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Molecular systematics and evolution of the *Synallaxis ruficapilla* complex (Aves: Furnariidae) in the Atlantic Forest

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ABSTRACT

The Neotropical *Synallaxis ruficapilla* complex is endemic to the Atlantic Forest and is comprised of three species: *S. ruficapilla*, *S. whitneyi*, and *S. infuscata*. This group is closely related to the *Synallaxis moesta* complex that occurs in the Andes, Tepuis, and Guianan shield. Here we used mitochondrial and nuclear gene sequences to infer the phylogeny and the time of diversification of the *S. ruficapilla* and *S. moesta* complexes. We also included samples of an undescribed population of *Synallaxis* that resembles other populations of the *S. ruficapilla* complex. Our results showed that different geographical lineages within the *S. ruficapilla* complex are reciprocally monophyletic, but the northern form (*S. infuscata*) grouped with an Andean taxon. This suggests that at least two lineages of this group independently colonized the Atlantic Forest. Specimens of the undescribed population formed a monophyletic clade with deep divergence. Estimated diversification dates were within the late Pliocene to Pleistocene (2.75–0.16 million of years ago). This suggests that at this time there was a higher connectivity between habitats in the rugged landscapes of the circum-Amazonian bioregions. The observed Pleistocene diversification within the Atlantic Forest is congruent in space and time with studies of other co-distributed organisms, and may be associated with climate changes and tectonic activity during this period.

1. Introduction

The genus *Synallaxis* Vieillot (Spinetails) is endemic to the Neotropical region and occurs from southern Mexico to Argentina. This is the most species-rich genus in the family Furnariidae, with 33 recognized species (Remsen, 2003). Recently, an additional species of *Synallaxis* was described (Hilty and Ascanio, 2009). Overall, the systematics of the genus is rather obscure, as many species are similar in morphology (Ridgely and Tudor, 1994), and some species include two to ten subspecies that could represent full species themselves (Remsen, 2003). Most *Synallaxis* species inhabit dense understory vegetation in riparian thickets, edge habitat and secondary forest, with some exceptions occurring inside continuous forest (Ridgely and Tudor, 1994; Sick, 1997). Remsen (2003) suggests a close relationship between *Synallaxis* and the genera *Certhiaxis*, *Schoeniophylax*, *Gyalophylax*, and *Siptornopsis*. Recent phylogenetic hypotheses (Irestedt et al., 2009; Derryberry et al., 2011) confirm these relationships.

The *S. ruficapilla* complex (Fig. 1) comprises *S. ruficapilla*, *S. whitneyi*, and *S. infuscata* (Pacheco and Gonzaga, 1995) and is endemic to the highly endangered and megadiverse Atlantic Forest – AF (Myers et al., 2000; Ribeiro et al., 2009). Pacheco and Gonzaga (1995) suggested that this species complex is monophyletic based on similarities in plumage and song and notably their nearly identical scolding call. In a recent near-complete species-level phylogeny of the Furnariidae (Derryberry et al., 2011) the *S. ruficapilla* and *S. moesta* complexes are placed together as a monophyletic clade, but with low node supports for the relationships within the clade. However, this phylogeny only included one species from the *S. ruficapilla* complex (*S. ruficapilla*) while the *S. moesta* complex was represented by all three species (*S. moesta*, *S. macconnelli*, and *S. cabanisi*), which occur in the Andes, Tepuis, and Guianan shield (Fig. 1; Ridgely and Tudor, 1994; Remsen, 2003).

Recently Stopiglia et al. (2012) suggested that *S. whitneyi* is a junior synonym of *S. ruficapilla* based on plumage, morphometric, and song characters. Thus, according to these authors, there are only two species in the *S. ruficapilla* complex: *S. ruficapilla* and *S. infuscata*. They also argued that only the number of rectrices is

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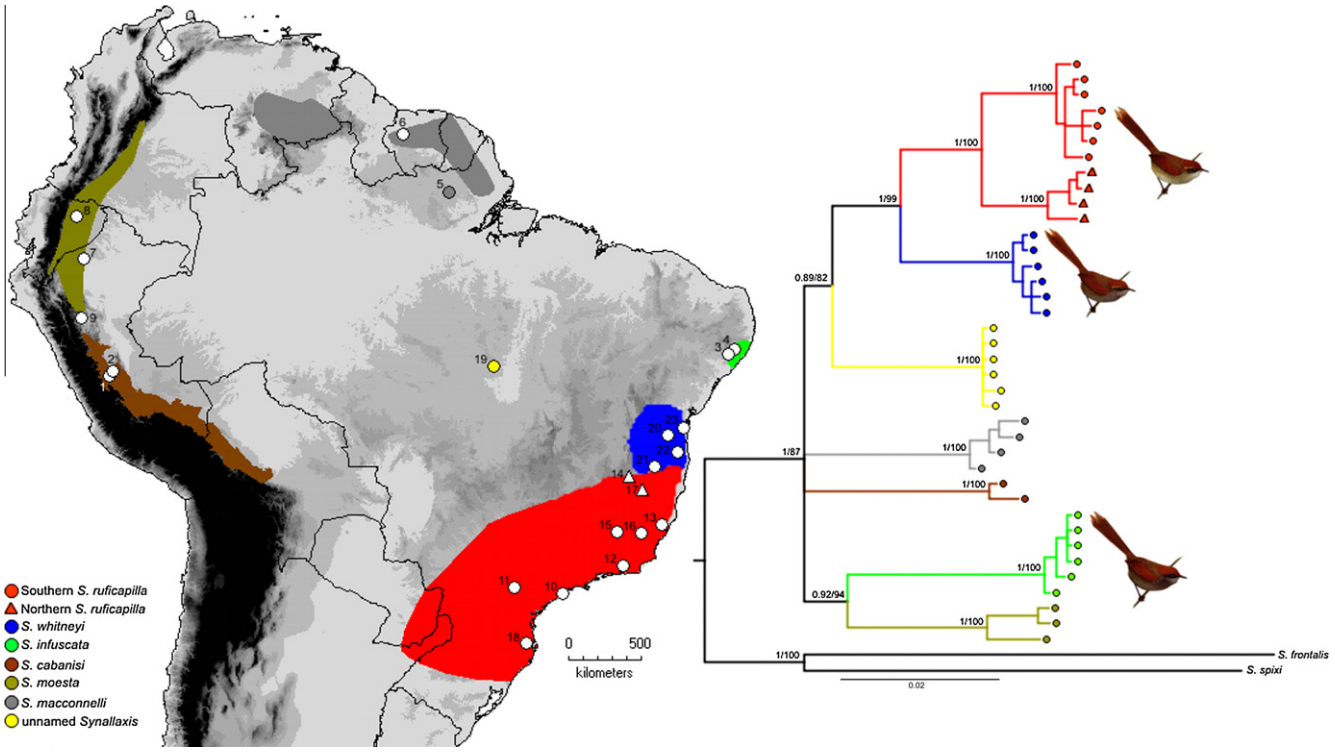

Fig. 1. Map showing localities sampled for species of the *S. ruficapilla* and *S. moesta* complexes, and mitochondrial tree obtained by Bayesian inference. Circles and triangles on the map indicate collecting sites and their colors follow the legend. Locality numbers in the map follows Table 1. Species distributions follow Ribon et al. (2002), Ridgely and Tudor (1994), and Vasconcelos and Silva (2004). The gradient of gray colour in the map represents the elevation gradient (the darker the higher is the altitude). The topology was obtained by Bayesian inference based on 2043 bp of cytb and ND2. Node supports are posterior probabilities and bootstrap values for Bayesian inference and maximum likelihood, respectively. The colors and symbols in the tree refer to the map and legend. Bird illustrations show the three species of the *S. ruficapilla* complex. *Source*: Pacheco and Gonzaga, 1995.

diagnostic for both species and that other phenotypic traits show great individual variation and no constant population differences. They also analyzed six specimens of *Synallaxis* sp. (vouchers in MZUSP; see Table 1) recently collected in Amazon (Vila Rica, Mato Grosso state in Brazil) and geographically disconnected from the AF. Accordingly to Stopiglia et al. (2012) these specimens overlap with *S. ruficapilla* in both morphology and song, suggesting that these birds, which do not occur in the AF, are *S. ruficapilla*. However, another possibility apparently not addressed by Stopiglia et al. (2012), is that this disjunct Amazonian population of the *S. ruficapilla* complex represents an independent lineage deserving a separate taxonomic status. Thus, the species limits within the *S. ruficapilla* complex are still uncertain.

Notwithstanding, defining limits between species it is not a trivial task, mainly due to ambiguity in the species concept in biology (de Queiroz, 2007). Because of retention of ancestral polymorphism and incomplete lineage sorting it is particularly difficult to establish the taxonomic status of recently diverged populations (Carstens and Dewey, 2010). However, the use of molecular data as an additional means to delimitate species has become a robust tool (Carstens and Dewey, 2010; Kubatko et al., 2011), especially when a species tree is inferred based on the coalescence of multiple independent loci (Liu and Pearl, 2007; Liu, 2008; Heled and Drummond, 2010).

Here, we infer the phylogenetic relationships within the *S. ruficapilla* complex, as well as its relationships with the *S. moesta* complex, using mitochondrial and nuclear sequences. Furthermore, we investigate the taxonomic status of the disjunct population of the *S. ruficapilla* complex from Vila Rica. In addition, we evaluated the species status of *S. whitneyi*, which was questioned by Stopiglia et al. (2012), and that might represent an independent evolutionary lineage. Our results will help to trace the evolutionary history of South American forests. Yet, in the context of conservation, we add data to discuss the taxonomic status of the threatened taxa *S. whitneyi* and *S. infuscata* (Roda, 2008; Silveira, 2008).

2. Materials and methods

2.1. Taxon sampling and molecular methods

We sampled 38 individuals from all taxa of the *S. ruficapilla* and *S. moesta* complexes (Ridgely and Tudor, 1994; Ribon et al., 2002; Vasconcelos and Silva, 2004; Stopiglia et al., 2012; Table 1, Fig. 1), including the disjunct population of the *S. ruficapilla* complex from Vila Rica (state of Mato Grosso, MT) in Brazil (Fig. 1). This sampling covered a wide geographical range of AF species. One specimen each from *S. frontalis* and *S. spixi* were used as outgroups, following Derryberry et al. (2011). The samples were obtained from fresh tissue (muscle or blood) and museum study skins (Table 1).

DNA was extracted from fresh samples following Bruford et al. (1992), while the laboratory procedures for museum study skin samples followed Irestedt et al. (2006). We sequenced two mitochondrial genes – cytochrome b (cytb) and NADH dehydrogenase subunit 2 (ND2); and tree nuclear introns – β-fibrinogen intron 5 (FIB5), myoglobin intron 2 (myo2), and glyceraldehyde-3-phosphodehydrogenase intron 11 (G3PDH). For fresh samples all loci were amplified as one product each, whereas additional internal primers were designed for the study skin samples to allow the amplification of shorter fragments (~200–250 bp each). All primers used in this study are described in Table 2.

PCR conditions were an initial denaturation step at 94 °C for 3 min and 30 s; followed by 35 or 40 cycles at 94 °C for 35 s,

Table 1
Samples of *Synallaxis* spp. used in this study: species, sampling locality, collection, tissue code, voucher number, and GenBank accession numbers for each marker.

Species	Locality	Collection*	Tissue code	Voucher	cytb	ND2	FIB5	myo2	G3PDH
Synallaxis cabanisi	1. Depto. Pasco, Cacazú, Peru	MZUSP[a]	–	MZUSP73261	KC437438	KC437514	KC437538	KC437470	KC437587
Synallaxis cabanisi	2. Depto. Pasco, 41 km on Villa Rica – Puerto Bermudez highway, Peru	LSU[b]	B2013	B2013	KC437445	HM449848[f]	KC437550	KC437480	–
Synallaxis infuscata	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA[b]	LGEMA13568	MZUSP88696	KC437425	KC437501	KC437537	KC437464	KC437568
Synallaxis infuscata	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA[b]	LGEMA13573	MZUSP88694	KC437426	KC437502	KC437546	KC437465	KC437569
Synallaxis infuscata	3. Usina Serra Grande, São José da Lage, Alagoas, Brazil	LGEMA[b]	LGEMA13574	MZUSP88695	KC437427	KC437503	KC437547	KC437466	KC437570
Synallaxis infuscata	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA[b]	LGEMA13575	MZUSP88699	KC437428	KC437504	KC437548	KC437467	KC437571
Synallaxis infuscata	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA[b]	LGEMA13576	MZUSP88698	KC437429	KC437505	–	KC437468	KC437572
Synallaxis infuscata	4. Rebio Pedra Talhada, Quebrangulo, Alagoas, Brazil	LGEMA[b]	LGEMA13577	MZUSP88697	KC437408	KC437485	KC437521	KC437448	KC437573
Synallaxis macconnelli	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG[b]	CN855	66294	KC437439	KC437515	KC437555	KC437477	–
Synallaxis macconnelli	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG[b]	CN928	66295	KC437440	KC437516	KC437556	KC437476	KC437580
Synallaxis macconnelli	5. Rebio Maicuru, Almeirim, Pará, Brazil	MPEG[b]	CN993	66293	KC437441	KC437517	KC437552	KC437478	KC437581
Synallaxis macconnelli	6. Sipaliwini, Balchuis Gebergte, ~78 km S Apura on Nickene River, Suriname	LSU[b]	B55261	B55261	KC437444	JF975204[f]	KC437551	KC437481	–
Synallaxis moesta	7. Rio Napo, Ecuador	NRM[a]	–	NRM552855	KC437431	KC437507	–	–	–
Synallaxis moesta	8. San José, Ecuador	NRM[a]	–	NRM552854	KC437432	KC437508	–	–	–
Synallaxis moesta	9. Depto. San Martín; ~33 km NE Florida, Peru	LSU[b]	B44663	B44663	KC437446	JF975205[f]	KC437549	KC437479	–
Synallaxis ruficapilla	10. Juquitiba, São Paulo, Brazil	LGEMA[b]	LGEMA1182	ITA211[d]	KC437414	KC437491	KC437527	KC437454	KC437558
Synallaxis ruficapilla	11. Wencenslau Braz, Paraná, Brazil	LGEMA[b]	LGEMA1367	ITA288[d]	KC437415	KC437492	KC437528	KC437455	KC437559
Synallaxis ruficapilla	12. Parque Nacional Serra dos Órgãos, Teresópolis, Rio de Janeiro, Brazil	LGEMA[b]	LGEMA13015	MZUSP82584	KC437416	KC437493	KC437529	KC437456	KC437560
Synallaxis ruficapilla	13. Estação Biológica Santa Lucia, Santa Teresa, Espírito Santo, Brazil	MNRJ[b]	229	MNA4873	KC437418	KC437495	KC437531	KC437458	KC437562
Synallaxis ruficapilla	14. José Gonçalves de Minas, Minas Gerais, Brazil	UFMG[c]	B2414	–	KC437419	–	KC437539	–	–
Synallaxis ruficapilla	15. RPPN Serra do Caraça, Minas Gerais, Brazil	MNRJ[b]	LGEMA15756	CPA365[e]	KC437420	KC437496	KC437532	KC437459	KC437563
Synallaxis ruficapilla	16. Parque Nacional do Caparaó, Alto Caparaó, Minas Gerais, Brazil	LGEMA[b]	LGEMA13530	MZUSP87024	KC437421	KC437497	KC437533	KC437460	KC437564
Synallaxis ruficapilla	17. Poté, Minas Gerais, Brazil	LGEMA[b]	LGEMA13536	MZUSP87020	KC437422	KC437498	KC437534	KC437461	KC437565
Synallaxis ruficapilla	17. Poté, Minas Gerais, Brazil	LGEMA[b]	LGEMA13545	MZUSP87019	KC437423	KC437499	KC437535	KC437462	KC437566
Synallaxis ruficapilla	17. Poté, Minas Gerais, Brazil	LGEMA[b]	LGEMA13549	MZUSP87017	KC437424	KC437500	KC437536	KC437463	KC437567
Synallaxis ruficapilla	18. Nova Trento, Santa Catarina, Brazil	LGEMA[b]	LGEMA14592	MZUSP91242	KC437430	KC437506	KC437540	KC437471	KC437579
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	LGEMA[b]	LGEMA416	MZUSP78834	KC437407	KC437484	KC437520	KC437447	KC437557
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP[a]	–	MZUSP78833	KC437433	KC437509	KC437541	KC437472	KC437582
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP[a]	–	MZUSP78830	KC437434	KC437510	KC437542	KC437473	KC437583
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP[a]	–	MZUSP78835	KC437435	KC437511	KC437543	KC437474	KC437584
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP[a]	–	MZUSP78832	KC437436	KC437512	KC437544	KC437475	KC437585
Unnamed *Synallaxis*	19. Fazenda Ipê, Vila Rica, Mato Grosso, Brazil	MZUSP[a]	–	MZUSP78831	KC437437	KC437513	KC437545	KC437469	KC437586
Synallaxis whitneyi	20. Serra da Ouricana, Boa Nova, Bahia, Brazil	LGEMA[b]	LGEMA13401	DZUFMG6009	KC437417	KC437494	KC437530	KC437457	KC437561
Synallaxis whitneyi	21. Rebio Mata Escura, Jequitinhonha/ Almenara, Minas Gerais, Brazil	LGEMA[b]	LGEMA13711	MZUSP86006	KC437409	KC437486	KC437522	KC437449	KC437574
Synallaxis whitneyi	22. RPPN Serra Bonita, Camacan, Bahia, Brazil	LGEMA[b]	LGEMA13720	MZUSP86015	KC437410	KC437487	KC437523	KC437450	KC437575
Synallaxis whitneyi	22. RPPN Serra Bonita, Camacan, Bahia, Brazil	LGEMA[b]	LGEMA13736	MZUSP86031	KC437411	KC437488	KC437524	KC437451	KC437576
Synallaxis whitneyi	23. Plantações da Michelin, Ituberá/ Camamu, Bahia, Brazil	LGEMA[b]	LGEMA13763	MZUSP86057	KC437412	KC437489	KC437525	KC437452	KC437577
Synallaxis whitneyi	23. Plantações da Michelin, Ituberá/ Camamu, Bahia, Brazil	LGEMA[b]	LGEMA13764	MZUSP86058	KC437413	KC437490	KC437526	KC437453	KC437578
Synallaxis frontalis	Serra de São Vicente, Mato Grosso, Brazil	MNRJ[b]	32	MNA2246	KC437443	KC437519	KC437553	KC437482	KC437588

Table 1 (*continued*)

Species	Locality	Collection*	Tissue code	Voucher	cytb	ND2	FIB5	myo2	G3PDH
Synallaxis spixi	Estação Biológica Santa Lucia, Santa Teresa, Espírito Santo, Brazil	MNRJ[b]	155	MNA4799	KC437442	KC437518	KC437554	KC437483	KC437589

Locality numbers follow map in Fig. 1.

* LGEMA – Laboratório de Genética e Evolução Molecular de Aves, Instituto de Biociências, Universidade de São Paulo, Brazil; MZUSP – Museu de Zoologia, Universidade de São Paulo, Brazil; UFMG – Laboratório de Biodiversidade e Evolução Molecular, Instituto de Ciências Biomédicas, Universidade Federal de Minas Gerais, Brazil; DZUFMG – Departamento de Zoologia, Universidade Federal de Minas Gerais, Brazil; MNRJ – Museu Nacional, Universidade Federal do Rio de Janeiro, Brazil; MPEG – Museu Paraense Emílio Goeldi, Brazil; NRM – Swedish Museum of Natural History, Sweden; and LSU – Museum of Natural Science, Louisiana State University, USA.

[a] Study skin samples.

[b] Chest tissue sample with specimen's voucher.

[c] Blood sample without specimen's voucher.

[d] Field numbers, MZUSP pending numbers.

[e] Field number, MNRJ pending number.

[f] Sequences from Derryberry et al. (2011).

Table 2
Primers used in this study. Primers used with fresh samples are in italics.

Primer name	Gene	Sequence 5′ → 3′	Reference
Cytb-SynR1	cytb	GTATTTCATGTCTCTTTGAATAGGTA	This study
Cytb-SynR1b	cytb	GTCTCTTTGAATAGGTAAGAGCC	This study
Cytb-SynF2	cytb	ACATCGGACGAGGCTTCTACTA	This study
Cytb-SynR2	cytb	TGTCAACTGAAAATCCTCCTCATG	This study
Cytb-SynF3	cytb	CCTATATCGGACAAACACTCGT	This study
Cytb-SynR3	cytb	GAATGTCTTTGGTTGAGAAGTAAG	This study
Cytb-SynF4	cytb	TCTCATCAAACTGCGATAAAATCC	This study
Cytb-SynR4	cytb	TTTATTTGGAATTGATCGTAGAATGG	This study
Cytb-SynF5	cytb	CCTGAATGATACTTCCTATTTGCAT	This study
Cytb-SynR5	cytb	TCTACTGGTTGGCTGCCTACT	This study
Cytb-SynF6	cytb	ATATTCTGAATCCTAGTTACAAACCTT	This study
L14841	cytb	CCATCCAACATCTCAGCATGATGAAA	Lougheed et al. (2000)
H16065	cytb	GTCTTCAGTTTTTGGTTTACAAGAC	Lougheed et al. (2000)
ND2-synR1	ND2	TAGAAAGTATTTGGTTGTGGCTTCA	This study
ND2-synF2	ND2	ATCCTTCCCCTAATCTCAAAACC	This study
ND2-synR2	ND2	AATCAGAAGTGGAATGGGACTAG	This study
ND2-synF3	ND2	TAACAACAGCCATTTCAATCAAACT	This study
ND2-synR3	ND2	TTTTCGGATTTGGGTTTGGTTTAG	This study
ND2-synF4	ND2	CTGCCTTAGGAGGATGAATAGG	This study
ND2-synR4	ND2	TTAGTGAAGGGATTTTTGTTCATGC	This study
ND2-synF5	ND2	ACCAAAATTCTTAAACTATCCTCAATAA	This study
ND2-synR5	ND2	ATCGGAGGTAGAAGAAAAGTCCT	This study
ND2-synF6	ND2	CAACAATCATTGCCCTCCTCTC	This study
Lmet	ND2	TATCGGGCCCATACCCCGAAAAT	Hackett (1996)
H6312	ND2	CTTATTTAAGGCTTTGAAGGCC	Cicero and Johnson (2001)
FIB5	FIB5	CGCCATACAGAGTATACTGTGACAT	Marini and Hackett (2002)
FIB6	FIB5	GCCATCCTGGCGATTCTGAA	Marini and Hackett (2002)
FIB-FurnL	FIB5	AAGGAGAGCCTGGCTCATTCCTTA	Fjeldså et al. (2007)
FIB-FurnH	FIB5	GAAGTTGAAGGAATGCCCTGGTCT T	Fjeldså et al. (2007)
Myo2	myo2	GCCACCAAGCACAAGATCCC	Slade et al. (1993)
Myo3F	myo2	TTCAGCAAGGACCTTGATAATGACTT	Heslewood et al. (1998)
Myo309L	myo2	CATAAGACCTGTCAGTGGCTGGA	Irestedt et al. (2006)
Myo345H	myo2	TCCTCCAGGGTTTGCTCTAAAATTGT	Fjeldså et al. (2007)
G3PL890	G3PDH	ACCTTTAATGCGGGTGCTGGCATTGC	Friesen et al. (1997)
G3PH950	G3PDH	CATCAAGTCCACAACACGGTTGCTGTA	Friesen et al. (1997)

annealing temperature for 40 s and 72 °C for 1 min; plus a final extension step at 72 °C for 9 min. Annealing temperatures were: cytb and ND2 56 °C; FIB5 and myo2 52–58 °C (2 cycles at 58 °C, 2 cycles at 56 °C, 2 cycles at 54 °C and 34 cycles at 52 °C); G3PDH 64 °C. PCR and sequencing protocols followed Batalha-Filho et al. (2012) and Irestedt et al. (2006) for fresh and study skin samples, respectively.

2.2. Sequence edition, alignment and recombination

Electropherograms were inspected and assembled in contigs using CodonCode Aligner v. 3.7 (CodonCode Inc.). Heterozygous sites in nuclear introns were coded according to IUPAC code when double peaks were present in both strands of the same individual's electropherograms. Nuclear sequences that contained heterozygous indels were analyzed using the algorithm Process Heterozygous Indels in CodonCode Aligner v. 3.7. Sequences were aligned using the CLUSTAL W method (Higgins et al., 1994) in MEGA5 (Tamura et al., 2011). All alignments were inspected and corrected visually. We used PHI test in SPLITSTREE4 (Bruen et al., 2006; Huson and Bryant, 2006) to check for recombination in the nuclear gene sequences. This test was used due to its power to distinguish recombination events from recurrent mutation (Bruen et al., 2006). To apply the coalescent species tree analysis we resolved the gametic phase of nuclear genes using the PHASE algorithm (Stephens et al., 2001) with default settings in DnaSP 5 (Librado and Rozas, 2009) assuming a threshold of posterior probability of 0.9. However, we kept the unphased sequences with probabilities below this threshold in the analysis.

2.3. Phylogenetic analyses

The best fit model for each gene was selected using MrModeltest 2.2 (Nylander, 2004) based on the Akaike information criterion (AIC). Two partitions (cytb + ND2) were considered in the Bayesian and maximum likelihood analyses using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and RAxML (Stamatakis et al., 2008), respectively. Both analyses were carried out at CIPRES Science Gateway (Miller et al., 2010). Bayesian runs consisted of 10 million generations for 2 independent runs with 4 chains of Markov chain Monte Carlo (MCMC) each. The first million generations were discarded as burn-in, after which trees were sampled every 500 generations. Chain convergence (Effective Sample Size – ESS values >200) was checked using the likelihood plots for each run using Tracer 1.5 (http://beast.bio.ed.ac.uk/Tracer). The Potential Scale Reduction Factor was also used to check chain convergence and burn-in; values close to one indicate good convergence between runs (Gelman and Rubin, 1992). We used RAxML under the GTRAC model; invariable sites and gamma distribution were estimated for each partition during the run. Node supports of the maximum likelihood analyses were estimated by 1000 bootstrap replications. We also obtained trees in RAxML for each nuclear gene.

2.4. Species tree and dating

In order to combine the information of all genes in a single tree and accommodate inconsistencies between gene and species trees (Degnan and Rosenberg, 2006; Kubatko and Degnan, 2007) we used the Bayesian coalescent method of species tree in *BEAST (Bayesian Inference of Species Trees from Multilocus Data; Heled and Drummond, 2010) in the Bioportal at University of Oslo (Kumar et al., 2009). The main mitochondrial lineages (Fig. 1) were assigned as "species" in the analysis. We also estimated the divergence times using the multispecies coalescent method of *BEAST (in BEAST 1.6.2, Drummond and Rambaut, 2007), as this method seems to be more accurate than those based on gene trees (McCormack et al., 2011). We generated the input file in BEAUTi considering four genes (mitochondrial + FIB5 + myo2 + G3PDH) and the substitution models selected by MrModeltest (Table 3). We used relaxed clock with an uncorrelated lognormal distribution (Drummond et al., 2006) and Yule process for all genes. Piecewise linear and constant root were used as population size model priors. Given the very sparse fossil record of furnariids, and the general problem of correlation of furnariid splitting events to specific past events (Derryberry et al., 2011), we used the mutation rate of mitochondrial genes (under a normal distributed prior) available for birds to estimate divergence dates (Lovette, 2004; Weir and Schluter, 2008). This mutation rate of 1.05% (±0.05) per lineage per million years was estimated by Weir and Schluter (2008) based on 90 calibration points and 12 orders of birds, and included a range of 12 million years. We then used BEAST to estimate the mutation rates for the nuclear introns using a log-normal distributed prior under default settings for each partition. We did two independent runs with 100 million generations each, with parameters sampled every 10,000 steps and a burn-in of 30%. We checked for convergence between runs and analysis performance using Tracer 1.5, and accepted the results if ESS values were >200. The resulting trees were combined in TreeAnotator and the consensus species tree with the divergence times was visualized in FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 3
Best fit models for *S. ruficapilla* and *S. moesta* groups and outgroups estimated for each partiton.

Partition (genes)	Model of evolution
cytb	HKY + I
ND2	HKY + Γ
Mitochondrial (cytb + ND2)	GTR + Γ
FIB5	HKY + I
myo2	GTR + I + Γ
G3PDH	HKY + I + Γ

3. Results

3.1. Phylogenetic inferences

3.1.1. Mitochondrial dataset

Our mitochondrial dataset comprised 2043 characters for 40 individuals, including outgroups: 1002 of cytb and 1041 of ND2. For the ingroup 120 and 125 sites were variable in cytb and ND2, respectively. No indels, unexpected stop codons, or ambiguous peaks in the electropherograms were found in these sequences, suggesting that they were of mitochondrial origin. The best fit model estimated for each gene is shown in Table 3.

Bayesian and maximum likelihood analyses indicated absence of monophyly of the *S. ruficapilla* complex, with *S. infuscata* being paraphyletic with other AF species (Fig. 1). Besides, specimens of the undescribed *Synallaxis* from Vila Rica-MT (hereafter unnamed *Synallaxis*) formed a clade with a deep divergence from the other species (Fig. 1). Yet, unnamed *Synallaxis* appeared as sister to the AF species *S. ruficapilla* and *S. whitneyi*, but with moderate node support (0.89 of posterior probability and 82% of bootstrap; Fig. 1). The relationships between the *S. ruficapilla* and *S. moesta* complexes were recovered as a polytomy (Fig. 1). Interestingly, the results revealed that the AF *S. infuscata* is sister to the Andean *S. moesta* with moderate support (0.92 of posterior probability and 94% of bootstrap; Fig. 1).

Our mitochondrial trees also revealed genetic structure in *S. ruficapilla*. Two clades were observed: a southern clade that included almost the entire geographical distribution of species, and an unexpected northern clade with samples from the north of state of Minas Gerais (Poté and José Gonçalves de Minas) where the AF meets the Cerrado biome (Fig. 1 and Table 1).

3.1.2. Nuclear dataset

We obtained sequences of all three nuclear introns for all species, except G3PDH for *S. moesta* (Table 1). In the FIB5 ingroup alignment (546 characters) 34 sites were polymorphic, and there were five indels in eight individuals ranging in length from 1 to 29 bp. We found 16 polymorphic sites in the myo2 ingroup alignment (587 characters) and one indel in 11 individuals of 1 bp long. For the alignment of the ingroup G3PDH (323 characters) there were 26 polymorphic sites and two indels (1 bp and 2 bp long, respectively) in six individuals. PHI test showed that there is no significant evidence for recombination events in any of the nuclear genes ($p > 0.1$).

Gene trees of nuclear introns did not recover the same genetic structure in the ingroup as found in the mitochondrial genes. However, a poorly supported clade that includes unnamed *Synallaxis*, *S. ruficapilla*, and *S. whitneyi* is observed in the G3PDH tree, but the last two taxa were not reciprocally monophyletic (Supplementary Fig. S1).

3.1.3. Species tree

The species tree generated by *BEAST was partially congruent with the mitochondrial one, but with poorly supported nodes (Fig. 2). The only exceptions were the central-southern AF clade (*S. ruficapilla* and *S. whitneyi*) that was moderately well supported, and its sister relationship with unnamed *Synallaxis* that was well supported.

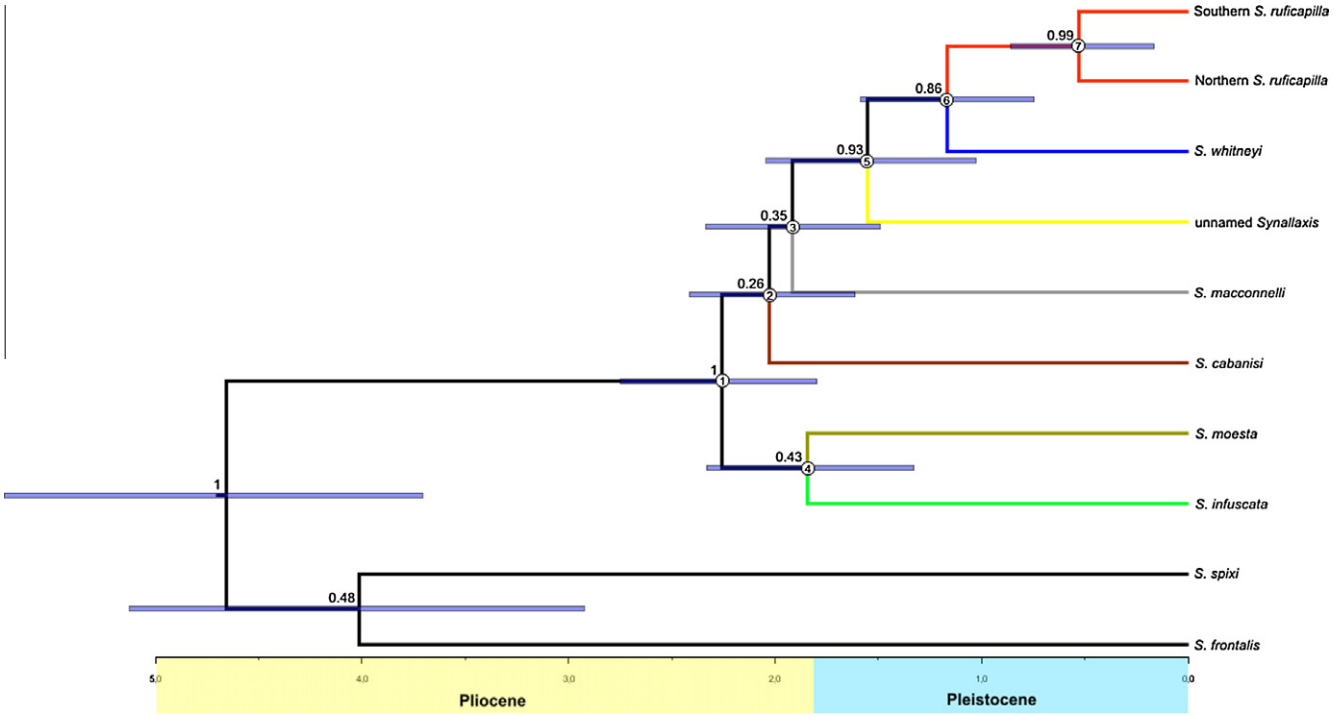

Fig. 2. Chronogram of species tree with divergence times generated by *BEAST. Number at the nodes are clade posterior probabilities. White circles with numbers at nodes are divergence times between clades according to Table 4. Bars on the nodes represent 95% of high posterior density of divergence times. Branch colors follow Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Divergence times and confidence intervals [95% of high posterior density (HPD)] between major mitochondrial lineages of *S. ruficapilla* and *S. moesta* groups estimated by *BEAST. Node numbers follow Fig. 2.

Node	Time mya (95% of HPD)
1	2.24 (2.75–1.80)
2	2.02 (2.40–1.61)
3	1.91 (2.33–1.49)
4	1.85 (2.33–1.33)
5	1.56 (2.04–1.03)
6	1.17 (1.58–0.74)
7	0.53 (0.86–0.16)

3.2. Divergence times

The dated chronogram based on the coalescent multilocus analysis in ∗BEAST, revealed a recent diversification. The splitting events occurred from the late Pliocene to early Pleistocene (2.75–0.16 million of years ago [mya]; Fig. 2; Table 4). The oldest divergence event was the split between the clade including *S. infuscata* and *S. moesta* at 2.24 mya. (95% of high posterior density [HPD]: 2.75 – 1.80 mya). The youngest diversification event was the separation of northern and southern clades in *S. ruficapilla* at 0.53 mya (95% of HPD: 0.86–0.16 mya).

4. Discussion

4.1. Systematics of the S. ruficapilla and S. moesta complexes

The mitochondrial phylogeny revealed eight well supported lineages including all previously described species and one unnamed *Synallaxis* (Fig. 1). Besides, it revealed a previously undetected cryptic diversity within *S. ruficapilla*, where a dichotomy separating a northern clade (comprising specimens from the northern state of Minas Gerais) and a southern clade (specimens from the remaining localities sampled in this study) where observed. *S. whitneyi* was found to be monophyletic and appeared as sister to *S. ruficapilla*. Specimens of unnamed *Synallaxis* also formed a clade with a deep differentiation from its sister group (*S. whitneyi* and *S. ruficapilla*). This result reinforces the hypothesis that these specimens represent an undescribed taxon. *S. infuscata* and *S. moesta* were reciprocally monophyletic and unexpectedly recovered as sister species with a moderate node support. This is not congruent with a previous suggestion that *S. infuscata* should group with the other two AF species, *S. ruficapilla* and *S. whitneyi* (Pacheco and Gonzaga, 1995). *S. cabanisi* and *S. macconnelli* were also monophyletic, but their relationships to the other clades could not be resolved and are best regarded as belonging to an unresolved polytomy.

Our data also suggest that the specimens from Vila Rica-MT belong to an undescribed taxon of *Synallaxis*. Additional specimens possibly related to this new taxon were collected in other localities in the Amazon, in Coroatá, Maranhão (Oren, 1991, specimens in Louisiana State University, Museum of Natural Science) and São Félix do Xingu (specimens in Museu Paraense Emílio Goeldi, Brazil), previously identified as *S. infuscata* and *S. macconnelli* respectively. The number of rectrices of these specimens suggests that they belong to the *S. ruficapilla* complex (Stopiglia et al., 2012). Also, Zimmer et al. (1997) mentioned specimens of *S. cabanisi* in Alta Floresta in the state of Mato Grosso (approx. 550 km west from Vila Rica-MT), but according to Whitney (1997) these birds possibly belong to an undescribed species of the *S. ruficapilla* complex. Therefore, further efforts of field surveys in these regions will be important to obtain specimens of unnamed *Synallaxis* to determine whether all these records represent the same cryptic taxon as identified in our study, as well as determining the geographic distribution and taxonomic status of this lineage.

Overall, our mitochondrial results were incongruent with the analyses by Stopiglia et al. (2012) that used song and morphological characters. This taxonomic revision of the *S. ruficapilla* group concluded that only two species should be accepted within the

complex: (i) *S. ruficapilla* that includes *S. whitneyi* and the unnamed *Synallaxis*, and; (ii) *S. infuscata*. According to Stopiglia et al. (2012), *S. whitneyi* is a synonym of *S. ruficapilla*, and, according to the same authors, the species in the complex are primarily diagnosed by their number of rectrices: *S. ruficapilla* (including *S. whitneyi* and unnamed *Synallaxis*) with eight rectrices, and *S. infuscata* with ten rectrices.

The absence of a clear phenotypic distinction between the lineages detected by our mitochondrial data set could be the result of retention of ancestral polymorphism, as the majority of the *Synallaxis* species share a very similar plumage (Remsen, 2003). This could also be due to morphological stasis and insufficient time to evolve morphological differentiation as the estimated population divergence date is relatively recent (late Pliocene to early Pleistocene). Yet, the initially described plumage differentiation (Pacheco and Gonzaga, 1995) could represent high flexibility in ecophenotypes with no bearing on the historical population structure. Another possible explanation is selection of phenotypic characters such as plumage and song in *S. ruficapilla*, *S. whitneyi*, and unnamed *Synallaxis*, which would make it difficult to find diagnosable states for each of these taxa.

The nuclear gene trees did not recover the same population structure as observed in the mitochondrial tree (Supplementary Fig. S1). Possible explanations for the lack of reciprocal monophyly in the nuclear DNA includes incomplete lineage sorting in these genes due to a recent diversification (Pinho et al., 2008), and the longer time of coalescence of nuclear DNA compared to the mitochondrial genome (approximately 4 times longer; Palumbi et al., 2001; Zink and Barrowclough, 2008). Also, there is the possibility of gene flow between some lineages observed here, as we found a possible contact zone (data not shown) between *S. ruficapilla* (northern and central to southern AF) and *S. whitneyi*. Moreover, this possible gene flow may also have contributed for the lack of diagnosability in vocal and morphological characters. Further analyses of gene flow modeling based on a larger dataset will help to depict this scenario.

Recent estimates (Degnan and Rosenberg, 2006; Liu and Edwards, 2009; Heled and Drummond, 2010) showed that multilocus phylogenies based on classical concatenation of genes are less accurate than inferences based on coalescent species tree approaches to recover the phylogenetic relationships among taxa. Moreover, estimates of coalescent species tree are efficient to accommodate inconsistencies between gene trees due to incomplete lineage sorting in recently diversified populations (Heled and Drummond, 2010). Therefore, in this study we implemented the Bayesian coalescent species tree by ∗BEAST to depict the systematic relationships between *S. ruficapilla* and *S. moesta* complexes. Our species tree was similar to the mitochondrial tree revealing the same clades with good node support values. Although all species showed long terminal branch-lengths, they were poorly supported for some species (*S. macconnelli*, *S. cabanisi*, *S. moesta*, and *S. infuscata*) and failed to resolve the polytomies observed in the mitochondrial tree (Fig. 2).

4.2. Biogeography

The majority of the species (∼17) of genus *Synallaxis* occurs in the Andes and Amazon, and just five are endemic to the AF (the three species from this study plus *S. spixi* and *S. cinerascens*; Remsen, 2003). Thus, assuming that the Andes and the Amazon are the possible centers of origin of the genus, the AF species seem to have originated from at least three independent lineages as observed in a recent phylogeny of the Furnariidae family (Derryberry et al., 2011). Interestingly, our study showed that the *S. ruficapilla* complex endemic to the AF is not monophyletic, which suggests that there were additional colonization events and subsequently isolation of geographical lineages in this biome. Similar patterns of non-monophyly of AF taxa have been observed in small mammals (Costa, 2003) and birds (Pessoa, 2007; Maldonado-Coelho, 2010; but see Weir and Price, 2011). Furthermore, the *S. ruficapilla* and *S. moesta* complexes together exhibit a circum-Amazonian pattern as described by Remsen et al. (1991).

The dating of our phylogeny suggests a recent diversification event (late Pliocene to early Pleistocene), with the two populations of *S. ruficapilla* separated during the climatically most unstable part of the late Pleistocene (Fig. 2). Recently, multi-taxon analysis in conjunction with fossil data have suggested an ancient biota diversification in South America (mainly before the Pleistocene) with the uplift of the Andes and marine incursions as the driving forces of diversification in the continent, instead of Pleistocene glacial cycles (Hoorn et al., 2010; Hoorn and Wesselingh, 2010). However, other studies argued that although the uplift of the Andes and marine incursions were important in the diversification of South American organisms, Pleistocene climate changes may also have played a key role in the diversification process (Rull, 2008, 2011), and also documented a continuous diversification from the Neogene until the Pleistocene (Rull, 2011). Despite the controversies about the tempo of diversification in South America, our data suggest a Plio-Pleistocene diversification for this group, supporting a higher connectivity between thicket and forest habitats in the rugged landscapes in the circum-Amazonian bioregions at this time.

The phylogenetic reconstructions suggest that at least two distinct lineages in the *S. ruficapilla* and *S. moesta* groups originated independently in the AF (Figs. 1 and 2): one was the *S. ruficapilla* and *S. whitneyi* clade in central-southern AF, and another was *S. infuscata* in northern AF. As *S. infuscata* is not the sister species of the other AF species and groups as sister of the Andean species *S. moesta* (even though with moderate node support), its origin seems to reflect a historical connection between the Andes and the AF (Silva, 1994; Percequillo et al., 2011). Yet, our observation that the unnamed *Synallaxis* is sister of the *S. ruficapilla*/*whitneyi* clade raises the possibility that this is a relictual population from the time when the Andean and south-east Brazilian upland habitats were connected (Silva, 1995). This lineage could then have survived in eastern Amazon after the end of historical connections between these two biomes. This result may support previously proposed historical routes during the Pleistocene connecting South American forests through the dry diagonal (Por, 1992; Willis, 1992; Oliveira et al., 1999; Behling et al., 2000; Auler and Smart, 2001; Costa, 2003; Auler et al., 2004; Wang et al., 2004; Batalha-Filho et al., 2013), specially the Amazon and the AF.

In addition, our results show a Pleistocene diversification within the AF with two vicariant events that lead to separation of three lineages (Figs. 1 and 2): an older split between *S. ruficapilla* and *S. whitneyi* at 1.17 mya (95% of HPD: 1.58–0.74 mya) and a more recent event separating the two groups of populations of *S. ruficapilla* at 0.53 mya (95% of HPD: 0.86–0.16 mya). While the AF holds lineages of ancient (mid-Tertiary) taxa (Fjeldså et al., 2012; Fouquet et al., 2012), the regional biota also underwent significant diversification during the Pleistocene, reflecting climatic-vegetational changes during the Quaternary (Cabanne et al., 2008; Carnaval and Moritz, 2008; Carnaval et al., 2009; d'Horta et al., 2011; Martins, 2011; Maldonado-Coelho, 2012). The cladogenetic events observed here show spatial and temporal congruence with previous studies of AF organisms (Cabanne et al., 2008; Carnaval et al., 2009; Ribeiro et al., 2010; Thomé et al., 2010). These studies evoked the refuge hypothesis (Haffer, 1969; Vanzolini and Williams, 1970) or tectonical activities (Silva and Straube, 1996; Thomé et al., 2010) as main causes of vicariance. Both these hypotheses may be relevant for the speciation events observed here, and phylogeographic studies are being conducted to help to outline the evolutionary history of this group within the AF.

4.3. Implication to conservation

Data from the present study provided significant arguments for the conservation of the threatened species *S. whitneyi* and *S. infuscata* (Roda, 2008; Silveira, 2008). The phylogenetic results confirmed that these are independent evolutionary units, which are reciprocally monophyletic (Fig. 1). Stopiglia et al. (2012) suggested that morphology and songs provide no clear diagnostic differences between *S. ruficapilla* and *S. whitneyi*, but our results favor the hypothesis that each of them is a distinct evolutionary significant unit (ESU). At least *S. ruficapilla* and *S. whitneyi* lineages seem to represent well supported phylogenetic species, but extra efforts are needed to find out what happens in potential zones of contact, and to investigate if phenotypic or vocal differences can be detected, that can provide reliable practical diagnosis.

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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.ympev.2013.01.007.

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