Multilocus phylogeography of the Wedge-billed Woodcreeper *Glyphorynchus spirurus* (Aves, Furnariidae) in lowland Amazonia: Widespread cryptic diversity and paralogy reveal a complex diversification pattern

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**A R T I C L E   I N F O**

Article history:
Received 1 March 2012
Revised 25 September 2012
Accepted 27 September 2012
Available online 9 October 2012

Keywords:
Amazonia
Cryptic species
*Glyphorynchus spirurus*
Historical biogeography
Multilocus phylogeography
ISSR genomic fingerprinting

**A B S T R A C T**

Amazonian rivers function as important barriers to dispersal of Amazonian birds. Studying population genetics of lineages separated by rivers may help us to uncover the dynamics of biological diversification in the Amazon. We reconstructed the phylogeography of the Wedge-billed Woodcreeper, *Glyphorynchus spirurus* (Furnariidae) in the Amazon basin. Sampling included 134 individuals from 63 sites distributed in eight Amazonian areas of endemism separated by major Amazonian rivers. Nucleotide sequences were generated for five genes: two mtDNA genes (1047 bp for cyt b and 1002 bp for ND2) and three nuclear genes (647 bp from the sex-linked gene ACO, 319 bp from the intron of G3PDH, and 619 bp from intron 2 of MYO). In addition, 37 individuals were randomly selected from the Rondônia and Inambari areas of endemism for genomic fingerprinting, using five ISSR primers. Our results reveal allopatric and well-supported lineages within *G. spirurus* with high levels of genetic differentiation (p-distances 0.9–6.3%) across opposite banks of major Amazonian rivers. The multilocus phylogenetic reconstructions obtained reveal several incongruences with current subspecies taxonomy. Within currently recognized subspecies, we found high levels of both paralogy and genetic differentiation, indicating deep divergences and strong isolation consistent with species-level differences. ISSR fingerprinting supports the existence of genetically differentiated populations on opposite sides of the Madeira River. Molecular dating suggests an initial vicariance event isolating populations from the Guiana center of endemism during the Late Miocene/Early Pliocene, while more recent events subdivided Brazilian Shield populations during the Lower Pleistocene.

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1. Introduction

The Amazon basin holds more avian species than any other region on Earth (Mittermeier et al., 2003). As in other regions, avian species are not randomly distributed across the Amazon (Haffer, 1974). Instead, the evolutionary history of the South American continent is characterized by particular regions known as “areas of endemism” (Haffer, 1974; Cracraft, 1985), which harbor distinctive assemblages of species and populations which ranges are delimited by major rivers, such as the Amazon, Negro, Solimões, Madeira, and Tapajós. The finding that different avian assemblages occur on opposite banks of large Amazonian rivers is a phenomenon noted since first naturalists visited the region over 150 years ago. In fact, the so called “river-barrier hypothesis” resulted from one of the earliest attempts to account for the diversification of the Amazonian biota (Wallace, 1853; Hellmayr, 1910; Snethlage, 1913; Sick, 1967).

Although the notion of areas of endemism has contributed over the years to the study of biogeography in Amazonia and elsewhere, other phylogeographic studies have challenged the limitations of this approach, particularly concerning the degree of variability among lineages in their responses to common vicariant barriers (Aleixo and Rossetti, 2007; Burney and Brumfield, 2009; Antonelli et al., 2010). Furthermore, all areas of endemism in Amazonia and elsewhere in the Neotropics have been delimited historically based on the distributions of taxa which were diagnosed morphologically and without an explicit phylogenetic framework (Haffer, 1969, 1974; Cracraft, 1985). Thus, since the mid-1990s comparative statistical phylogeography has provided a new paradigm for fundamental questions concerning the diversification of the Amazonian and Neotropical biota as a whole (Patton et al., 1994; Costa, 2003; Ribas et al., 2012).

Recent advances in the fields of palynology, paleontology, climatology, and phylogenetics have provided new insights into the diversification of the organisms and environmental changes in...
lowland Amazonia (for a review see Hoorn et al., 2010). For example, evidence of a dramatic change in the course of the Amazonian rivers during the late Pliocene was reported by Espurt et al. (2010), which was mediated by neotectonics following the establishment of the Fitzcarrald Arch. This continental-wide drainage reorganization of the Amazon Basin is now assumed as the main driver of avian speciation in Amazonia (Fernandes et al., 2012; Ribas et al., 2012).

The Wedge-billed Woodcreeper (Glyphorynchus spirurus, Furnariidae) has a wide distribution ranging from Central America, west to the Andes, throughout central Amazonia, and south along the Atlantic coast of Brazil (Marantz et al., 2003). It is a common species occurring in different types of lowland habitats, including both terra firme and seasonally flooded lowland forests (várzea and igapó), and up to about 1500 m in the Andes (Stotz et al., 1996). Taxonomically, G. spirurus represents a polytypic species with an uncertain number of recognized subspecies, depending upon the taxonomic authority. The most recent review recognized thirteen subspecies (Marantz et al., 2003), most endemic or associated with particular areas of endemism (Haffer, 1974; Cracraft, 1985). These characteristics make G. spirurus an ideal model organism to investigate the influence of biogeographical barriers (e.g., rivers) on population structure, and test hypotheses concerning the origin of the remarkable Amazonian biodiversity.

Marks et al. (2002) have already analyzed rather short fragments of three mitochondrial genes of G. spirurus to examine the phylogenetic relationships among Neotropical areas of endemism as defined by Cracraft (1985). The authors discovered several inconsistencies between their reconstructed molecular phylogeny and patterns of morphological differentiation providing the basis for subspecific diagnoses in G. spirurus. They also documented a more complex pattern of diversification between the Madeira and Tapajós rivers in central Amazonia, whereby smaller rivers in this interfluve also appear to delimit the ranges of divergent and parapatric phylogroups (see also Sardelli, 2005; Fernandes, 2007 and Fernandes et al., 2012). However, some critical nodes of those phylogenetic trees (Marks et al., 2002) were poorly supported, and the alternative methods of phylogeny reconstruction (maximum parsimony and maximum likelihood) showed incongruent tree topologies. Moreover, the historical relationships among Amazonian areas of endemism found by Marks et al. (2002) contrasted strongly with those suggested for other bird lineages (Patané, 2009; Ribas, 2005, 2009).

Several unlinked genes are required for coalescent theory-based analyses. Superior results can be expected as more loci are added to the analyses (Maddison, 1997). Coalescent methods should provide better estimates of speciation times by mitigating the influence of deeply coalescent alleles (Drummond and Rambaut, 2007), and providing better estimates of species trees than supermatrix methods based on concatenation of molecular data-sets (Heled and Drummond, 2010; Liu and Pearl, 2007; Liu et al., 2008; Rannala and Yang, 2003; Wilson and Balding, 1998; Wilson et al., 2003). More genes may also increase the possibility of detecting anomalous loci such as those affected by paralogous gene copies or selection that might otherwise mislead phylogeny and divergence time estimations.

We investigated the phylogeographic and population genetics structure of the Wedge-billed Woodcreeper G. spirurus using a multilocus approach and a larger dataset than other previous avian phylogenetic studies carried out in the Amazon basin. The present investigation addresses the following questions: (1) Does current taxonomy accurately reflect evolutionary patterns and genetic structure of G. spirurus populations? (2) Do small rivers also delimit populations within larger interfluvis? (3) Does the genetic structure of G. spirurus populations indicate potential zones of secondary contact?, and (4) Which diversification hypotheses better explain the historical patterns of differentiation revealed by the multilocus phylogenies and population genetics analyses of G. spirurus lineages?

2. Materials and methods

2.1. Taxon sampling

Altogether, tissue samples of G. spirurus were obtained from 63 localities distributed in eight Amazonian areas of endemism (sensu Cracraft, 1985). Sampling localities encompassed opposite sides of the Andes, all major Amazonian river basins, and the Atlantic Forest on the Brazilian coast (Fig. 1). A total of 134 individuals was sequenced, with the Rondônia Amazonian area of endemism comprising the largest sample size (see Table 1). Detailed information on specimens, sampling sites, as well as voucher and GenBank accession numbers are provided in Appendix A.

2.2. Extraction, amplification, sequencing of DNA and ISSR genomic fingerprinting

DNA was extracted from breast muscle (0.2 g, approximately) and blood using a standard phenol chloroform protocol (Sambrook et al., 1989). Two mitochondrial genes (cytochrome b [cyt b] and nicotinamide adenine dinucleotide dehydrogenase subunit 2 [ND2]), and three nuclear genes (intron 2 of the myoglobin gene [MVO], intron 11 of the glyceraldehyde-3-phosphohydrogenase gene [G3PDH], and the sex-linked gene for aconitase [ACO]) were amplified and sequenced (Table 2).

Details of DNA extraction and PCR amplifications were described in Fernandes et al. (2012). Sequencing was carried out using an ABI 3730 automated capillary sequencer (Applied Biosystems) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 by STARSEQ GmbH (Mainz, Germany). To distinguish mutations from PCR or sequencing errors, both strands of each sample were sequenced.

Genomic fingerprinting with inter-simple-sequence-repeats (ISSR) PCR were performed with 30–60 ng of template DNA in 25 μl reaction volumes containing: 10 pmol of the 5′-anchored microsatellite repeat primer, 0.1 mM of dGTP, dCTP, and dTTP, 0.045 mM dATP, 1 μCi (α-33P)-dATP (Amersham Biosciences), 0.6 units of Taq DNA polymerase (Pharmacia Biotech, Freiburg) and 2.5 μl of 10× amplification buffer (10 mM Tris–HCl pH 8.5, 50 mM KCl and 1.5 mM MgCl2). Thermocycling was performed with a T-gradient thermocycler (Bioterm). Following the initial 10 min denaturation at 94 °C, the program consisted of 35 cycles of 60 s at 94 °C, 60 s at 52–60 °C, 120 s at 72 °C and 10 min at 72 °C for final elongation. DNA fragments were separated by vertical polyacrylamide gel electrophoresis (gel length 40 cm) for 2–3 h at 65 W using a Base Acer Sequencer (Stratagene). After drying, the denaturing gels were exposed for 24 h to X-ray films (BioMax MR Film, Kodak) to produce autoradiograms. The ISSR primer sequences used are shown in Table 3.

2.3. Phylogenetic analyses based on mtDNA

The phylogenetic analyses of mtDNA marker genes were performed using Bayesian inference (BI) as implemented in MrBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003) and maximum likelihood (ML) via RAXMLGUI vs. 0.93 (Silvestro and Michalak, 2011). The evolutionary models were selected with JMODELTEST (Posada, 2008). Two independent runs of 108 generations each were conducted and trees were sampled every 500 generations. The first 500 samples were discarded as burn-in. Nodal support values in the maximum likelihood tree were assessed using 1000 bootstrap
iterations. The mitochondrial genes (cytb and ND2) were analyzed both independently and concatenated in a single data matrix. In the combined data set, a mixed model Bayesian analysis was employed using the GTR + I + G model for ND2 and the HKY + G model for cyt b. The genera *Nasica*, *Dendrocolaptes*, *Dendrexetastes*, *Hylexetastes*, *Xiphocolaptes*, *Xiphorhynchus*, *Lepidocolaptes*, *Drymornis*, and *Dendroplex* were used as outgroups (Derryberry et al., 2011; Moyle et al., 1987).

To visualize genealogical relationships among individuals, haplotype networks were constructed using the median-joining algorithm in the program NETWORK 4.5.1.0 (Forster et al., 2007). The mean pairwise uncorrected p-distance (Nei, 1987) within and among lineages was calculated using MEGA 4.0 (Tamura et al., 2007).

### 2.4. Molecular clock dating and species tree from multilocus data

#### 2.4.1. Molecular clock dating based on mtDNA

Molecular dating was carried out using the mtDNA genes with two partitions (cyt b and ND2), each with individual models chosen

---

*Table 1*

Provenance, DNA markers, and sample size of *G. spirurus* populations analyzed in this study.

<table>
<thead>
<tr>
<th>Area of endemism</th>
<th>Cyt b (1047 bp)</th>
<th>ND2 (1002 bp)</th>
<th>ACO1 (647 bp)</th>
<th>G3PDH (319 bp)</th>
<th>MYO (619 bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocó</td>
<td>2</td>
<td>–</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Imerí</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Inambari</td>
<td>16</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Rondônia</td>
<td>85</td>
<td>16</td>
<td>21</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>Pará</td>
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<td>2</td>
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<tr>
<td>Guiana</td>
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</tr>
<tr>
<td>Napo</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Atlantic Forest</td>
<td>1</td>
<td>–</td>
<td></td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>28</td>
<td>35</td>
<td>31</td>
<td>27</td>
</tr>
</tbody>
</table>

*Fig. 1.* Geographic distribution of tissues of the Wedge-billed Woodcreeper (*Glyphorynchus spirurus*) and Neotropical areas of avian endemism (sensu Cracraft, 1985) sampled in this study. Polygons involving certain localities (©) denote clades of haplotypes as shown in Figs. 2 and 3. The small map to the right depicts the entire range of *G. spirurus*.

---

*Table 2*

List of primers used in PCR amplifications and cycle sequencing.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Reference or sequences (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyt b L14990</td>
<td>Kocher et al. (1989)</td>
</tr>
<tr>
<td>cyt b L15389</td>
<td>Hackett (1996)</td>
</tr>
<tr>
<td>cyt b H15710</td>
<td>Helm-Bychowski and Cracraft (1993)</td>
</tr>
<tr>
<td>cyt b HXIPH and L15505</td>
<td>Aleixo (2004)</td>
</tr>
<tr>
<td>ND2 L5216 and H6313</td>
<td>Sorenson et al. (1999)</td>
</tr>
<tr>
<td>myo2</td>
<td>Slade et al. (1993)</td>
</tr>
<tr>
<td>myo3f</td>
<td>Heslewood et al. (1998)</td>
</tr>
<tr>
<td>myo39f</td>
<td>Irestedt et al. (2006)</td>
</tr>
<tr>
<td>myo344h</td>
<td>Irestedt et al. (2006)</td>
</tr>
<tr>
<td>G3PDH (f)*</td>
<td>GCCGCGCTTTGCGAATCCGGTTA</td>
</tr>
<tr>
<td>G3PDH (r)*</td>
<td>GGCCAGTTCATCCCTAATTCAGTC</td>
</tr>
<tr>
<td>ACO Ai115fb</td>
<td>CCCGCTCTATATCTATGT</td>
</tr>
<tr>
<td>ACO Ai15ra*</td>
<td>CACCAGATAACATAGATG</td>
</tr>
</tbody>
</table>

---

*Table 3*

List of ISSR primers and annealing temperatures used in this study.

<table>
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<tr>
<th>Primers</th>
<th>Annealing temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td>(GA)9C</td>
<td>60</td>
</tr>
<tr>
<td>(GACA)4</td>
<td>52</td>
</tr>
<tr>
<td>(CT)4(CA)5</td>
<td>54</td>
</tr>
<tr>
<td>(GA)BT</td>
<td>60</td>
</tr>
<tr>
<td>CTC(6)3AA</td>
<td>54</td>
</tr>
</tbody>
</table>

---

*Primers designed for this project.*

*Primers designed for this project.*

*According to Cracraft (1985).*

---

*Fig. 1.* Geographic distribution of tissues of the Wedge-billed Woodcreeper (*Glyphorynchus spirurus*) and Neotropical areas of avian endemism (sensu Cracraft, 1985) sampled in this study. Polygons involving certain localities (©) denote clades of haplotypes as shown in Figs. 2 and 3. The small map to the right depicts the entire range of *G. spirurus*.
by JMODELTEST 0.1.1 (Posada, 2008), Norman et al. (2007) suggest a rate of 2.8% for ND2 gene. This mutational rate, however, has been proposed only for a particular group of birds (honeyeater); therefore, it may not represent a universal rate among avian taxa. Thus, we applied a cyt b mutational rate of 2.21% sequence divergence per million years (0.01105 substitutions/site/lineage/million years) since this represents an average rate for the cyt b gene based on several independent calibrations (Weir and Schluter, 2008). We performed two runs setting the clock model as strict and relaxed. Two independent simultaneous runs of 10,000,000 generations were performed, sampling once every 1000 trees in BEAST v1.6.1 (Drummond and Rambaut, 2007). Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was determined by examining marginal probabilities plotted as a time series in TRACER 1.5 (Rambaut and Drummond, 2007).

2.4.2. Multilocus molecular clock dating and species tree
In this study, we estimated species trees using the method proposed by Heled and Drummond (2010) in ’BEAST v1.6.1 (Drummond and Rambaut, 2007). This method employs substitution models commonly used in phylogenetics, but also uses coalescence to provide joint inferences of a species tree phylogeny and divergence times from a collection of gene trees across a set of closely related species.

The Bayesian haplotype reconstruction for the nuclear loci (ACO, G3PDH, and MYO) was performed using PHASE 2.1 ( Stephens et al., 2001). As we are evaluating the historical relationships among areas of bird endemism of G. spirurus, the haplotypes of the five genes (Table 1) were grouped in different trait sets, defined by the area of endemism where populations were sampled. The model that best fit the data was estimated in JMODELTEST (Posada, 2008) and used for each gene a priori. In addition to the substitution model, the clock model and tree topologies were estimated independently for each gene except for the mtDNA genes. Due to a lack of recombination among mitochondrial genes in most organisms (including birds), mtDNA genes should be considered as linked in this kind of analyses (Heled and Drummond, 2010). We applied the same cyt b mutational rate used in previous analysis. Setting this mutational rate for the mitochondrial partition allowed us to estimate the rate of substitution of the three nuclear partitions.

Two runs were performed setting the clock model as strict and relaxed. Two independent simultaneous runs of 10^7 generations were carried out, sampling once every 1000 trees. Posterior probabilities of the nodes were computed for all Bayesian analyses across the sampled trees after burn-in. The number of generations required to reach stationarity of the posterior distribution was evaluated with TRACER 1.5 (Rambaut and Drummond, 2007).

2.5. Population genetics and historical demography

2.5.1. Cytochrome b sequencing
Population genetics analyses were carried out using the cyt b sequences just for those populations with the largest sample sizes, i.e., those including two subspecies (G. s. castelnaui and G. s. inornatus), two areas of endemism (Inambari and Rondônia), and opposite banks of three Amazonian rivers (Madeira River and two of its right bank tributaries, Aripuanã and Jiparanã). Population groups were defined as major haplotypes classes recovered in the phylogenetic analyses.

Using the cyt b sequences we estimated the nucleotide diversity (π) and haplotype diversity (h) among populations in ARLEQUIN 3.1 (Excoffier et al., 2005) and DNASP 4.0 (Rozas et al., 2003). Tajima’s D and Fu’s Fst statistic were calculated in order to test population size fluctuations. Significance was determined based on 100 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, we performed an a posteriori analysis of population genetic structure using AMOVA (Excoffier et al., 1992).

We reconstructed historical population size dynamics using Gaussian Markov random field (GMRF) skyride plot method (Minin et al., 2008) as implemented in BEAST v1.6.1 (Drummond and Rambaut, 2007). The GMRF skyride plot method is a nonparametric analysis that uses the waiting time between coalescent events in a gene tree to estimate changes in effective population size over time. It differs from the related Bayesian skyline plot (Drummond et al., 2005) by not requiring the specification of a user-defined prior on the number of population size changes (Ho and Shapiro, 2011). In this study, the GMRF skyride plots were constructed using the cyt b mutational rate of 2.21% sequence divergence per million years (0.01105 substitutions/site/lineage/million years) and time-aware smoothing. The analysis of the log file generated under the relaxed clock model gave ucl.mean and ucl.stdev parameters close to zero. Thus, we made use of the strict molecular clock model. All other parameters were identical to the molecular clock described above.

2.5.2. Clustering of individuals based on ISSR markers
Genomic fingerprints by ISSR-PCR (Gonzalez et al., 2008; Gonzalez and Wink, 2010) were performed for the populations from the Rondonia and Inambari areas (the geographic regions with the largest sampling sizes). ISSR bands were scored as dominant markers, 1 = band present and 0 = band absent. In order to examine the clustering pattern of all individuals, a principal coordinate analysis (PCoA) was performed using FAMD 1.25 (Schlüter and Harris, 2006) with a Jaccard coefficient. The potential number of clusters (K) of individuals was inferred using a Markov chain Monte Carlo algorithm implemented in STRUCTURE 2.2.2 (Falush et al., 2007; Pritchard et al., 2000). In order to estimate the value of K, a series of five independent runs (with K = 1–10) were performed using an admixture model and correlated allele frequencies. The proportion of membership to the inferred clusters was assessed for each individual. The burn-in period was set up to 50,000 followed by 500,000 cycles. In order to evaluate the statistical significance of gene flow between species/subspecies we made use of the USEPOPINFO option (Pritchard et al., 2000). We set GENBACk to 2 generations and MIGRPRIOR to 0.05.

3. Results

3.1. Phylogenetic analyses based on mtDNA

The Bayesian inference and maximum likelihood trees based on cyt b sequences of 134 individuals yielded similar tree topologies, with minor differences occurring in the position of the Inambari area of endemism. Both methods of phylogeny reconstruction recovered sixteen clearly defined genetic lineages (Figs. 2 and 3).

The expanded Bayesian mtDNA and maximum likelihood (~lnL = 6934.670513) gene trees (based on combined cyt b and ND2 sequences; Fig. 4) were identical and consisted of an entirely resolved phylogeny for all Cis-Andean populations of G. spirurus. With the caveat that this phylogeny includes a slightly smaller subset of individuals and areas than that based solely on cyt b sequences, only three nodes are supported by Bayesian posterior probabilities smaller than 0.95, including two with nearly significant posterior probabilities of 0.9 (Fig. 4). A high degree of population subdivision was found in three areas of endemism: Rondônia (four clades apparently confined, at least in the northern part of their ranges, by the Aripuanã and Jiparanã rivers), Napo (two
clades apparently confined by the Japurá River), and Inambari (clades apparently confined by the Purus River; Fig. 1).

Subspecies delimitations based on morphological characteristics (Marantz et al., 2003) generally agree with the well-supported clades in the molecular phylogeny, with some notable exceptions. The subspecies G. s. inornatus, a taxon thought to be endemic to the Rondônia area of endemism, is not monophyletic. Other subspecies recovered as being non-monophyletic include castelnaudii, paraven sis, and rufigularis (Figs. 2–4). The mean pairwise uncorrected genetic $p$-distances of mtDNA between major clades from different areas of endemism ranged from 1.0% to 6.3%, while the mean genetic $p$-distance within these clades varied between 0.0% and 0.8% (Fig. 2).

The median-joining haplotype networks based on cyt $b$ sequences recovered 16 phylogroups that had already been identified in the Bayesian phylogeny. These clades display non-overlapping geographic distributions and are separated by larger-than-average numbers of mutational steps (Fig. 5).

3.2. Molecular clock dating and species trees

3.2.1. Dating with mtDNA

The strict and relaxed mtDNA molecular clock models resulted in trees with very similar divergence times. We present the tree generated with relaxed-clock model because the values evaluated with TRACER 1.5 (Rambaut and Drummond, 2007) were higher.

According to the relaxed-clock model the earliest split among G. spirurus lineages took place in the Pliocene (5.8–3.4 Mya; mean 4.5 Mya; Fig. 6), separating Guiana populations from all remaining lineages. Subsequent splits (Inambari and Rondônia/Pará) occurred in the middle–lower Pliocene (3.6–2.2 Mya; Fig. 6). The latest diversification involved the subspecies from the north of the Amazon River (Napo and Imerí regions) and the subspecies from the Brazilian Shield (Rondônia and Pará) during the Pleistocene.

3.2.2. Multilocus clock dating and species trees

The species tree estimated with ‘BEAST resulted in a topology with overall lower posterior probability values and more recent divergence time estimates compared to those retrieved from the concatenated mtDNA analysis. In terms of topologies and divergence times, we obtained similar results using either strict or the relaxed molecular clock models. The analysis of the log file generated under the relaxed clock model gave ucldev.mean and ucldev.stddev parameters close to zero for the cyt $b$, ACO, G3PDH and MYO genes, suggesting that the data for these four genes evolving a clock like fashion (Drummond et al., 2007). However, the ucldev.stddev parameter was very high (8.5) for the ND2 on this analysis. Thus, we present the tree generated with the relaxed-clock model.

As in the mtDNA dating analysis, the multilocus approach reveals that areas of endemism from the Brazilian Shield (Pará and Rondônia) cluster together; similarly, the phylogeny also recovered the subspecies G. s. inornatus (the taxon endemic to the Rondônia area) as paraphyletic (Fig. 7). However, the position of G. s. castelnaudii...
(Inambari), which clustered together with northern Amazonian lineages, differs from the mtDNA phylogeny, but with low support value (0.3).

3.3. Population genetics and historical demography

3.3.1. Cytochrome b sequencing

Analyses conducted based on cyt b sequences to detect demographic changes suggest stable demographic histories for Inambari 1, 2, 3 and Rondônia 4 populations, but demographic expansion for Rondônia 1, 2, 3 populations. Values of Tajima’s D and Fu’s Fs statistic were not significantly negative for Inambari 1, 2, 3 and Rondônia 4 populations. Conversely, Tajima’s D and Fu’s Fs statistics were significantly negative for Rondônia 1, 2 populations. We observed higher values of haplotype diversity (h) in comparison to nucleotide diversity (π) in all areas, except Inambari 3, 4, 5 where both parameters were equally high (Table 4). The AMOVA analysis indicates that most of genetic variance is partitioned...
among groups (90.5%, \(p < 0.001\)) corroborating the population structure revealed in the phylogenetic analyses.

The Bayesian Skyride Plots (BSPs) estimated for the \(G. \text{spirurus}\) clades agree with the neutrality tests in inferring histories of demographic fluctuations during the Middle and Late Pleistocene.

Rondônia 1 population is inferred as expanding between 0.30 and 0.25 Mya followed by a slight decline and another expansion in the last 0.10 Mya (Fig. 8). Rondônia 2 population also presents an expansion–decline–expansion historical demography as Rondônia 1. A small and gradual population expansion was also recovered for Rondônia 3 population during the last 0.10 Mya, while the other two clades in western Amazonia (Inambari) and in the northern portion of Madeira/Tapajós interfluve (Rondônia 4) appeared to have maintained a relatively stable size during the Pleistocene.

3.3.2. Clustering of individuals based on ISSR fingerprints

Genomic fingerprints were performed with 37 individuals randomly selected from Rondônia and Inambari (the most densely sampled regions) including two subspecies (\(G. \text{s. castelnaudii}\) and \(G. \text{s. inornatus}\)) using five different ISSR primers. These primers generated a total of 40 scorable bands.

The PCoA analyses support the existence of genetically differentiated populations on opposite sides of the Madeira River, but no strong differentiation among phylogeographic provinces of populations from the same river bank (i.e., Rondônia and Inambari areas of endemism; Fig. 9). These results were corroborated by the Bayesian probability assignment analyses, which indicated that the individuals could be partitioned into two main clusters, capturing most of the genetic structure in the data (\(\ln L = -458.8\)). These distinctive clusters include populations from opposite sides of the Madeira River, with one individual from the Inambari area of endemism (individual 2; Appendix B) providing evidence for mixed ancestry with 40% posterior probability (Fig. 10).

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>(N)</th>
<th>(h)</th>
<th>(\pi)</th>
<th>(D)</th>
<th>(F_{ST})</th>
</tr>
</thead>
<tbody>
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<td>0.016</td>
<td>1.07</td>
<td>0.50</td>
</tr>
<tr>
<td>Rondônia 1</td>
<td>51</td>
<td>0.97</td>
<td>0.0037</td>
<td>-1.45</td>
<td>-11.03</td>
</tr>
<tr>
<td>Rondônia 2</td>
<td>10</td>
<td>0.87</td>
<td>0.001</td>
<td>-1.83</td>
<td>-4.52</td>
</tr>
<tr>
<td>Rondônia 3</td>
<td>10</td>
<td>0.95</td>
<td>0.002</td>
<td>-0.57</td>
<td>-3.80</td>
</tr>
<tr>
<td>Rondônia 4</td>
<td>8</td>
<td>1.0</td>
<td>0.003</td>
<td>-0.41</td>
<td>-3.08</td>
</tr>
</tbody>
</table>

- \(N\) = number of individuals sampled; \(h\) = gene diversity; \(\pi\) = nucleotide diversity; \(D\) = Tajima’s \(D\) statistic; \(F_{ST}\) = Fu’s \(F_{ST}\) statistic.
- Significant values (\(p < 0.05\)).
4. Discussion

4.1. Phylogeographic structure, taxonomy, and species limits

Thirteen of the 16 lineages of *G. spirurus* recognized in this study are confined by Amazonian rivers along most of their distribution ranges (Fig. 1), suggesting a major role for rivers as vicariant barriers for diversification in Amazonia. However, our data also show that modern drainage limits of major Amazonian river basins are not always in agreement with the distribution of corresponding clades.

Altogether, only half of the 16 lineages of *G. spirurus* revealed in this study are formally recognized taxonomically, indicating a very

Fig. 8. Bayesian Skyride Plots based on ca. 1047 bp of cyt b gene showing the demographic history for different lineages of Glyphorynchus spirurus (see Fig. 2). The black line represents the median value whereas the gray area denotes 95% Bayesian credibility intervals. N = number of samples analyzed. See Fig. 1 for clades geographic distributions.

Fig. 9. Geographical distribution of samples used in ISSR fingerprinting and principal coordinate analysis (PCoA) plot for all possible pairs of ISSR loci comparisons.
high degree of cryptic diversity and the need of an in-depth taxonomic revision contemplating a thorough morphological analysis to identify phenotypically diagnosable units and define new taxa. Additionally, the high number of genetically differentiated populations of G. spirurus, in addition to the existence of two main markedly different song types, and several phenotypically distinct subspecies have prompted the speculation that more than a single species is involved (Marantz et al., 2003; Remsen, 2012).

Both the cyt b (Figs. 2 and 3) and expanded mtDNA phylogeny (Fig. 4) support the reciprocal monophony of the following populations and taxa, which fit the definition of species under a plethora of different evolutionary and lineage-based species concepts (de Queiróz, 1998): (1) Guiana (G. [s.] spirurus); (2) Imeri (G. [s.] rufgularis); (3) Rondônia 4 (G. [s.] inornatus); (4) Inambari 4 (G. [s.] albigularis); (5) Pará 2 (G. [s.] paraensis); and Atlantic Forest (G. [s.] cuneatus). At least 10 additional reciprocally monophyletic mtDNA lineages of G. spirurus which would agree with evolutionary species definitions have yet to be described (as discussed in detail below), and thus cannot be treated as species-level taxa herein.

However, when a multilocus species tree is considered (with the caveat that a much smaller subset of individuals and areas, and only five genes were sampled for this analysis), an overall low statistical support for the mtDNA clades is obtained (Fig. 7). In fact, the multilocus phylogeny provides strong support only for the monophyly of those populations sampled more densely (i.e., Brazilian shield populations from the Rondônia and Pará areas of endemism) with respect to all remaining Amazonian populations, as well as for the split separating Rondônia 2 from Rondônia 1 and 3 populations. Thus, under a multilocus approach, our data allows the recognition of only three species among the Cis-Andean taxa of G. spirurus, as follows: G. spirurus (the oldest name applicable to sampled populations of the Guiana, Inambari, Imeri, and Napo areas of endemism); G. inornatus (the oldest name applicable to populations Rondônia 4 and Pará); and an undescribed taxon including populations Rondônia 1, 2, 3. It is expected that because of shared polymorphism and bigger effective population sizes, nuclear genes will sort out later among monophyletic populations than mitochondrial genes (Heled and Drummond, 2010). Thus, the multilocus perspective on intra-specific limits in G. spirurus provided herein may reflect a conservative estimation of species limits in this genus. In contrast, the mitochondrial phylogenies probably mirror more recent events of gene flow cessation due to isolation by distance or in response to a build-up of new barriers. When different species criteria are considered, the conclusions inferred from a multilocus phylogeny are best translated in terms of biological species, whereas those obtained from mtDNA phylogeny are more easily reconciled with lineage-based species definitions (i.e., the phylogenetic species concept). Considering the different sampling intensities underlying our mitochondrial and multilocus phylogenies, we believe that we have provided a more accurate redefinition of interspecific limits in G. spirurus under a lineage-based rather than biological species concept approach. More extensive future multilocus sampling regimes should provide a better framework for the revision of interspecific limits in G. spirurus under a biological species concept perspective. This will allow the reconciliation of species and mitochondrial trees to support the recognition of distinct biological and evolutionary species.

4.2. Comparison with previous studies

The phylogeographic structure recovered here differs in some aspects from the findings of Marks et al. (2002) and contrasts strongly with currently recognized taxonomy and subspecies limits in G. spirurus (Marantz et al., 2003). On the other hand, our findings are consistent with a major biogeographic pattern detected for several other avian lineages, whereby populations of the Brazilian shield in south-central Amazonia are reciprocally monophyletic with respect to other populations (Rivas et al., 2005, 2009, 2012; Alexio and Rossetti, 2007; Patané, 2009).

Marks et al. (2002) found that the subspecies G. s. albigularis, a taxon distributed throughout northern Bolivia and southern Peru in the southern part of the Inambari area of endemism, was not monophyletic, being closely related to some populations of G. s. inornatus, an endemic taxon to the Madeira/Tapajós interfluve (Marantz et al., 2003). According to Marks et al. (2002), the Madeira River (the second largest river in the Amazon) did not delimit monophyletic populations of G. spirurus, and their mitochondrial DNA phylogeography was inconsistent with patterns of morphological differentiation between these subspecies. However, our results do not support these findings. Based on mitochondrial and nuclear markers, including ISSR fingerprints, we found a clear genetic break coincident with the Madeira River, which separates populations from the Inambari and Rondônia areas of endemism into separate clades. Our larger sampling and multilocus approach showed in fact that the Madeira River does represent an effective biogeographical barrier separating monophyletic populations of G. spirurus. Similarly, all specimens sequenced carrying the white throat typical for G. s. albigularis clustered together in a clade (MPEG 59810, MPEG 59828, MPEG 58874, FJ899295, FJ899301; Figs. 2 and 3), indicating that this taxon corresponds to a valid and distinct evolutionary unit in G. spirurus. In contrast, populations thought to belong to G. s. castelnaudii proved to be paraphyletic (Figs. 2 and 3). The type locality of castelnaudii (Santa Maria, lower Huallaga River in central Peru; Peters, 1951) indicates that this name should be applied only to the clade containing Napo 3 and Inambari 1 populations (Figs. 1–3). On the other hand, Inambari 2 and 3 populations form a well-supported clade sister to G. s. albigularis (Inambari 4; Figs. 2 and 3), but differing from it genetically (2.7% of average cyt b uncorrected p-distance) and phenotypically (rusty rather than white throat). These discrepancies between our study and that of Marks et al. (2002) are probably attributed to the smaller sequencing regime employed in the latter study (a combined 1171 bp for partial sequences of cyt b, ND2, and ND3 mtdNA genes), which can yield less consistent phylogeny estimates when compared to bigger datasets such as the one provided herein.

Fig. 10. Bayesian clustering analysis-based DNA bands generated from ISSR fingerprinting; black = mixed ancestry, dark gray = Inambari, and light gray = Rondônia. See Fig. 9 for groups’ geographic distributions.
In agreement with Marks et al. (2002) but unlike the generalized area cladogram showed by Bates et al. (1998), where the Chocó populations are sisters to all Amazonian populations, our cyt b phylogeny shows the Chocó population nested between the Napo and Imeri populations. This could be attributed to the continuity of the distribution of G. spirurus across the Chocó, Napo and Imeri areas of endemism (Fig. 1). In fact the species is found continually along the Andean foothills from Santa Cruz in Bolivia to western Venezuela and then across the Andean foothills of northern Colombia. On the other hand, the sister relationship and relatively low (ca. 1%) uncorrected p-distance values between Pará 2 and in the Atlantic Forest populations suggest that the latter may represent a recent dispersal from southeastern Amazonia (see Batalha-Filho et al., 2012).

4.3. Phylogenetic affinities and population structure within areas of endemism

Our phylogeographic analyses revealed four non-monophyletic lineages in the Madeira/Tapajós interfluve (Rondônia area of endemism; Fig. 1). We also found that G. s. inornatus (the name previously thought to apply to all populations in this interfluve; Marantz et al., 2003) is paraphyletic (Figs. 2–4 and 6). The type locality of G. s. inornatus (Parnintins, in the lowest eastern bank of the Madeira River in central Amazonian Brazil; Peters, 1951) indicates that this name should be applied only to the northernmost population of the Madeira/Tapajós interfluve (Rondônia 4). This clade is sister to the lineages distributed to the east of the Tapajós and the Atlantic Forest rather than those immediately to the south in the same interfluving. Therefore, no name exists that could be applied to the monophyletic populations found in the southern portion of the Madeira/Tapajós interfluve (i.e., clades Rondônia 1, 2, and 3), which represent a legitimate and independent evolutionary lineage (Figs. 2–4 and 6).

As already suggested by other authors, these patterns reinforce the notion that the Madeira/Tapajós interfluve has a complex history and holds a rich evolutionary diversity (Willis, 1969; Haffer, 1997; Marks et al., 2002; Sardelli, 2005; Cohn-Haft et al., 2007; Fernandes, 2007; Isler et al., 2007; Tobias et al., 2008; Fernandes et al., 2012). Originally considered to be a single and uniform area of endemism (Cracraft, 1985; Dinerstein et al., 1995), the area between the rivers Madeira and Tapajós may contain new phylogeographical provinces delineated by the G. spirurus clades recovered in this study as well as several other animals (van Roosmalen et al., 1998; Hall and Harvey, 2001).

Our dataset also reveals a complex and fine-scale pattern of population differentiation in two additional areas of endemism in Amazonia: Napo and Pará. When a larger dataset was analyzed (concatenated cyt b and ND2 sequences), Napo 1 populations became sister to Imeri populations rather than to Pará 2 (Fig. 4). Ribas et al. (2012) also found populations of Psophia trumpeters associated with the Napo area of endemism to be paraphyletic, even though their biogeographic affinities with populations from other areas of endemism differed from the pattern recovered for G. spirurus herein and by Marks et al. (2002). Populations of G. spirurus in the Pará area of endemism (sensu Cracraft, 1985) are paraphyletic. The populations of G. s. paraensis distributed east of the Xingu River (Pará 2) are sister to the disjunct Atlantic Forest population (to which the name caneus applies; Marantz et al., 2003), rather than those populations of G. s. paraensis found west of the Xingu in the same area of endemism (Figs. 2 and 3; Marks et al., 2002).

Similarly, Milá et al. (2009) found genetic substructures in the Chocó area of endemism for G. spirurus, also suggesting an unrecognized phylogeographic structure and cryptic speciation in this Trans-Andean South American population. Therefore, the number of cryptic lineages in the polytypic G. spirurus, which is distributed throughout most of the Neotropics, is yet unknown and will only be revealed by additional phylogeographic and taxonomic studies.

4.4. Contact zones

Our data showed that only two of the six areas of endemism recognized by Cracraft (1985) in Amazonia contain monophyletic populations of G. spirurus (Imeri and Guiana). In contrast, Napo, Inambari, Rondônia, and Pará populations showed extensive paraphyly, with many populations having a closer relationship with clades inhabiting adjacent, rather than the same, areas of endemism (Figs. 2–4). Most phylogeographic studies have shown that areas of endemism in Amazonia and elsewhere are often inhabited by non-monophyletic populations (Aleixo and Rossetti, 2007), but the degree of paraphyly documented for G. spirurus appears to be much higher than that recovered for most avian lineages. Some of the few lineages studied so far share the same Trans-Amazonian distribution pattern: Mionectes olegineus (Miller et al., 2008), Ramphastos tucaucus and Ramphastos vitellinus (Patané et al., 2009), and Psophia spp. (Ribas et al., 2012). These studies have discovered just a few Amazonian areas of endemism to be inhabited by non-monophyletic populations of those lineages, including Guiana (Miller et al., 2008), Inambari (Patané et al., 2009), and Napo (Ribas et al., 2012). The concept of areas of endemism does not accurately describe the differentiation patterns of G. spirurus uncovered in our study. Perhaps these areas of endemism are not cohesive biogeographic units in which lineages became isolated and differentiated in response to a common history (Cracraft, 1985), and a different framework for thinking about diversification processes in the Amazon needs to be considered. As shown here for G. spirurus, this can result in the recognition of areas of endemism and taxa inconsistent with traditional lineages of diversification (Aleixo and Rossetti, 2007; Burney and Brumfield, 2009; Antonelli et al., 2010).

For instance, the Imeri clade distributed mostly to the east of the Negro River is also found across this river’s western bank and upper course near São Gabriel da Cachoeira in Brazil (Fig. 1). However, the most extreme case involves the Rondônia 1 clade in the southern part of the Madeira/Tapajós interfluve, whose range is mostly delimited independently of major Amazonian rivers, including both major tributaries of the Tapajós, i.e., Juruena and Teles Pires (Fig. 1). Similarly, the Rondônia 3 clade is also distributed across the Juruena River into the Juruena – Teles Pires interfluve, where it meets with Rondônia 1 birds in an area not bisected by any major river. Other contact zones apparently not maintained by rivers include those between: (1) Rondônia 3 and 4 birds in the northern part of the Madeira/Tapajós interfluve; (2) Rondônia 1 and Pará 1 birds in the southern portion of the Tapajós/Xingu interfluve; and (3) Inambari 3 and 4 birds in the southern part of the Inambari area of endemism (Fig. 1).

When the mitochondrial data alone are considered, none of these contact zones set away from major rivers discussed above involve sister clades of G. spirurus, and thus can be regarded as secondary (i.e., resulting from dispersal of at least one of the lineages involved; Figs. 2–4). However, when the multilocus species phylogeny is considered (Fig. 7), only the contact zone between Rondônia 1 and 3 birds can possibly be interpreted as primary rather than secondary, but the Bayesian posterior probability associated with this sister relationship is weak (0.8). More detailed studies are needed at the headwaters of major Amazonian tributaries and along the base of the Andes to determine the degree of interactions among genetically divergent populations.

4.5. The role of rivers

According to the mitochondrial phylogenies, the modern course of the following rivers separate sister clades of G. spirurus: lower
Amazon (Guiana and all remaining populations; Figs. 2 and 3), Mid-lower Negro (Imeri and Napo 1 populations; Figs. 2–4), Japurá (Napo 1 and 2 populations; Fig. 3), Marañón (Napo 3 and Inambari 1 populations; Figs. 2 and 3), Purus (Inambari 2 and 3 populations), Madeira (all Inambari and Rondônia/Pará populations; Fig. 3), lowermost Tapajós (Rondônia 4 and all Pará populations; Figs. 2–4), and Xingu (Pará 1 and 2 populations; Figs. 2 and 3).

Hence, rivers appear to play an important role as primary diversification barriers in *G. spirurus*, whereas contact zones not coincident with river courses result from secondary contact probably via dispersal. In *G. spirurus*, the dissociation between riverine barriers and clade limits is present foremost in the Napo (upper Amazon/Negro interfluvie) and Rondônia (Madeira/Tapajós interfluvie) areas of endemism, both known to have suffered major drainage reorganization patterns during the Pleistocene in response to neotectonics (Willis, 1969; Latrubesse and Franzinelli, 2005; Almeida-Filho and Miranda, 2007) and postulated megafans (Latrubesse, 2002).

According to the mitochondrial data, the confidence intervals for the timing of diversification involving Napo populations is entirely in the Pleistocene (0.8–1.7 Mya), whereas those associated with Rondônia populations encompass the Late Pliocene and most of the Pleistocene (1.1–2.7 Mya; Fig. 6). However, when the multilocus species tree is considered, ages associated with the diversification of Rondônia populations are younger and confidence intervals completely overlap with the Pleistocene (0.01–2.1 Mya), thus supporting the hypothesis of cladogenesis in response to relatively recent drainage reorganization in those two areas of endemism. Interestingly, so far, drainage reorganization patterns in response to tectonism in Amazonia have been described only for a third area of endemism (easternmost Pará; Rossetti and Valeriano, 2007), where local populations of *G. spirurus* were shown to track both spatially and temporally the pattern of drainage change in the lower Tocantins River valley.

Tectonically mediated mega-drainage capture as documented for the Negro, Madeira, and Tocantins river basins (Latrubesse and Franzinelli, 2005; Almeida-Filho and Miranda, 2007; Rossetti and Valeriano, 2007) as well as megafans (Latrubesse, 2002), can yield diversification patterns in which sister lineages differentiating in response to a river will no longer be separated by this same vicariant barrier over time (Wilkinson et al., 2010). This can apparently explain the observed instances of mismatch between modern drainage limits and the distribution of *G. spirurus* clades in Amazonia, as discussed above, as well as the existence of so many parapatric populations in hypothesized areas of endemism. The fact that most, if not all, contact zones away from riverine barriers involve non-sister lineages of *G. spirurus*, and that the opposite is true for the majority of contact zones matching river barriers, provide together strong evidence for the paramount role played by rivers as primary diversification barriers in Amazonia, even when they courses change over time.

Unfortunately, phylogenetic relationships among main *G. spirurus* lineages received low statistical support and are to some extent contradictory among the different phylogeny estimates obtained (Figs. 2–4 and 7), preventing a more detailed discussion on the spatial patterns of diversification involving those splits. However, confidence intervals associated with the timing of splits involving *G. spirurus* lineages in Amazonia span the Late Neogene (Pliocene; i.e., 5.8 Mya for mitochondrial and 4.3 Mya for multilocus data) through the Quaternary (Late Pleistocene; i.e., 0.8 Mya for mitochondrial and 0.01 Mya for multilocus data; Figs. 2 and 3). This is consistent with the most recent models based on geological and paleontological evidence proposed for the historical development of the Amazon drainage (Mora et al., 2010; Espurt et al., 2010; Latrubesse et al., 2010). According to these models, the continuous subduction of the Nazca Ridge under the South American Plate during the Miocene caused the Amazonian foreland basins located on the eastern Andean foothills in Bolivia, Brazil, Peru, Ecuador, and Colombia to evolve from a depositional to a predominantly erosional state, draining sediments eastward and hence creating the modern transcontinental Amazon drainage flowing towards the Atlantic between the Late Pliocene (Espurt et al., 2010; Latrubesse et al., 2010; Mora et al., 2010) and Early Pleistocene (Espurt et al., 2010; see also Campbell et al., 2006).

On the other hand, population structure within Rondônia may or may not reflect population changes caused by glaciations during the Pleistocene when the Amazon rainforest is thought to have been fragmented. Despite the historical demographic dynamics detected in some populations of *G. spirurus*, there is evidence that demographic changes did not involve all populations in the same area of endemism, supporting a secondary and perhaps more local role for Pleistocene climate change as a diversification promoter in Amazonia.

### 4.6. Conservation implications

The phylogeography of *G. spirurus* corroborates the existence of several cryptic unnamed taxa, most of them restricted to particular sectors of Amazonia, such as several mini-interfluves of the Rondônia area of endemism. As discussed by Aleixo (2009) and implied by some previous studies (Cohn-Haft et al., 2007; Fernandes, 2007; Fernandes et al., 2012), both biogeographic studies and conservation policies should take taxonomic uncertainties into account when respectively delimiting proper analytical and conservation targets. Disregarding the significance of entire cryptically diverse sectors of Amazonia under the current high anthropogenic development pressure may lead to inconsistent interpretations of the diversification history of its organisms and the irretrievable loss of valuable but yet unrecognized species.

### Acknowledgments

We thank the curators and curatorial assistants of the following collections for allowing us to study and sequence skins and tissues under their care: Instituto Nacional de Pesquisas da Amazônia (INPA), Museu Paraense Emílio Goeldi (MPEG), Universidade Federal de Mato Grosso (UFMT), Mario Cohn-Haft (curator of birds, INPA) provided essential logistic support in Manaus during the field-work. During data collection and analysis AMF was supported by an overseas doctoral fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 290034/2008-12) and is currently supported by a post-doctoral fellowship from the same agency (CNPq 150842/2012-0). The laboratory work was conducted in the Institute of Pharmacy and Molecular Biotechnology, Heidelberg University. Support to AA’s research is provided by CNPq (#310593/2009-3, “INCT em Biodiversidade e Uso da Terra da Amazônia” # 574008/2008-0, and # 471342/2011-4), Luciano Naka and Catherine Bechtoldt provided useful and inspiring comments. We also thank Susanne Renner for help with the molecular clock dating analysis. Theodor C.H. Cole proofread and helped to improve the manuscript. Permits for the collection of specimens were provided by IBAMA (Instituto Brasileiro do Meio Ambiente).

### 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.2012.09.033](http://dx.doi.org/10.1016/j.ympev.2012.09.033).