *M. semitorquatus*.

The Bayes Factor strongly supported the seven partitions model over the one partition model ($\ln B_F = 1023.3$), three partitions model ($\ln B_F = 915.4$) and four partitions ($\ln B_F = 7.6$). This result suggests that recognizing the specificity of the substitution dynamics of each locus and codon position is important in accurately modeling the evolution of mtDNA, with the most important parameter being the specific codon position. Two primary *Micrastur* clades were recovered in the mitochondrial tree (Fig. 1). These two clades correspond to the traditional species groupings: *M. semitorquatus* and *M. ruficollis*. We found substantial genetic differentiation among species (minimum net divergence: 5% between *M. gilvicollis* and *M. mintoni*, maximum net divergence: 16.5% between *M. mintoni* and *M. semitorquatus*) when compared to within species divergence (maximum: 0.9% within *M. gilvicollis*). *M. buckleyi* was recovered as the sister group of the *M. mirandollei–M. semitorquatus* clade with strong support (PP = 1.0, ML = 100). In contrast, the relationships among the four taxa within the *ruficollis* species group only received weak support (PP = 0.55–0.70, ML = 31–40).

3.2. Nuclear data

The level of resolution differed among the nuclear gene trees but in most cases, the monophyly of each the two primary species groups (*M. semitorquatus* and *M. ruficollis*) and of each species was recovered with strong support (Supplementary Figs. S1–S7). No conflict was detected among the different nuclear loci concerning inter-specific relationships within the genus *Micrastur*.

The concatenated nuclear data set was in agreement with the mitochondrial data in several respects. The monophyly of each species group was confirmed based on all nuclear loci with high support (PP = 1.0, ML = 100), but relationships among species within each group differed (Fig. 2). For example, *M. buckleyi* was the sister-group of *M. mirandollei* in the nuclear tree (PP = 1.0, ML = 98) whereas it was sister to the *M. mirandollei–M. semitorquatus* clade in the mtDNA tree (PP = 1.0, ML = 98). The relationships among the four species from the *M ruficollis* group also differed between the mitochondrial and nuclear trees. Yet there was no significant conflict among the two trees as the relationships among the four species were never supported.

3.3. Concatenated and species tree

We could not achieve satisfactory mixing and convergence on the concatenated mitochondrial and nuclear data set using MRBAYES 3.1.2, despite changing some prior values (e.g. branch length, rate multiplier) and chain parameters (temperature, number of chains, number of swaps, and proposals). All parameters but the rate multiplier achieved satisfactory mixing and convergence and values were normally distributed whereas the rate multiplier posterior distributions were ragged. Hence we will not consider the results of the concatenated analyses using MRBAYES 3.1.2. In the concatenated approach using BEAST and RAxML, all nodes within the Falconidae received posterior probabilities or bootstrap values greater than 0.95 or 80, respectively.

The topology resulting from the *BEAST coalescent-based analyses of all loci (species tree approach) was well resolved, with 13 of the 15 nodes within the Falconidae having posterior probabilities of 1.0 (Fig. 3).

The topology resulting from the concatenated BEAST analyses was very similar to the *BEAST species tree approach but noticeable differences were found concerning the relationships among taxa within the *M. ruficollis* species group. The two nodes that were poorly supported in the species tree approach involve relationships among the four species within the *M. ruficollis* species group. These nodes were fully resolved and highly supported in the concatenated approach, with *M. mintoni* diverging first in this group (PP = 0.99, ML = 80) and a sister group relationship between *M. gilvicollis* and *M. plumbeus* (PP = 1.0, ML = 87). Given the short branch lengths involved in this part of the tree, it is possible that the concatenated approach will result in a highly supported but incorrect topology (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007). Hence, we consider the relationships among *M. mintoni*, *M. ruficollis*, *M. plumbeus* and *M. gilvicollis* as unresolved. Although we could not resolve the relationships among the four species in this clade, we can conclude that the multiple speciation events probably occurred rapidly, in less than 0.5 myr. In the second primary clade within *Micrastur*, *M. buckleyi* was sister to the *M. mirandollei–M. semitorquatus* clade (PP = 1.0).

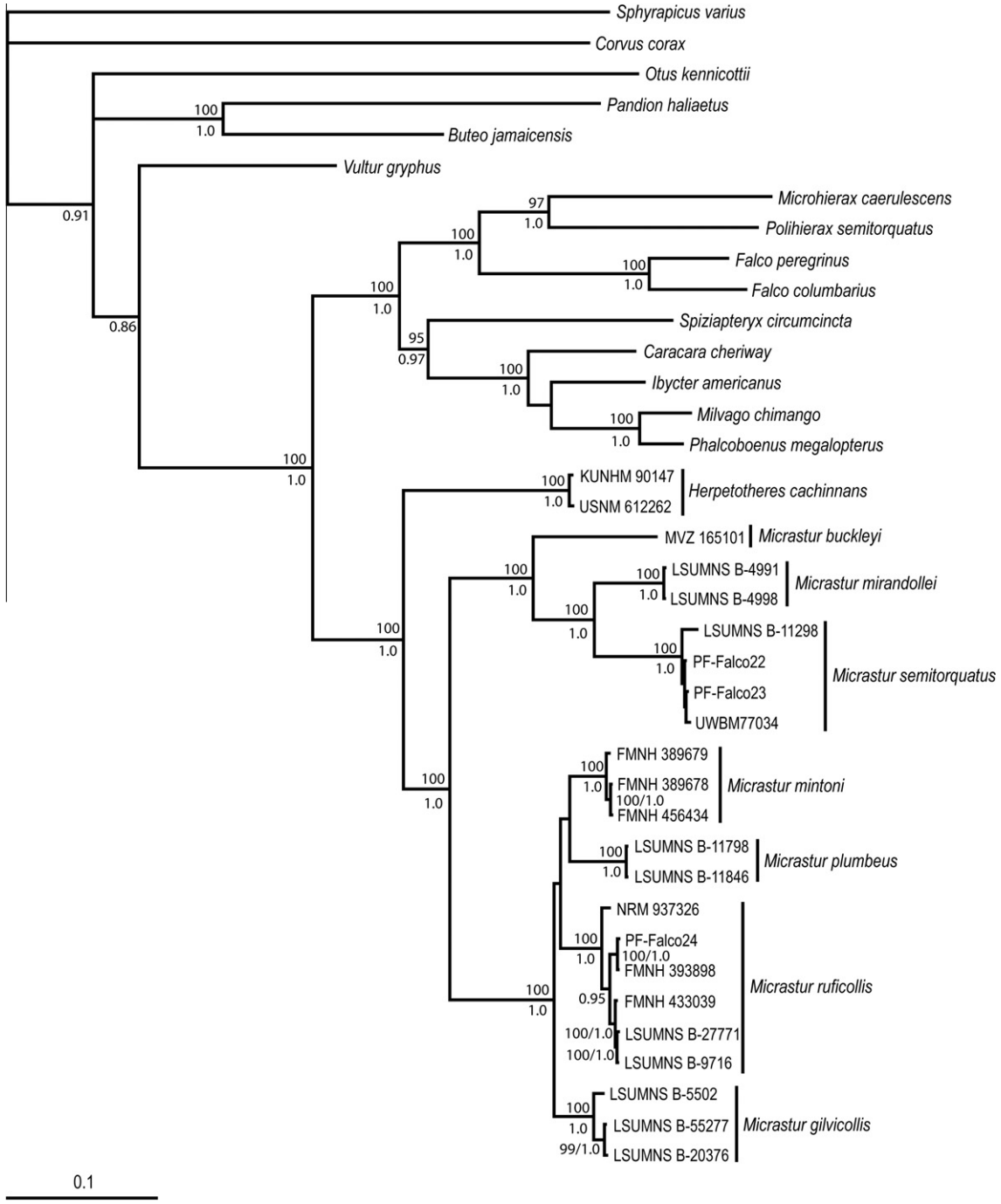

Fig. 1. Fifty percentage majority consensus rule tree resulting from the Bayesian analyses of the mitochondrial data set (seven partitions). Numbers close to the nodes refer to posterior probabilities and maximum likelihood bootstrap support values higher than 0.85% and 60%, respectively.

3.4. Divergence times estimates

All speciation events within the genus *Micrastur* are inferred as occurring within the last 2.5–3.6 myrs, at or before the Pliocene/Pleistocene boundary, with the basal split within the genus being 7 myrs old (Fossil calibration point; 95% Highest Posterior Density – HPD: 5.5–8.8) (Table 3, Fig. 1). Within species divergences were not older than 0.7 myrs (*M. ruficollis*, 95% HPD: 0.5–1.0). The split between *Herpetotheres* and *Micrastur* was estimated to have occurred around 11 mya (95% HPD: 8.8–13.4). The first split within the Falconinae and Polyborinae occurred ca 12.1 mya (95% HPD: 10.4–14.0) and 14.2 mya (95% HPD: 11.8–16.5), respectively. Divergence times obtained using the 6.1%/Myr rate are younger than the estimates obtained using the fossil calibration point by a factor of two and the 95% HPD of the two calibrations do not overlap (Table 3). Hence, the 6.1%/Myr rate does not conform to the Falconidae fossil record. Using the fossil data, the traditional 2%/Myr mitochondrial rate is not included within the 95% HPD for both ND1 (mean rate: 0.0182 s/s/myr/lineage, so 3.6%, 95% HPD: 0.014–0.027) and ND2 (mean rate: 0.0167 s/s/myr/lineage, so 3.4%, 95% HPD: 0.013–0.020).

4. Discussion

Our study provides a well-supported phylogeny of the genus *Micrastur* and confirms the distinction at the species level of two

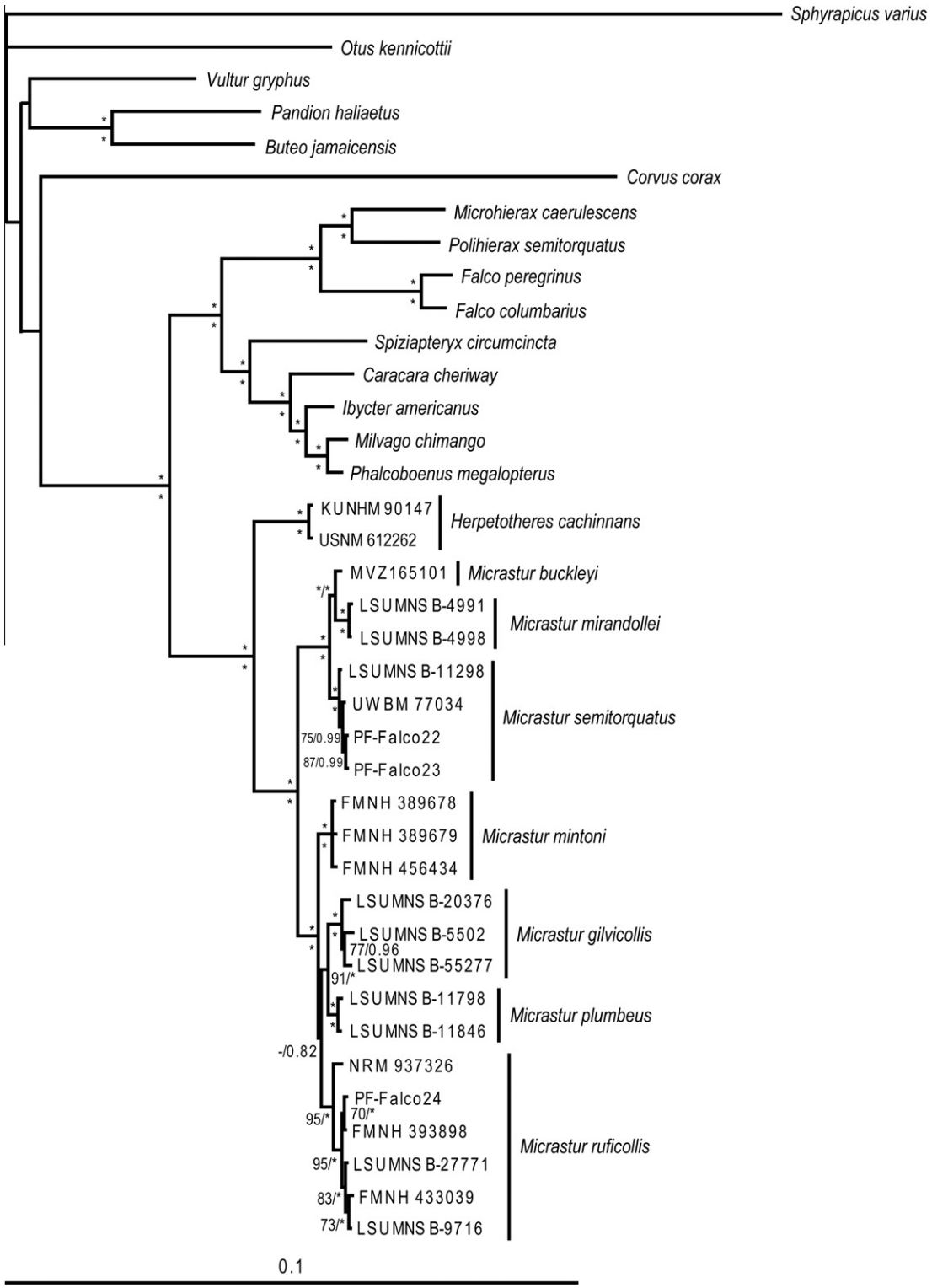

Fig. 2. Fifty percentage majority consensus rule tree resulting from the Bayesian analyses of the nuclear data set. Asterisks indicate posterior probabilities and maximum likelihood bootstrap support values of 1.0 and 100, respectively. Numbers close to the nodes refer to posterior probabilities and maximum likelihood bootstrap support values higher than 0.80% and 60%, respectively.

taxa (*M. buckleyi* and *M. mintoni*) that were recently described or debated. We recovered two primary clades, in accordance with overall size and plumage pattern: the first clade includes three species of large size (*M. buckleyi*, *M. mirandollei*, *M. semitorquatus*) with uniform underparts whereas the second clade consists of four species (*M. gilvicollis*, *M. mintoni*, *M. plumbeus* and *M. ruficollis*) of medium size with barred underparts. The levels of phylogenetic resolution differed between the two clades; relationships are all resolved in the *M. semitorquatus* clade, with *M. buckleyi* being the sister-group of the *M. semitorquatus* and *M. mirandollei*, whereas a polytomy was recovered in the *M. ruficollis* clade. Given the amount of data we collected, we consider the lack of resolution

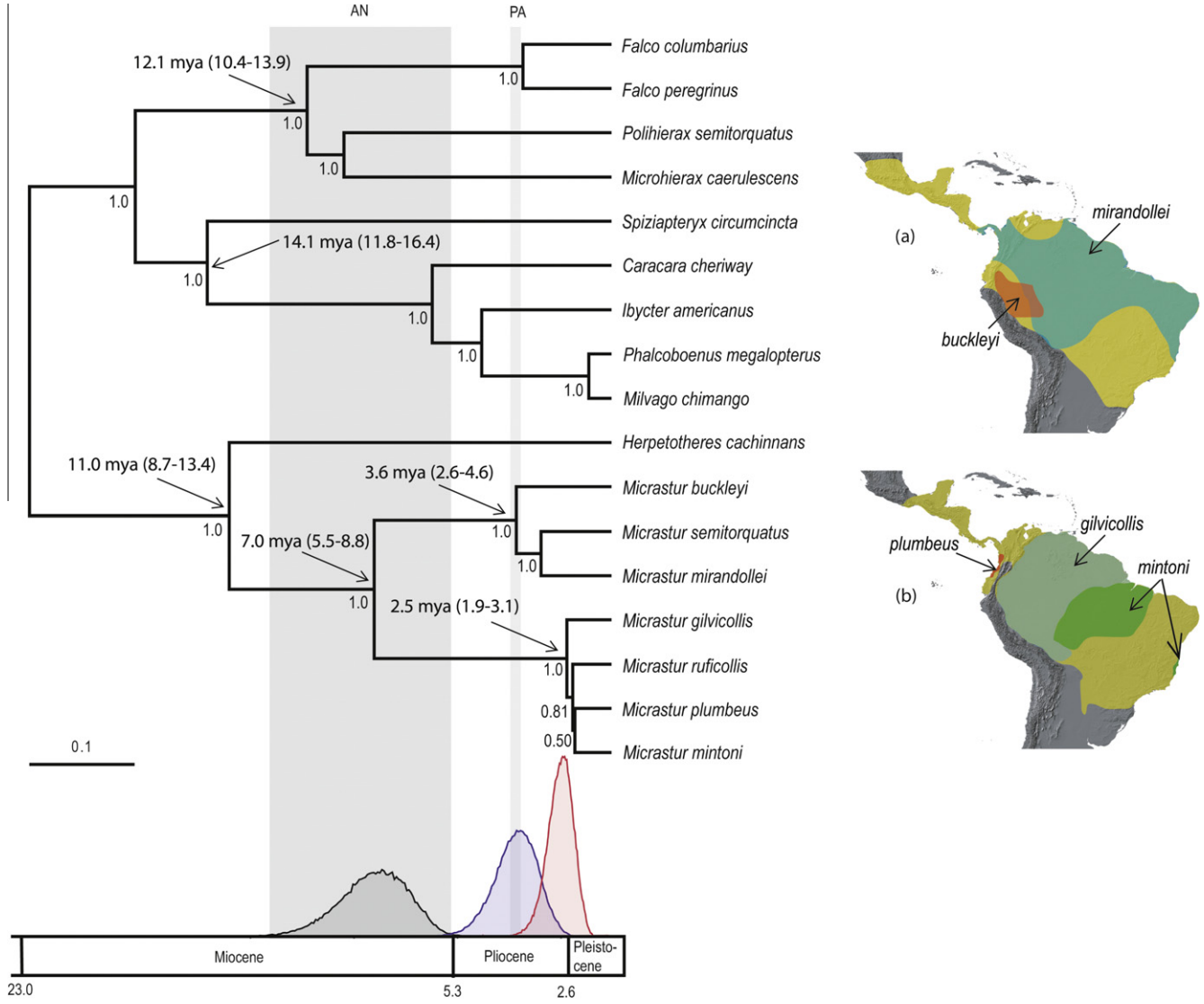

Fig. 3. Species tree obtained using the coalescent approach implemented in *BEAST. Values close to node represent posterior probabilities. We assumed an uncorrelated lognormal clock model for all loci and used the best fit substitution model for each partition. Each locus was specified its own specific substitution and clock models. The divergences times were obtained using BEAST V. 1.5.4 using an uncorrelated lognormal molecular clock model for each locus and a fossil calibration point (Falconidae/Polyborinae split; 16.3 myrs ago, see the *Divergence times estimates* section for further explanations). The prior for the fossil calibration point was a lognormal distribution (zero set off 16.3, lognormal mean: 0.8, lognormal standard deviation: 0.61). The posterior distribution of divergence times for the genus *Micrastur*, *M. semitorquatus* species group and *M. ruficollis* species group are indicated. The AN and PA acronyms represent the timing of the last rising of the Andes and closure of the Panama seaway, respectively. (a) Distribution of the species from the *M. semitorquatus* clade. The distribution of *M. semitorquatus* encompasses the distribution of the two other species plus the extra colored area. (b) Distribution of the species from the *M. ruficollis* clade. The distribution of *M. ruficollis* encompasses the distribution of the two other species plus the extra colored area. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the *M. ruficollis* group to be the result of rapid diversification events leading to four lineages rather than insufficient information in the data. One species from each clade (*M. semitorquatus* and *M. ruficollis*) has a distribution range that encompasses the distribution of other species from their clades. Both of these widely distributed species have broader habitat tolerances than their closest relatives and can be found in secondary growth (Bierregaard, 1994b,c). Both *M. semitorquatus* and *M. ruficollis* reach Central America, a major area of endemism for birds in which no *Micrastur* species is endemic. A possible explanation is that both species experienced range expansion from Central America and colonized South America. The diversification pattern we found within *M. ruficollis* indicates a basal divergence for an individual from Paraguay, which is somewhat differentiated from other *M. ruficollis* individuals (mitochondrial uncorrected p-distances: 1.4%). Hence, *M. ruficollis* might have expanded northwards. The time to most recent common ancestor (TMRCA) for the mitochondrial haplotypes are 0.3 mya (95% HPD: 0.2–0.5 mya) and 0.7 (95% HPD: 0.5–1.0) for *M. semitorquatus* and *M. ruficollis*, respectively. The TMRCAs only slightly overlap but the extent of geographic sampling in our study differs between species, as the range of *M. ruficollis* was better sampled. Further phylogeographic studies are needed to test the hypothesis of a northward range expansion and concordant timing.

We recovered different timing for the divergences within the two primary clades, the *M. semitorquatus* clade started to diversify about 3.6 mya whereas the *M. ruficollis* clade diversified about 2.5 mya although the 95% HPD overlap. Assuming that the fossil record for Falconidae is representative, we can formerly reject one of the five primary hypothesis that aimed to explain the biodiversity in the Neotropics: (1) the classical Pleistocene refuge hypothesis as all speciation events occurred prior to the Pleistocene, and (2) the Miocene marine incursions hypothesis (16–21 mya and 11 mya,

Table 3
Divergence time estimates for the primary lineages within the Falconidae using BEAST 1.5.4. Numbers between brackets represent the 95%HPD. Estimates were obtained using a fossil calibration point (*Pediohierax ramenta*), a molecular clock rate for ND2 (6.1%, Arbogast et al., 2006). All loci were allowed to have their own independent substitution matrix and molecular uncorrelated lognormal clock model. The asterisk highlights the location of the calibration point.

Clade	Fossil calibration	Molecular clock
Falconidae	22.5 (19.9–25.4)	11.3 (9.3–13.3)
Falconinae	12.1 (10.4–13.9)	6.1 (4.9–7.3)
Polyborinae	14.1 (11.8–16.4)	7.1 (5.7–8.6)
Falconinae/Polyborinae*	18.0 (16.3–19.8)	9.0 (7.4–10.6)
Herpetotherinae	11.0 (8.7–13.4)	5.6 (4.4–6.9)
Micrastur	7.0 (5.5–8.8)	3.6 (2.8–4.5)
Micrastur clade 1	3.6 (2.6–4.6)	1.8 (7.4–10.6)
M. semitorquatus	0.4 (0.2–0.5)	0.2 (0.1–0.3)
Micrastur clade 2	2.5 (1.9–3.1)	1.3 (1.0–1.6)
M. gilvicollis	0.5 (0.3–0.8)	0.3 (0.1–0.3)
M. ruficollis	0.7 (0.5–1.0)	0.4 (0.2–0.5)

Lovejoy et al., 2006), (3) the Andean uplift hypothesis (last uplift of the Andes: 6 mya, Garzione et al., 2008), and (4) the riverine barrier hypothesis as most drainage systems were present at the time the two primary clades diversified (7–8 mya, Potter, 1997; Hoorn et al., 2010). The lack of Pleistocene speciation events could be due to the higher frequency of climatic cycles that were driving habitat expansion and contraction, in which time is insufficient to allow strong genetic incompatibilities to build (Jansson and Dynesius, 2002). Our study adds thus further support to the idea that very few speciation events in the lowlands occurred during the Pleistocene in the Neotropics, and that most of the speciation events occurred at or before the Pliocene/Pleistocene boundary (Eberhard and Bermingham, 2005; Ribas et al., 2005, 2007, 2009; Brumfield and Edwards, 2007; Santos et al., 2009; Steiner et al., 2005). This situation contrasts with inferences from temperate region studies (e.g. Hewitt, 2004; Weir and Schluter, 2004). Finally, the last primary hypothesis invoked to explain speciation patterns in Amazonia, namely ecological gradient in general (Endler, 1982) and elevational gradients in particular, have not been supported in recent studies (e.g. Patton and Smith, 1992; Dingle et al., 2006; Parra et al., 2009). Hence it is likely that no single common factor can explain the exceptional diversity encountered in the Neotropics. Rather, a combination of all these processes, along with stochastic factors (e.g. extinction), may have driven the diversification of neotropical taxa (Santos et al., 2009).

Several hypotheses have been proposed for the limits and relationships of taxa distributed in major neotropical areas of endemism (e.g. Cracraft, 1985). With the exception of *M. plumbeus*, endemic to the Choco, and *M. buckleyi*, endemic to the Napo, the distribution of *Micrastur* species does not fit any single recognized area of endemism for birds. Yet, a closer look indicates that the distribution of *M. mintoni* encompasses the bioregions Para, Belem and Rondonia and the distributions of *M. gilvicollis* roughly corresponds to the Guyana, Napo, Inambari and Imeri regions. This implied relationship among areas is in agreement with the pattern recovered in a broad-scale analysis for passerines (Bates et al., 1998). The single exception involves the Atlantic Coast of Brazil, as no endemic lineage of *Micrastur* occurs there (although a presumably extinct population of *M. mintoni* was described from this area, Whittaker, 2002). The Atlantic Forest region has been referred to as a biogeographic hybrid by Cracraft and Prum (1988); some taxa are well differentiated from those of other regions whereas other taxa are closely related to those from the Para and Belem areas. This hypothesis has been corroborated by molecular studies on parakeets, where some taxa represent Pliocene age lineages (3 mya, *Brotogeris*, Ribas et al., 2009), whereas others diverged during the Pleistocene (0.3 mya, Ribas et al., 2007).

Although our estimates do not fit any of the traditional hypotheses for speciation patterns, our divergence time estimates are in strong agreement with those recently recovered for other bird taxa (e.g. Eberhard and Bermingham, 2005; Ribas et al., 2005, 2009). For example, the split involving the divergence of *M. buckleyi* (restricted to Western Amazonia and Eastern Andes), estimated to have occurred about 3.6 mya (Fig. 1), corresponds to estimates obtained for other lineages of birds, including *Phaeothlypis* woodwarblers (Lovette, 2004), *Gypopsitta* parakeets (Ribas et al., 2005) and the *Xyphorhynchus paradalotus*/*ocellatus* complex (Aleixo and Rossetti, 2007). The *M. ruficollis* clade diversified at the Plio-Pleistocene boundary, when several other lineages of birds diversified, including woodcreepers (Marks et al., 2002; Aleixo and Rossetti, 2007). The Pliocene was rich in climatic and tectonic factors that may have enhanced speciation events, including a globally cooler and drier climate due to the formation of the Northern Hemisphere ice-sheets (Zachos et al., 2001) and the final closure of the Panama seaway (Coates, 1992), which may have been an crucial in the isolation of *M. plumbeus*, endemic to the Choco region. Further reconstructions of Pliocene environments in the Amazonian basin are necessary to assess the existence of Pliocene refugia.

Our study confirms the species status of the seven taxa currently recognized as species, *M. gilvicollis* and *M. ruficollis* are not only clearly diagnosable by morphological and vocalization data (Schwartz (1972) but also by molecular data. All seven species have been recovered as monophyletic by the mitochondrial data, by some of the nuclear gene trees (e.g. RAG1) and in the concatenated nuclear data set. Yet, two species (*M. gilvicollis* and *M. ruficollis*) appear paraphyletic in some of the nuclear gene trees, a pattern that can result from lineage sorting processes resulting from the greater effective population size and slower mutation rate of the nuclear genome (Hudson and Coyne, 2002). It is also worth noting that *M. gilvicollis* and *M. ruficollis* have the highest within-species mitochondrial divergence (ca 1.3%). Our sampling within these two species includes the extremes of their distribution. Hence, whereas there are some indications of genetic differentiation at the nuclear and mitochondrial level, further studies with denser sampling within these two species are needed to disentangle discrete genetic structure from a pattern of isolation by distance. Finally, it is worth emphasizing that *M. plumbeus* (IUCN status Vulnerable) is genetically distinct from all other species and that conservation efforts should be directed towards the habitat Chocó where this species in confined.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.05.008.

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