



Phylogeography of the chestnut-tailed antbird (*Myrmeciza hemimelaena*) clarifies the role of rivers in Amazonian biogeography

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ABSTRACT

Aim We examined patterns of spatial and temporal diversification of the Amazonian endemic chestnut-tailed antbird, *Myrmeciza hemimelaena* (Thamnophilidae), to evaluate the diversification of a widespread avian taxon across rivers that potentially represent major natural barriers.

Location Lowland Amazonia.

Methods Sequences of the mitochondrial *ND2* and cytochrome *b* genes were investigated from 65 individuals distributed throughout the entire range of *M. hemimelaena*, and including the two currently valid subspecies *M. h. hemimelaena* and *M. h. pallens*. Based on a combination of phylogeographic tools, molecular dating, and population genetic methods, we reconstructed a spatio-temporal scenario of diversification of *M. hemimelaena* in the Amazon.

Results The data revealed three genetically divergent and monophyletic groups in *M. hemimelaena*, which can also be distinguished by a combination of morphological and vocal characters. Two of these clades correspond to the previously described taxa *M. h. hemimelaena* and *M. h. pallens*, which are separated by the upper Madeira River, a main Amazonian tributary. The third clade is distributed between the middle reaches of the Madeira River and the much smaller tributaries Jiparaná and Aripuanã, and, although currently treated as *M. h. pallens*, clearly constitutes an independent evolutionary lineage probably deserving separate species status. Molecular clock and population genetic analyses indicate that diversification in this group occurred throughout the Pleistocene, with demographic fluctuations assumed for *M. h. hemimelaena* and *M. h. pallens*.

Main conclusions The findings implicate rivers as barriers driving diversification in the *M. hemimelaena* complex. Levels of mitochondrial DNA divergence and associated morphological and vocal traits support its division into at least three separate species with comparatively small ranges. The existence of a previously unrecognized lineage in the *M. hemimelaena* complex, and the high degree of population structuring found in *M. h. hemimelaena* underscore the pervasiveness of cryptic endemism throughout Amazonia and the importance of DNA-based taxonomic and phylogeographic studies in providing the accurate estimates of diversity that are essential for conservation planning.

Keywords

Amazonia, antbirds, conservation biogeography, historical biogeography, *Myrmeciza hemimelaena*, phylogeography, Pleistocene, rivers, speciation, Thamnophilidae.

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INTRODUCTION

Amazonian landscape evolution has probably been influenced by a combination of tectonic events that mould river attributes and orbital-induced climatic changes (Haffer & Prance, 2001; Rossetti *et al.*, 2005). Thus, both river formation and glacial cycles have historically been considered major drivers of biotic diversification in Amazonia (Haffer, 1997a). Originally proposed by Wallace (1852), the *riverine barrier hypothesis* states that closely related taxa on opposite river banks diversified because of the riverine barrier effect (Wallace, 1852; Hellmayr, 1910; Sick, 1967). Thus, one of its predictions is that, given sufficient time, isolation will generate reciprocally monophyletic sister populations/species across major Amazonian rivers. In contrast to this hypothesis, and despite the fact that major rivers are the primary distributional boundaries for a large proportion of Amazonian vertebrates, the importance of river dynamics as a cause of allopatric speciation has not gained wide acceptance, mainly because some major rivers located in western Amazonia (e.g. the Juruá and Purus) do not appear to have acted as primary diversification barriers for some *terra-firme* vertebrate taxa (Antonelli *et al.*, 2010). However, recent molecular phylogenies of Neotropical birds revealed splits consistent with the establishment of major Amazonian rivers during the Pliocene/early Pleistocene (Tobias *et al.*, 2008; Patel *et al.*, 2011; Ribas *et al.*, 2012). Therefore, phylogeographic data amassed so far for several lineages of Amazonian vertebrates provide mixed support for the riverine barrier hypothesis (Antonelli *et al.*, 2010).

The competing *refuge hypothesis* postulates that forest cover in Amazonia was reduced and fragmented during glacial maxima throughout the late Pliocene and Pleistocene (Haffer, 1969; Vanzolini & Williams, 1970; Prance, 1973). Savanna and seasonally dry forest blocks are assumed to have functioned as barriers separating isolated populations of humid forest species, thus facilitating allopatric speciation (Haffer, 1969, 1997b; Prance, 1973). However, phylogeographic studies addressing predictions derived from the refuge hypothesis (namely, frequent and recurrent episodes of population contraction and expansion during the Pleistocene; Hewitt, 1999; Moritz *et al.*, 2000) are still very limited (Aleixo, 2004; Cheviron *et al.*, 2005; Ribas *et al.*, 2012). In addition, the paucity of fully resolved, densely sampled time-calibrated phylogenies for Amazonian organisms makes it difficult to assess the generality of any diversification hypothesis proposed so far for the region, underscoring the need for additional phylogeographic studies aimed at evaluating the role of Amazonian landscape evolution and climatic oscillations on diversification of the local biota.

Antbirds (Aves: *Thamnophilidae*) are particularly good models for studying diversification in the Neotropics because they (1) are usually common; (2) inhabit the forest understorey and hence are poorer dispersers and more prone to developing phylogeographic structuring than canopy birds (Burney & Brumfield, 2009); and (3) exhibit morphological, vocal, and genetic differentiation across major geographic

barriers such as mountain ranges and rivers (Zimmer & Isler, 2003).

The Amazonian endemic chestnut-tailed antbird, *Myrmeciza hemimelaena* Sclater, 1857, is one of the few species that has already been the focus of experimental studies as a model system for understanding Amazonian diversification (Seddon & Tobias, 2007). It occurs on opposite sides of rivers of various sizes south of the Amazon/Marañón/Solimões rivers from eastern Peru eastwards to the western bank of the Xingu River in Brazil, reaching as far south as north-eastern Bolivia in eastern Santa Cruz province (Zimmer & Isler, 2003). Two subspecies are currently recognized: *M. h. hemimelaena* (west of the Madeira River and south to the La Paz province in Bolivia), and *M. h. pallens* (east of the Madeira River; Zimmer & Isler, 2003). *Myrmeciza castanea*, its sister species, ranges from southern Colombia through eastern Ecuador and north-eastern Peru; it was originally described as a subspecies of *M. hemimelaena* (Zimmer, 1932), but was later recognized as a distinct species based on bioacoustics and morphometrics (Isler *et al.*, 2002; Zimmer & Isler, 2003). Both *M. hemimelaena* and *M. castanea* occur in the same overall habitat type throughout their ranges, namely upland *terra-firme* forest, often in areas where those forests grow on predominantly sandy soils (Zimmer & Isler, 2003).

More restricted bird distributions and new endemic species have recently been found to be confined to the Jiparaná/Aripuanã interfluvium in the Madeira River basin (Sardelli, 2005; Fernandes, 2007; Isler *et al.*, 2007; Tobias *et al.*, 2008), showing the existence of smaller Amazonian areas of endemism in what have been termed 'mini-interfluvia' (Cohn-Haft *et al.*, 2007). Several avian lineages inhabiting this area are represented by closely related taxa replacing each other on opposite banks of comparatively small rivers (Cohn-Haft *et al.*, 2007; Isler *et al.*, 2007). Molecular studies have shown the existence of genetically distinct populations with restricted ranges bounded by the Jiparaná and Aripuanã rivers (Sardelli, 2005; Fernandes, 2007). Such discoveries have important implications for defining and recognizing new areas of endemism in the Amazon Basin.

A putatively new species of *Myrmeciza* was observed and collected on several field trips to the Jiparaná/Aripuanã interfluvium area in 2003–2004, and evidence for species-level divergence based on vocal and morphological features was reported by B. Whitney and M. Cohn-Haft (Instituto Nacional de Pesquisas da Amazônia, Manaus, pers. comm.; see also Isler *et al.*, 2002; Whittaker, 2009), details of which are yet to be communicated formally. Our task has been to provide a comparative molecular analysis to evaluate the existence of a purported new species in the context of a broader phylogeographic study focused on the *Myrmeciza hemimelaena* complex. The samples of the proposed new species investigated in this study were from specimens collected in the Jiparaná/Aripuanã interfluvium area by Cohn-Haft *et al.* (2007) and A. Fernandes in 2009 (deposited at the Instituto Nacional de Pesquisas da Amazônia) and by the staff of the Museu Paraense Emílio Goeldi (MPEG) in 2004 (deposited therein; see Appendix S1 in Supporting Information).

Here, we present phylogeographic and population genetic analyses of the *M. hemimelaena* complex to elucidate its evolutionary history in lowland Amazonia. We sought to evaluate the role of putative riverine barriers and climatic oscillations on its diversification. Finally, we revise current interspecific limits in this group by delimiting genetically differentiated populations known also to differ morphologically and vocally, including the purported new taxon from the Jiparaná/Aripuanã interfluvium.

MATERIALS AND METHODS

Sampling design

A total of 65 individuals were sampled from 47 localities covering the entire range and all currently described subspecies of *M. hemimelaena* (Fig. 1; Appendix S1). All tissues were either collected directly by us in the field or obtained from the following institutions: Field Museum of Natural History, Chicago, USA (FMNH); Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil (INPA); Laboratório de Genética e Evolução Molecular de Aves, São Paulo, Brazil (LGEMA); Louisiana State University Museum of Natural Science, Baton Rouge, USA (LSUMZ); Museu Paraense Emílio Goeldi, Belém, Brazil (MPEG); Universidade Federal de Mato Grosso, Cuiabá, Brazil (UFMT); and Natural History Museum of Denmark, Copenhagen, Denmark (ZMUC). Tissue samples were subsequently vouchered together with research specimens (mostly skins). Included in the analysis are samples collected close to the type localities of *M. h. hemimelaena*, *M. h. pallens* and *M. c. castanea* (used as outgroups; Fig. 1). As additional outgroups we employed sequences of *Cercomacra serva* and *C. tyrannina* downloaded from GenBank, because the most complete phylogeny estimated so far for the Thamnophilidae recovered, with high statistical support, these

two species as the immediate sister clade to *M. hemimelaena* (Brumfield *et al.*, 2007).

Extraction, amplification and sequencing of DNA

DNA was extracted from breast muscle (*c.* 0.2 g) using a standard phenol/chloroform protocol (Sambrook *et al.*, 1989). The mitochondrial cytochrome *b* (*cyt b*) and NADH dehydrogenase subunit 2 (*ND2*) genes were amplified using published mitochondrial DNA (mtDNA) primers: for *cyt b*, L14993 and H16064 (Fernandes, 2007); and for *ND2*, H6313 and L5215 (Brumfield *et al.*, 2007). Two additional primers, *forward* 5'-CCCTAGGCGGTTGAGCCGGA-3' and *reverse* 5'-TTGTGTTGAGAGTAAGGAAGATGGGGA-3', were designed particularly for this project. Population genetic analyses of *M. hemimelaena* were based on sequences of *cyt b* (*c.* 984 bp) for 65 individuals and of *ND2* (*c.* 1041 bp) for 41 individuals. Phylogeographic analyses used a concatenated data set of 41 individuals for which both *cyt b* and *ND2* sequences were available (*c.* 2025 bp).

Polymerase chain reaction (PCR) amplifications were performed with 50- μ L reaction volumes containing 1 \times PCR buffer (Bioron, Ludwigshafen, Germany), 100 μ M dNTPs, 0.2 units of Taq DNA polymerase (Bioron), 200 ng of DNA, and 5 pmol of primers. The optimal annealing temperature was determined by gradient PCR in a Tgradient thermocycler (Biometra, Göttingen, Germany). Thermal cycling involved: (1) an initial denaturing step at 94 °C for 5 min; (2) 35 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C; and (3) a final 5-min extension at 72 °C. PCR products were precipitated with 4 M NH₄Ac and ethanol (1:1:6) and centrifuged for 15 min (16,000 g). Sequencing was carried out on an ABI 3730 automated capillary sequencer (Applied Biosystems, Carlsbad, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (STARSEQ GmbH, Mainz,

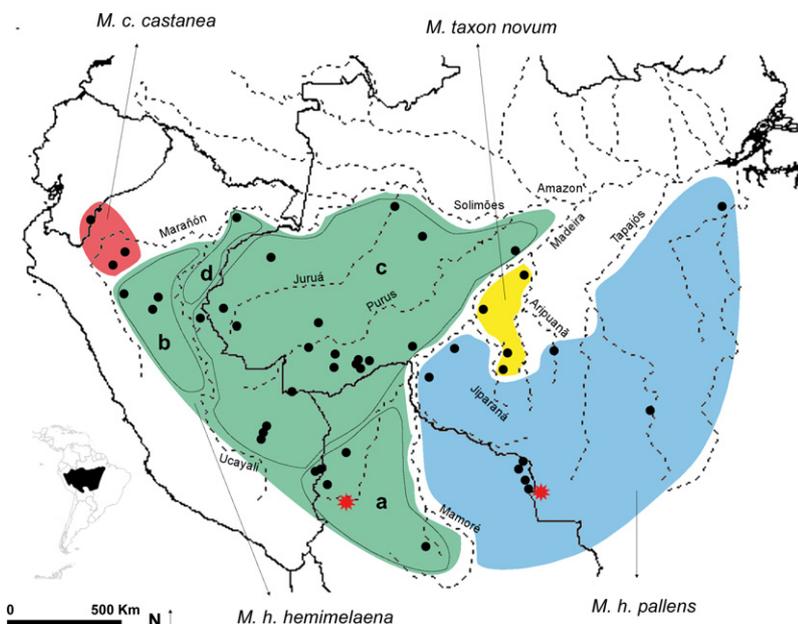


Figure 1 Origin of samples sequenced across the range of the *Myrmeciza hemimelaena* complex in Amazonia, and distribution of phylogroups recovered in the Bayesian phylogeny (Isler *et al.*, 2002; Zimmer & Isler, 2003). Asterisks depict the type localities of *M. hemimelaena* (Mapiri, La Paz, Bolivia; Gyldenstolpe, 1945) and *M. h. pallens* (Vila Bela da Santíssima Trindade, Mato Grosso, Brazil; Graf von Berlepsch & Hellmayr, 1905); a, b, c and d are clades of *M. h. hemimelaena*.

Germany). To confirm observed mutations, both strands of each sample were sequenced.

Phylogenetic analyses

Phylogenetic analyses were performed using Bayesian inference (BI) implemented in MRBAYES 3.1.2 (Ronquist & Huelsenbeck, 2003). The evolutionary models were selected with jMODELTEST using the Bayesian information criterion (BIC; Posada, 2008). Four different MRBAYES analyses were conducted, each with two parallel simultaneous runs, for a total of 5×10^6 generations each, with trees sampled every 500 generations. The mitochondrial genes (cyt *b* and *ND2*) were analysed independently and concatenated in a single data matrix. In the combined data set, a mixed-model Bayesian analysis was employed using the GTR + Propinv model for *ND2* and the HKY + G model for cyt *b*.

To visualize genealogical relationships among individuals, haplotype networks were constructed with maximum parsimony in the program TCS 1.18 (Clement *et al.*, 2000). Mean pairwise (*p*)-distances (Nei, 1987) within and among lineages were calculated using MEGA 4.0 (Tamura *et al.*, 2007).

Molecular dating

Estimates of the divergence time of the main lineages of *M. hemimelaena* were conducted using two data partitions (cyt *b* and *ND2*), each with individual models of molecular evolution chosen by jMODELTEST 0.1.1 (Posada, 2008). The widely used cyt *b* mutational rate of 2.1% sequence divergence per million years (0.0105 substitutions/site/lineage/million years) was applied (Weir & Schluter, 2008; Weir *et al.*, 2009). Because a clock-like evolution was rejected using a likelihood ratio test in PAUP* 4.0b10 (Swofford, 2002) and by the estimation of the parameter ucl.d.stdev in BEAST 1.6.1 (Drummond & Rambaut, 2007), we applied an uncorrelated lognormal relaxed clock. Two independent simultaneous runs of 2×10^7 generations were performed, sampling one tree in every 1000 in BEAST 1.6.1. 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 & Drummond, 2007).

Population genetics and historical demography

For population genetic analyses, groups were defined according to the major clades of haplotypes recovered in the phylogenetic analyses. Calculations of the following population parameters based only on cyt *b* sequences were calculated in ARLEQUIN 3.1 (Excoffier *et al.*, 2005): nucleotide diversity (π), haplotype diversity (*h*), and Tajima's *D* (Tajima, 1989) and Fu's *F_S* (Fu, 1997) statistics. Significance was determined based on 1000 coalescent simulations. To confirm and expand on the results obtained from the phylogenies, we performed a

posteriori analysis of population genetic structure using analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992). The defined groups were also the major clades recovered in the phylogenies.

We reconstructed historical population size dynamics using the Gaussian Markov random field (GMRF) skyride plot (Minin *et al.*, 2008), implemented in BEAST 1.6.1. The GMRF skyride plot 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 imposition of a user-defined prior on the number of population size changes in the history of the sample (Ho & Shapiro, 2011). The defined groups were the clades recovered in the phylogenies for which more than five individuals were available. The GMRF skyride plots were constructed using both mtDNA genes using the cyt *b* mutational rate of 2.1% sequence divergence per million years (0.01105 substitutions/site/lineage/million years), time-aware smoothing and strict molecular clock priors. All other parameters were identical to in the molecular dating described above.

RESULTS

Phylogenetic analyses

Phylogeographic analyses recovered a sister relationship and reciprocal monophyly between *M. castanea* and *M. hemimelaena*, which are separated by a genetic distance of 6.2% (uncorrected *P*-values within *M. hemimelaena*). Three clades of *M. hemimelaena* were identified on the basis of mtDNA data. They were found to correspond to vocally and morphologically distinct lineages (Figs 1 & 2; Isler *et al.*, 2002; Zimmer & Isler, 2003): (1) *M. h. hemimelaena* (west of the Madeira River), (2) *M. h. pallens* (east of the Madeira River ranging eastwards to the Xingu River), and (3) *M. h. pallens* (endemic to the Jiparaná/Aripuanã interfluvium). In addition to being monophyletic in mtDNA and separated by the highest average uncorrected genetic distance within *M. hemimelaena* (3.1%), populations of *M. h. pallens* from inside and outside the Jiparaná/Aripuanã interfluvium are also vocally and morphologically distinct (Isler *et al.*, 2002; Zimmer & Isler, 2003), thus indicating that these populations are best treated as different evolutionary units. Although our intent in this study does not extend to a formal taxonomic revision, for convenience we hereafter refer to populations found within the Jiparaná/Aripuanã interfluvium (Fig. 1) as *M. taxon novum*.

Significant phylogeographic structuring also exists within *M. h. hemimelaena* (Fig. 2), but in this case genetic distances are much smaller (0.8–1.4%), and the lack of congruent mtDNA, morphological and vocal diagnoses precludes a separation into distinct taxa (see also Zimmer, 1932; Isler *et al.*, 2002). Posterior probability values supporting the monophyly of these three main clades were high, as was the major split separating *M. h. hemimelaena* from *M. h. pallens*/

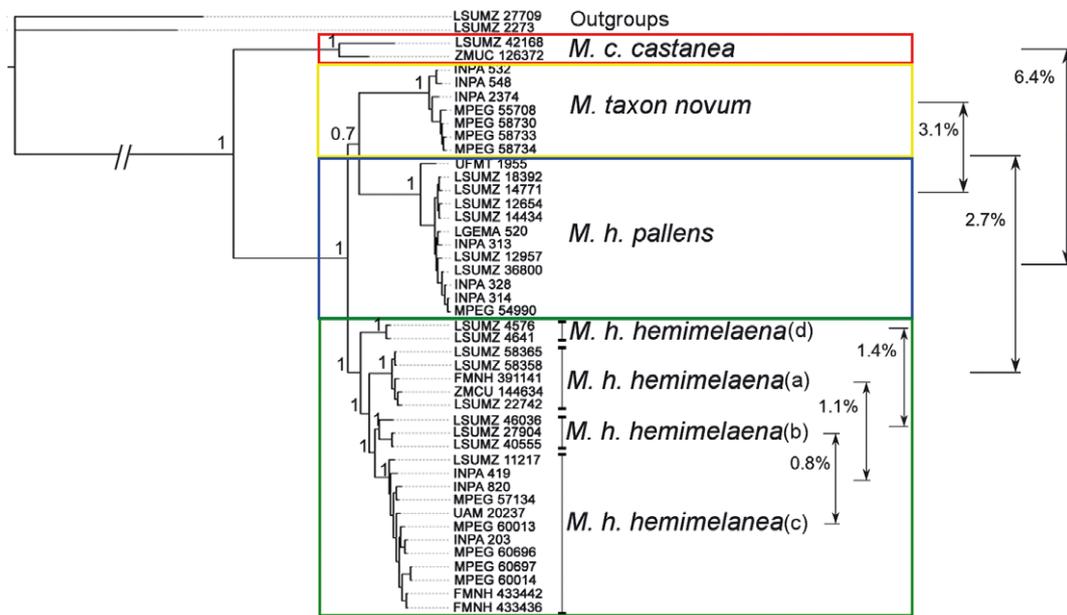


Figure 2 Bayesian phylogeny based on *ND2* and *cyt b* sequences ($n = 41$) showing the relationships among phylogroups of the *Myrmeciza hemimelaena* complex in Amazonia. Numbers next to nodes refer to posterior probabilities; percentages are the uncorrected pairwise (p)-distances; a, b, c and d are clades of *M. h. hemimelaena* as shown in Fig. 1.

M. taxon novum; on the other hand, support for the sister relationship between *M. h. pallens* and *M. taxon novum* was weak (Fig. 2).

Maximum parsimony haplotype networks (95% connection limit) mirrored the Bayesian phylogenetic tree in recovering the same three major clades corresponding to the three genetically and phenotypically diagnostic forms of *M. hemimelaena* (*M. h. hemimelaena*, *M. h. pallens* and *M. taxon novum*). These clades display non-overlapping geographic distributions and are separated by larger than average numbers of mutational steps (Fig. 3).

Molecular dating

According to the relaxed-clock model, diversification times of *Cercomacra* and the *M. hemimelaena* complex ranged from the late Miocene through the entire Pliocene between 2.7 and 8.8 Ma (mean 5.7 Ma; Fig. 4). The earliest split in the *M. hemimelaena* complex occurred during the Pliocene–early Pleistocene (1.8–4 Ma; mean 2.9 Ma) and involved *M. castanea* and all taxa grouped under *M. hemimelaena* (Fig. 4). Subsequent splits within *M. hemimelaena* were all clustered during the mid–early Pleistocene (1.8–0.3 Ma; Fig. 4).

Population genetics and historical demography

High values of haplotype diversity (h) and low values of nucleotide diversity (π) were obtained for the three major clades of *M. hemimelaena* identified in the maximum parsimony networks. Neutrality tests showed signs of population fluctuations for all three clades. Values of Fu 's F_S statistic were significantly negative for all lineages, whereas Tajima's D was

significantly negative only for *M. h. hemimelaena* (Table 1). AMOVA confirmed that most of the genetic variation in *M. hemimelaena* is partitioned geographically among the three main lineages identified in both the Bayesian phylogeny and haplotype networks (86%, $P = 0.0000$, $F_{ST} = 0.86$).

Most of the Bayesian skyride plots (BSPs) estimated for the three *M. hemimelaena* clades agreed with the neutrality tests in inferring histories of demographic fluctuations during the mid–late Pleistocene and Holocene. *Myrmeciza h. pallens* presents a clear pattern of population decline during the last 0.3 million years (Myr) in eastern Amazonia (Fig. 5). A slight signal of gradual population expansion was also recovered for *M. h. hemimelaena* (population a) in the Andean foothills south of the Amazon/Marañón River during the last 0.15 Myr (Fig. 5). The BSP for *M. h. hemimelaena* (population c) in western Amazonia shows an abrupt decline between 0.3 and 0.25 million years ago (Ma), followed by an expansion c. 0.2 Ma, then population stability between 0.2 and 0.1 Ma, and another expansion during the last 0.1 Myr. Finally, *M. taxon novum* appears to have maintained a relatively stable size during the last 0.4 Myr (Fig. 5).

DISCUSSION

Temporal and spatial patterns of diversification and historical demography in the *Myrmeciza hemimelaena* complex

Overall, the temporal diversification pattern estimated herein for the *M. hemimelaena* complex is at odds with the notion that avian diversity in Amazonia was generated mostly during the Pliocene and Pleistocene. Most Amazonian avian lineages

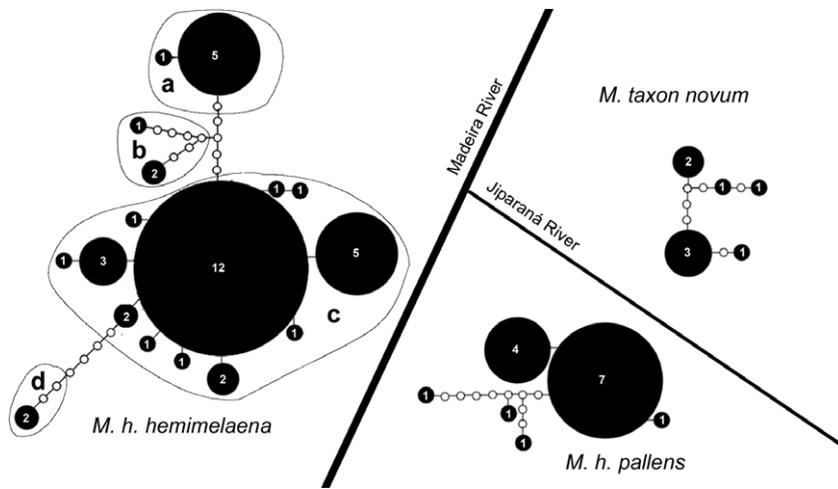


Figure 3 Haplotype networks (95% connection limit) based on *cyt b* sequences estimated for 66 individuals of the main three *Myrmeciza hemimelaena* vocal/morphological lineages separated by the Madeira and Jiparaná rivers in Amazonia. Each black circle represents a haplotype whose size corresponds to its total frequency in the respective lineage. Numbers inside black circles refer to the number of sampled individuals possessing that particular haplotype. Solid lines connecting haplotype clades represent single mutational steps. Empty circles correspond to unsampled haplotypes. Note that each of the three main clades (which were also recovered by the Bayesian phylogeny estimate; Fig. 1) is supported by a higher level distance in *r*cs, and hence they are not connected; a, b, c and d are clades of *M. h. hemimelaena* as shown in Fig. 1.

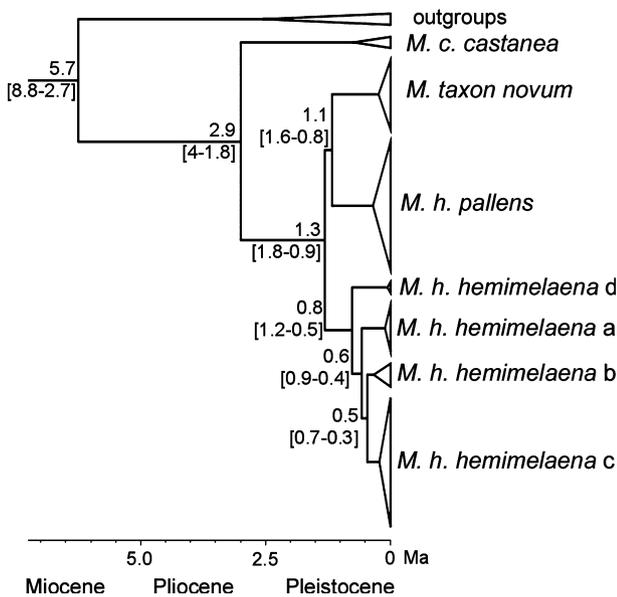


Figure 4 Bayesian divergence times of *Myrmeciza hemimelaena* lineages in Amazonia (in million years ago, Ma) estimated with a relaxed-clock model based on *ND2* and *cyt b* sequences ($n = 41$). Numbers within brackets indicate 95% posterior age intervals.

for which dated molecular phylogenies are available show the same pattern of ongoing diversification during those epochs (Aleixo & Rossetti, 2007; Tobias *et al.*, 2008; Patané *et al.*, 2009; Patel *et al.*, 2011; Ribas *et al.*, 2012). Therefore, as more studies with extensive taxonomic sampling and multiple character evidence become available, we predict that chronograms estimated for Amazonian lineages should point towards

Table 1 Genetic diversity and demographic population parameters for clades of the *Myrmeciza hemimelaena* complex in Amazonia based on *cyt b* sequences. Values given in bold are significant at $P < 0.05$

	<i>n</i>	<i>h</i>	π	<i>D</i>	<i>F_S</i>
<i>M. h. hemimelaena</i>	42	1.00	0.003	-1.40	-25.70
<i>M. h. pallens</i>	15	1.00	0.004	-1.12	-15.75
<i>M. taxon novum</i>	8	1.00	0.003	0.34	-4.50

n, number of sequences; *h*, haplotypic diversity; π , nucleotide diversity; *D*, Tajima's *D* (Tajima, 1989); *F_S*, Fu's *F_S* (Fu, 1997).

diversification scenarios concentrated in both the Pliocene and the Pleistocene. The relative importance of each of these epochs to the current patterns of diversity and endemism in Amazonia will become clearer with additional studies.

Rivers of various sizes conspicuously delimit the distribution of most species of the *M. hemimelaena* complex. The only exceptions are *M. castanea* and *M. hemimelaena*, which share a narrow and barrier-free contact zone south of the upper Marañón River in central Peru (Isler *et al.*, 2002). However, as shown in the Bayesian phylogeny (Fig. 2), the population of *M. hemimelaena* in this contact zone (population b) is sister to the *M. hemimelaena* population c, which is distributed throughout most of western Amazonia south of the Amazon and east of the Ucayali River (Fig. 1). Hence, the contact zone between *M. castanea* and *M. hemimelaena* can be interpreted as a secondary contact between non-sister lineages that diversified elsewhere, rather than as an area of primary differentiation. This interpretation challenges the role of the upper Marañón River in promoting cladogenesis in the *M. hemimelaena*

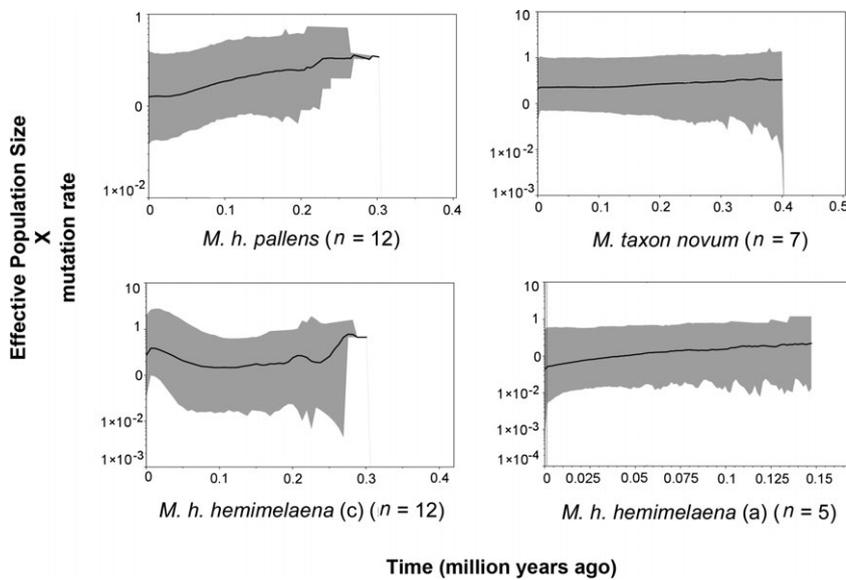


Figure 5 Demographic history of *Myrmeciza hemimelaena* lineages in Amazonia determined by Bayesian skyride plots based on *ND2* and *cyt b* sequences. The black line represents the median values; the shaded area denotes 95% Bayesian credible intervals; a and c are clades of *M. h. hemimelaena* as shown in Fig. 1.

complex. In contrast, even though the phylogenetic relationships among *M. h. hemimelaena*, *M. h. pallens* and *M. taxon novum* could not be resolved with confidence because of the low (0.7) nodal support for the sister relationship between *M. h. pallens* and *M. taxon novum*, any combination involving this triplet implies that at least parts of the Madeira River could have acted as a barrier involved in the primary diversification of lineages of the *M. hemimelaena* complex (Fig. 1). In the most likely scenario recovered by the Bayesian tree, the differentiation between *Myrmeciza h. hemimelaena* and *M. h. pallens*/*M. taxon novum* took place at *c.* 1.3 Ma across the entire course of the Madeira River (Fig. 4). Alternative tree topologies would imply that either only the upper (in the case of a sister relationship between *M. h. hemimelaena* and *M. taxon novum*) or only the middle (in the case of a sister relationship between *M. h. hemimelaena* and *M. h. pallens*) courses of the Madeira River might have acted as barriers of primary diversification (Fig. 1). Interestingly, the distributions of both *M. h. hemimelaena* and *M. h. pallens* extend farther to the south into the headwaters of the Madeira River in eastern Bolivia, where the few available records suggest that populations are likely to be separated by the Mamoré River, the largest tributary forming the Madeira River (Isler *et al.*, 2002; Zimmer & Isler, 2003; Fig. 1). However, as this is a fairly small river, they could equally well come into contact somewhere else, as also observed for the warbling antbird (*Hypocnemis cantator*) complex (Isler *et al.*, 2007).

Together, the Bayesian phylogeny estimate and the current distribution patterns of *M. h. hemimelaena* and *M. h. pallens* allow the interpretation that the Madeira River and possibly its headwaters have been functioning as a primary diversification barrier in the *M. hemimelaena* complex. The same can be said about the much smaller Jiparaná and Aripuanã rivers, which separate *M. taxon novum* from its most likely sister taxon (*M. h. pallens*; Fig. 1). On the other hand, large tributaries bisecting the range of *M. h. hemimelaena* population c in

western Amazonia (e.g. Juruá and Purus rivers) do not separate sister clades on their opposite banks, and thus do not appear to be directly involved with primary population differentiation in this species (Fig. 1).

BSPs (Fig. 5) and population genetics neutrality tests (Table 1) both point towards ubiquitous demographic fluctuations throughout the Pleistocene for all subspecies in the *M. hemimelaena* complex, except maybe *M. taxon novum*, for which only Fu's F_S statistic showed a significant departure from neutrality. Even though the power of those tests and analyses could be severely compromised by our limited sampling and reliance on mtDNA, the emerging pattern seems to be one of demographic instability throughout the Pleistocene, as predicted by the refuge hypothesis (Zink, 1997; Hewitt, 1999; Aleixo, 2004; Cheviron *et al.*, 2005). The historical demographic pattern shown by *M. h. hemimelaena* in western Amazonia and the foothills of the Andes (for which we have the most complete sampling, i.e. 42 individuals) matches models of climatic oscillation proposed world-wide for the mid–early Pleistocene (e.g. Head & Gibbard, 2005), as follows: (1) the abrupt population decrease after *c.* 0.3 Ma followed the onset of a glacial period; (2) the slight population increase before *c.* 0.2 Ma is consistent with the establishment of an interglacial warmer period; (3) the new population decrease starting after 0.2 Ma followed another glacial period lasting until 0.1 Ma, after which a new population rebound is apparent; and (4) the most recent demographic sink started after *c.* 0.02 Ma and can be tentatively linked to the Last Glacial Maximum (LGM) – apparently, the effective population size of *M. h. hemimelaena* has not yet recovered to pre-LGM levels (Fig. 5). If the BSP produced for *M. h. pallens* is interpreted at face value, a steady population decline after *c.* 0.3 Ma is apparent, as also found for *M. h. hemimelaena*, but, unlike in the latter species, no episodic population rebounds are visible (Fig. 5). However, *M. h. hemimelaena* and *M. h. pallens* populations share the same overall mid–early

Pleistocene decline. The interpretation of our historical estimates of effective population sizes for *M. taxon novum* is particularly complicated by our limited sampling of this taxon (Fig. 5).

The drivers of cladogenesis in the *Myrmeciza hemimelaena* complex

The spatial and temporal patterns of diversification and historical demography presented and discussed herein for the *M. hemimelaena* complex allow us to discuss the relative importance of the two main hypotheses accounting for the differentiation of the Amazonian biota: the *refuge* and *riverine barrier hypotheses* (see Haffer, 1997a and Antonelli *et al.*, 2010 for a synopsis of these and other hypotheses of Amazonian diversification).

Under the riverine barrier hypothesis, rivers would always be associated with areas of primary differentiation in the *M. hemimelaena* complex. However, as shown previously, western Amazonian rivers flowing through the Solimões sedimentary basin (e.g. Marañon, Juruá and Purus) have less stable courses and experience frequent lateral channel migration rarely observed in rivers located on the Brazilian and Guianan shields (e.g. Madeira, Jiparaná and Aripuanã; Aleixo, 2004; Bates *et al.*, 2004). Thus, to this day, discrepancies with the riverine barrier hypothesis involving *terra-firme* forest species, such as those grouped in the *M. hemimelaena* complex, appear to be restricted to a small subset of wide rivers flowing through the relatively young Solimões sedimentary basin in western Amazonia (da Silva & Patton, 1993; Patton *et al.*, 1994; Gascon *et al.*, 1998; Aleixo, 2004; Wanderley-Filho *et al.*, 2010).

Indeed, when molecular phylogenies of Amazonian birds associated with *terra-firme* forests are viewed together, the Madeira, Jiparaná and Aripuanã rivers have all been shown to delimit sister and monophyletic groups of many other lineages, thus demonstrating the generality of their role as likely vicariant agents; river width and water load alone are not necessarily correlated with a strong barrier effect (Aleixo, 2004; Sardelli, 2005; Fernandes, 2007; Nyári, 2007; Patel *et al.*, 2011). Varying degrees of barrier effects in the Amazon seem to be determined primarily by an interaction between geology and river width and discharge (Antonelli *et al.*, 2010). Again, the Bayesian phylogeny estimate obtained for the *M. hemimelaena* complex supports this broad conclusion because it fits a general pattern shown by several other avian lineages, whereby populations from the Brazilian shield are monophyletic, but those from the Solimões sedimentary basin are paraphyletic (Aleixo & Rossetti, 2007).

The timing and mode of the diversification of the *M. hemimelaena* complex is consistent with the most recent models based on geological and palaeontological evidence proposed for the historical development of the Amazon drainage (Espurt *et al.*, 2010; Latrubesse *et al.*, 2010; Mora *et al.*, 2010). According to these models, the continuous subduction of the Nazca Ridge under the South American

Plate during the Miocene caused the uplift of the Fitzcarrald Arch located in the Acre basin distributed in Brazil and Peru during Pliocene times (Espurt *et al.*, 2010). This, in turn, prompted the Amazonian foreland basins located on the eastern Andean foothills in Bolivia, Brazil, Peru, Ecuador and Colombia to evolve between the late Pliocene and early Pleistocene from a depositional to a predominantly erosional state, draining sediments eastwards and hence creating the modern trans-continental Amazon drainage flowing towards the Atlantic (Espurt *et al.*, 2010; Latrubesse *et al.*, 2010; Mora *et al.*, 2010; see also Campbell *et al.*, 2006). This major continental-wide drainage reorganization was responsible for the modern configuration of most Amazonian rivers, including those located far away from the sub-Andean foreland basins, such as the Tapajós, which also drains in an eastward direction, as do those major tributaries whose sources are located well into the foreland basins (e.g. Negro, Amazon/Marañon/Solimões and Madeira rivers). Thus, the first split in the *M. hemimelaena* complex involving *M. castanea* and *M. h. hemimelaena*, *M. h. pallens* and *M. taxon novum* and dated as of late Pliocene in age (Fig. 4) is consistent with this model of drainage formation. The fact that the Marañon River appears not to have acted as a primary barrier of diversification between *M. castanea* and the remaining species in the *M. hemimelaena* complex should not be interpreted as evidence undermining the riverine barrier hypothesis because populations of those two lineages co-occurring nowadays south of the Marañon are not sisters and either one or both could have dispersed more recently into this area across the river after a long period of differentiation in isolation (see above; Fig. 1). Currently, the entire Amazon and most of the Marañon rivers delimit the ranges of *M. castanea* and *M. hemimelaena* in northern Peru (Isler *et al.*, 2002; Zimmer & Isler, 2003), so their split could still be associated with the formation of a proto-Amazon river following the separation of the Amazonian foreland basins into a northern and a southern Amazonian foreland basin after the uplift of the Fitzcarrald Arch during the Pliocene (Espurt *et al.*, 2010).

Similarly, the splits involving the remaining species in the *M. hemimelaena* complex seem to be associated with the formation of the modern Madeira River basin following a major continental-wide drainage reorganization of the Amazon Basin after the establishment of the Fitzcarrald Arch. Exact dates for the establishment of the modern Madeira River basin are still uncertain but have not been thought of as older than the late Pliocene (Westaway, 2006), and thus the establishment of the basin could theoretically precede the major splits across the Madeira River between *M. h. hemimelaena* and *M. h. pallens/M. taxon novum* by c. 1 Myr (Fig. 4). Clearly, the absence of more detailed data-rich models accounting for the temporal development of the Madeira drainage prevents a more accurate assessment of the congruence between the ages of the estimated splits in the *M. hemimelaena* complex and the formation of the modern Madeira River basin. However, the documentation of two separate active large mega-fans of suggested late Pleistocene age involving both the Jiparaná and

the Aripuanã drainages (Latrubesse, 2002) is indicative of palaeodrainages in this interfluvium that are much wider and more complex than those found today. This history may explain patterns of primary diversification in this sector of Amazonia involving the *M. hemimelaena* complex as well as other avian and even primate lineages (van Roosmalen *et al.*, 1998; Sardelli, 2005; Fernandes, 2007). A second non-exclusive and somewhat related explanation could also involve tectonically mediated mega-drainage capture as shown for the Negro River (Almeida-Filho & Miranda, 2007), whereby either the Jiparaná or Aripuanã once captured a significant part of the Madeira or even the neighbouring Tapajós drainages or both, but later became mere tributaries following the major continental-wide drainage reorganization of the Amazon Basin during the late Pliocene (see also Willis, 1969). When applied to the Madeira drainage, future studies with a perspective similar to that in Almeida-Filho & Miranda (2007) should clarify the causes of the seemingly disproportional riverine barrier effect verified for the *M. hemimelaena* complex and other vertebrate lineages across the Aripuanã and Jiparaná rivers.

Unlike the riverine barrier hypothesis, the refuge hypothesis makes no spatial prediction about the location of splitting events involving lineages isolated in different climatic refuges (Patton & da Silva, 1998), but populations under this model of evolution are expected to show signs of demographic fluctuations during the Pleistocene (Zink, 1997; Hewitt, 1999; Aleixo, 2004; Cheviron *et al.*, 2005) and even before that (Haffer, 1997b), such that the temporal predictions of this hypothesis are very vague. As discussed above, when neutrality tests and BSPs were interpreted together, at least two lineages on the *M. hemimelaena* complex showed clear signs of demographic changes during the Pleistocene. Furthermore, it was possible to find a temporal match between the historical demographic dynamics of the most thoroughly sampled subspecies (*M. h. hemimelaena*) and glacial and interglacial cycles (Head & Gibbard, 2005). However, all these intense demographic fluctuations detected for *M. h. hemimelaena* and *M. h. pallens* during the Pleistocene post-dated their differentiation as separate species and appeared not to have contributed to most splitting events in the *M. hemimelaena* complex. Two possible exceptions, whose earliest 95% posterior age intervals fall into the time frame of the reconstructed historical population dynamics by the BSPs, could be the splits among three separate populations of *M. h. hemimelaena* (a, b and c) inhabiting different parts of western Amazonia between the Mamoré/Madeira and Amazon/Marañon rivers (Figs 1 & 4). However, mean time estimates for those splits are older than the BSP time frames (Figs 4 & 5), and their wide 95% posterior age intervals prevent a more detailed correlation with specific glacial/interglacial times. Thus, as also verified for two independent lineages of Amazonian birds, it appears that historical demographic dynamics detected in the *M. hemimelaena* complex during the mid-early Pleistocene were not accompanied by intense cladogenesis, as predicted by the refuge hypothesis (Aleixo, 2004; Ribas *et al.*, 2012).

As discussed above, the spatial and temporal nature of splitting events leading to major lineage differentiation in the *M. hemimelaena* complex is more easily explained by the formation of the modern Amazon River drainage during the late Pliocene. The data and interpretations presented herein provide evidence that tectonic activity leading to major drainage reorganization during the late Pliocene/early Pleistocene may have been an important driver of speciation in Amazonia.

Conservation implications

The phylogeography of the *M. hemimelaena* complex corroborates the existence of a new species restricted to the mini-interfluvium Jiparaná/Aripuanã. This agrees with previous studies suggesting that smaller rivers can delimit the distributions of birds and other vertebrates in Amazonia, and that this seems to be a common pattern rather than an exception. At least two other apparently new avian taxa have their distributions bounded by the Jiparaná and Aripuanã rivers. The warbling antbird complex (*Hypocnemis* spp., Thamnophilidae; Isler *et al.*, 2007; Tobias *et al.*, 2008) has vocally, morphologically and genetically distinct populations separated by these two rivers, and an undescribed taxon of the genus *Herpsilochmus* (Thamnophilidae) is also restricted to this interfluvium (A.M.F. and A.A., pers. obs.; M. Cohn-Haft, pers. comm.). The bar-breasted piculet (*Picumnus aurifrons*) shows distinct plumage coloration on opposite banks of the Aripuanã River (Cohn-Haft *et al.*, 2007). Genetic differentiation among morphologically indistinguishable populations bounded by the Jiparaná and Aripuanã rivers has also been described for Snethlage's tody-tyrant (*Hemitriccus minor*; Sardelli, 2005) and the wedge-billed woodcreeper (*Glyphorhynchus spirurus*; Fernandes, 2007). These two rivers also delimit the ranges of several taxa of primates (van Roosmalen *et al.*, 1998) and butterflies (Hall & Harvey, 2002). Therefore, the Jiparaná/Aripuanã interfluvium harbours a cryptically endemic fauna that is being accurately described only now with modern tools such as genetic and bioacoustic analyses that were unavailable a few decades ago. This has important conservation implications because this region is currently suffering high rates of deforestation (Fearnside, 2002, 2006). As discussed by Aleixo (2009) and implied by various previous studies (Cohn-Haft *et al.*, 2007; Fernandes, 2007), conservation policies should take taxonomic uncertainties into account in delimiting proper conservation targets. Disregarding the significance of entire cryptically diverse sectors of Amazonia under the current high anthropogenic development pressure may lead to the irretrievable loss of valuable unrecognized species.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 Collection locality, voucher number, institution of origin, and GenBank accession numbers for *Myrmeciza hemimelaena* and *M. castanea* samples used in this study.

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