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Continental-scale analysis reveals deep diversification within the polytypic Red-crowned Ant Tanager (*Habia rubica*, Cardinalidae) [☆]



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ABSTRACT

We explored the phylogeographic patterns of intraspecific diversity in the Red-crowned Ant Tanager (*Habia rubica*) throughout its continent-wide distribution, in order to understand its evolutionary history and the role of evolutionary drivers that are considered to promote avian diversification in the Neotropics. We sampled 100 individuals of *H. rubica* from Mexico to Argentina covering the main areas of its disjunct distribution. We inferred phylogenetic relationships through Bayesian and maximum parsimony methodologies based on mitochondrial and nuclear markers, and complemented genetic analyses with the assessment of coloration and behavioral differentiation. We found four deeply divergent phylogroups within *H. rubica*: two South American lineages and two Mexican and Middle American lineages. The divergence event between the northern and southern phylogroups was dated to c. 5.0 Ma, seemingly related to the final uplift of the Northern Andes. Subsequently, the two South American phylogroups split c. 3.5 Ma possibly due to the development of the open vegetation corridor that currently isolates the Amazonian and Atlantic forests. Diversification throughout Mexico and Middle America, following dispersion across the Isthmus of Panama, was presumably more recent and coincident with Pleistocene climatic fluctuations and habitat fragmentations. The analyses of vocalizations and plumage coloration showed significant differences among main lineages that were consistent with the phylogenetic evidence. Our findings suggest that the evolutionary history of *H. rubica* has been shaped by an assortment of diversification drivers at different temporal and spatial scales resulting in deeply divergent lineages that we recommend should be treated as different species.

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1. Introduction

The American continent possess the highest avian diversity of the world with over 4000 breeding species, more than 40% of the birds of the planet. However, this richness is disproportionally distributed among its two biogeographic regions: the Neotropics harbors 3370 species, over four times the Nearctic avifauna (732 bird species; Newton, 2003). This remarkable diversity, in association with a complex topography and an intricate array of highly varied and contrasting habitats, makes the Neotropics an exceptional

region for the study of the factors and processes that have promoted avian diversification.

One of the most traditional explanations that addresses the diversification of Neotropical taxa is the refuge hypothesis (Haffer, 1969), which proposes that genetic differentiation could be driven by the fragmentation of populations into remnant forest patches (i.e. the refuges) during the climatic oscillations associated with Quaternary glacial cycles. However, the refuge model has gradually lost its initial support as the main reason behind South American avian diversity (Batalha-Filho et al., 2012; Bush and Oliveira, 2006; Cheng et al., 2013; Lessa et al., 2003; Ribas et al., 2012). Currently, this hypothesis is mostly evoked as one of the factors promoting diversification below the species level (e.g. Cabanne et al., 2008) and as an explanation for the relatively low proportion of taxa with Pleistocene origin in the region (Lijtmaer

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et al., 2011; but see Rull (2015) for a comment on why Pleistocene speciation does not necessarily always mean refuge speciation). This contrasts with the evident relevance of glacial cycles in the Nearctic, where the direct advance of glacial ice sheets promoted vicariant speciation, resulting in a higher proportion of recently diverged species with limited phylogeographic structuring (Lijtmaer et al., 2011; Lovette, 2005). In southern Mexico and Middle America, Pleistocene climatic fluctuations, coupled with the particularly complex topography of the region, also seem to have played a major role in avian diversification due to the expansion and contraction of humid and dry habitats (Escalante et al., 1993; Smith et al., 2012; Weir, 2009).

Other factors have gained much more support and attention as key evolutionary drivers in the Neotropics, which could have acted alone or in combination with Pleistocene climatic oscillations. Among these factors is the orogeny of the Andes, which constitute a tremendous barrier to gene flow between lowland populations on each side of the mountain range (Fernandes et al., 2014; Miller et al., 2008; Weir and Price, 2011), but can also promote divergence between highland and lowland taxa and diversification of montane species (Gutiérrez-Pinto et al., 2012; Valderrama et al., 2014; Weir, 2006, 2009). In addition to the Andes, other factors that have been proposed as diversification agents include the establishment of the Amazonian drainage system and the effect of riverine barriers (Fernandes et al., 2014; Ribas et al., 2012), the closure of the Isthmus of Panama (Smith and Klicka, 2010; Weir et al., 2009), and the Neogene and Quaternary marine incursions (Nores, 1999).

Organisms with continent-wide distribution can be useful in providing insights into the tempo and mode of biodiversity generation since they allow assessing the impact of various diversification drivers at different spatial and temporal scales (e.g. Fuchs et al., 2011; Harvey and Brumfield, 2015; Smith et al., 2014; Weir and Price, 2011). In this context, the Red-crowned Ant Tanager (*Habia rubica*) constitutes an exceptional model for the study of avian diversification in the Neotropics. This species inhabits the understory and midlevel vegetation of lowland evergreen forests throughout a widespread but disjunct distribution comprising four major areas (Fig. 1): (1) southeastern Mexico and Middle America; (2) southwestern Mexico; (3) east of the Andes covering the Amazon Basin and the transition zone between the Andean Yungas and the Amazonian forest; and (4) the Atlantic Forest in eastern South America (Hilty, 2011). Here we investigate the patterns of intraspecific differentiation within *H. rubica*, which is also one of the most polytypic passerines in the Neotropics with 17 subspecies, several of which are only weakly differentiated and hard to recognize (Hilty, 2011). The species broad and disjunct distribution, together with the strikingly high number of subspecies described, suggests that hidden diversity within this species may exist. We therefore use genetic analyses based on a geographically comprehensive sampling of the species distribution, coupled with complementary vocalization and plumage coloration analyses, to elucidate the species evolutionary history and to understand the role of different factors that are thought to promote avian diversification in the region.

2. Materials and methods

2.1. Taxon sampling

We analyzed a total of 100 specimens of *H. rubica* from 48 collection sites corresponding to 27 major sampling localities (country states, departments or provinces) which cover the main disjunct areas of its distribution (Fig. 1; Appendix Table A1). Samples of *H. fuscicauda*, *Piranga flava*, and *Ramphocelus carbo* were

included as outgroups based on Klicka et al. (2007). Subspecies were determined according to their distribution range as detailed in Hilty (2011) and also following Navarro-Sigüenza and Peterson (2004) for Mexico and Middle America in particular. Our sampling covers seven subspecies of *H. rubica* (Hilty, 2011; Fig. 1), with most of the remaining subspecies being endemic to Costa Rica, Trinidad and small isolated areas of Venezuela, which precluded us from obtaining samples.

2.2. Laboratory procedures

Genomic DNA was obtained from muscle and blood tissue samples following a glass fiber-based extraction protocol developed by Ivanova et al. (2006). We amplified two mitochondrial genes [cytochrome *c* oxidase subunit I (COI, 694 bp), and cytochrome *b* (cyt *b*, 1049 bp)] and two nuclear introns [intron 5 of the autosomal β -fibrinogen gene (FIB5, 530 bp) and intron 9 of the Z-linked low density lipoprotein receptor gene (VLDL9R, 393 bp)]. See Appendix B for details about primers and thermocycling conditions. GenBank accession numbers for all sequences generated in this study are provided in Appendix Table A1.

Sequences were edited and aligned using CodonCode Alligner 4.0.4 (CodonCode Corporation, Dedham, MA). Heterozygous positions in the nuclear sequences were coded with the appropriate IUPAC ambiguity code (unphased dataset). We inferred haplotypes for the nuclear loci (phased dataset) using Phase 2.1 (Stephens and Donnelly, 2003) under default settings. Sites with assignment probabilities <0.80 were coded as missing data. The PHI test (Bruen et al., 2006) implemented in the software SplitsTree4 (Huson and Bryant, 2006) was used to verify the lack of recombination in the nuclear markers ($p > 0.05$ for both loci).

2.3. Phylogenetic analyses

Phylogenetic analyses were first performed on each marker separately and then on three concatenated datasets: (1) a mitochondrial dataset (mtDNA: COI + cyt *b*) with 116 individuals, (2) a nuclear dataset (nuDNA: FIB5 + VLDL9R) with 106 individuals, and (3) a multi-gene dataset (mtDNA + nuDNA) with 116 individuals with at least three markers sequenced. Since the results were highly congruent, we show only the topologies obtained from the concatenated datasets. Because previous molecular evidence suggested that *H. rubica* is closely related to species of the genus *Chlorothraupis* (Klicka et al., 2007; Barker et al., 2015), we mined from GenBank (www.ncbi.nlm.nih.gov) cyt *b* sequences of *Chlorothraupis carmioli*, *C. stolzmanni*, and *Habia gutturalis* to better assess the monophyly of *H. rubica*.

Phylogenetic gene trees were inferred through Bayesian methodology using MrBayes 3.2.2 (Ronquist et al., 2012). We conducted two independent runs of 10 million generations sampling trees every 100 generations under default priors for all parameters. The standard deviation of split frequencies between runs was always <0.01 indicating convergence. We assured that both runs reached stationarity and that we had a good sample of the posterior probability distributions using Tracer 1.5 (Rambaut and Drummond, 2007). We discarded the first 25% of the sampled trees as burn-in and the remaining 75,000 trees of each run were combined to generate a 50% majority rule consensus tree. We selected the best-fit model of nucleotide substitution for each locus prior to the analysis using the Bayesian information criterion (BIC) implemented in jModelTest 2.1.1 (Darrriba et al., 2012): HKY + G + I for COI, HKY + G for cyt *b*, and HKY for FIB5 and VLDL9R. All loci were placed in unlinked partitions allowing parameters to vary and to be estimated independently (except for topology and branch lengths). Gaps were coded as binary characters and placed in a separate partition.

To assess the sensitivity of inferred relationships to the reconstruction algorithm implemented, we performed a maximum parsimony (MP) analysis in TNT1.1 (Goloboff et al., 2003). We ran heuristic searches based on 1000 random addition sequences (RAS) coupled with the tree bisection reconnection (TBR) branch-swapping algorithm, saving 10 trees per replication. A strict consensus tree was estimated from all most parsimonious trees. To estimate node support we conducted a bootstrap analysis that consisted in 1000 pseudoreplicates of 100 RAS + TBR saving 10 trees per replicate.

Based on the analysis of the mtDNA and the multi-gene datasets we defined four reciprocally monophyletic and deeply divergent lineages to which we will refer throughout the manuscript. Further analyses are based on these lineages, with the exception of the clade from southwestern Mexico that had to be excluded from most of the analyses due to its small sample size ($n = 2$).

2.4. Genetic diversity, population structure and demographic signature

Uncorrected genetic distances among all individuals were estimated with MEGA 5.0 (Tamura et al., 2011) and used in a principal coordinate analysis (PCoA) performed in GenAlEx 6.5 (Peakall and Smouse, 2012). We conducted analyses of molecular variance (AMOVA) in Arlequin 3.5 (Excoffier and Lischer, 2010) with individuals grouped by sampling locality or subspecies within major phylogroups. The Φ -statistics were computed using uncorrected genetic distances and significance was tested through 2000 random permutations. The number of haplotypes (k), and the nucleotide (π) and haplotype (h) diversities of the main clades were estimated in DNASP 5.10 (Librado and Rozas, 2009). Results obtained with the phased and unphased nuclear datasets were almost identical, so we report only the former.

We used PopART 1.0 (<http://popart.otago.ac.nz>) to generate unrooted statistical parsimony mtDNA networks (Templeton et al., 1992) within major clades. We also tested the existence of isolation-by-distance with a Mantel test carried out at the individual level using a multi-gene distance matrix. The tests were performed in GenAlEx and significance was evaluated with 999 permutations.

To look for signs of population expansion or contraction within major clades, we calculated Tajima's D (Tajima, 1989) and Fu's F_s (Fu, 1997) statistics in DNASP. Significance was assessed by 10,000 coalescent simulations. We also estimated the exponential population growth parameter (g) using LAMARC 2.1.9 (Kuhner, 2006) in maximum likelihood mode (more details in Appendix B).

2.5. Species tree and divergence time estimation

We used *BEAST (Heled and Drummond, 2010) as implemented in BEAST 1.8.0 (Drummond et al., 2012) to estimate a time-calibrated species tree. We selected a subset of 54 individuals to be included in this analysis in order to reduce computational time but still sample all the genetic variation within and among lineages. Individuals were grouped by subspecies, which were in all cases represented by more than one individual (except for the subspecies *rhodinolaema* for which we counted with only one sample). All markers were placed in separate partitions under the models of nucleotide substitution selected with jModelTest. Substitution, clock and tree models were unlinked across partitions, with the exception of the tree models of mitochondrial loci which were linked. We specified a Yule speciation tree prior, a piecewise linear and constant root population size model, and a relaxed uncorrelated lognormal clock for each gene tree. For calibration we used four different substitution rates, one for each marker, and ran the analysis for 100 million generations sampling every 1000 generations (see Appendix B for details). We checked

for stationarity and adequate ESS values using Tracer 1.5. We discarded the first 10% of the sampled trees as burn-in and then estimated the 95% highest posterior density (HPD) intervals of divergence dates using TreeAnnotator 1.8 (Drummond et al., 2012). HPD intervals were summarized on the maximum clade credibility tree obtained with the same program using mean node heights. Finally, we used DensiTree 2.01 (Bouckaert, 2010) to generate a cloudogram resulted from the superimposition of a subsample of 18,000 post-burn-in trees.

2.6. Song analyses

We analyzed vocalizations of 79 males of *H. rubica* from the subspecies *rubicoides*, *peruviana*, *hesterna*, *rhodinolaema*, and *rubica*, representing three of the four major genetic lineages (Fig. 1; Appendix Table A2). All recordings were in 'wav' format and spectrograms were generated and analyzed using RavenPro 1.4 (<http://www.birds.cornell.edu/raven>). The conditions for the analyses were a 'Hamming' window type, a 512 fast Fourier transform length with 50% overlap, and a 'grayscale' color scheme. We analyzed one song per individual favoring the best signal-to-noise ratio and measured 11 variables on each spectrogram (five temporal variables, four frequency variables, the number of inflections, and the inflection rate; see Appendix B for details).

We evaluated differences of song variables among major phylogroups through a principal components analysis (PCA). Some of the principal components (PCs) extracted did not meet the assumptions of homoscedasticity and normality, so we used the nonparametric Kruskal–Wallis test followed by Mann–Whitney U -tests to assess differences among lineages in their PCs scores. All variables were standardized previous to the analyses and we considered that variables were significantly correlated with a PC only when their |factor loadings values| were ≥ 0.7 .

2.7. Coloration analyses

The avian visual system is remarkably different from that of humans. Among several dissimilarities, birds possess a fourth visual cone sensitive to short wavelengths in their retinas (300–700 nm) that makes them able to see ultraviolet (UV) colors (Cuthill et al., 2000). This, coupled with the presence of oil droplets (Bowmaker et al., 1997), also allows the birds visual system to have a higher color definition making plumage coloration assessments from a human perspective biologically inappropriate (Cuthill et al., 2000; Eaton, 2005). Given this, it is necessary to employ objective methods such as reflectance spectrophotometry to describe plumage coloration (Montgomerie, 2006) and models of avian visual perception to determine its potential as a signal in birds' interactions (Endler and Mielke, 2005; Stoddard and Prum, 2008; Vorobyev and Osorio, 1998).

In order to objectively describe plumage color variation in *H. rubica* we took reflectance spectra measurements on museum skins deposited at the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia". We measured 14 male and 13 female specimens of the subspecies *rubica*, three male and three female specimens from *peruviana*, and one specimen of each sex from *rubicoides*. This sample represents three of the four main genetic lineages (Fig. 1; Appendix Table A3). Reflectance spectra were obtained for four plumage patches (crown, back, throat and chest) and analyzed with the 'pavo' package (Maia et al., 2013) in R 3.1.1 (R Development Core Team, 2014) in order to estimate a perceptual distance (ΔS) in the avian color space between all individuals under the Vorobyev and Osorio (1998) visual model (see Appendix B for a detailed explanation). ΔS is expressed in terms of just noticeable differences (jnd) and values larger than 2 jnd are

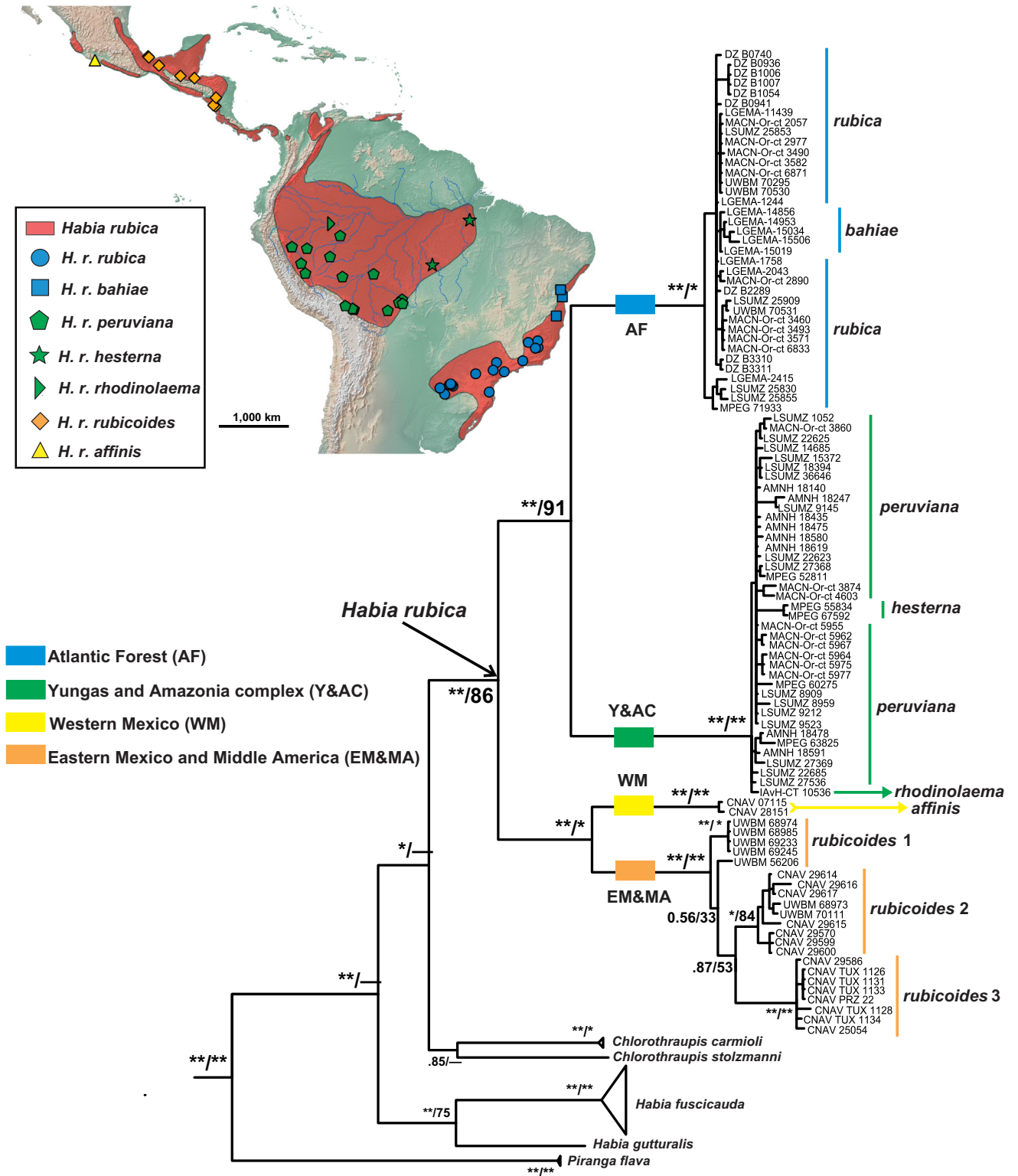


Fig. 1. Bayesian majority rule consensus tree obtained from the analysis of 1743 bp of the concatenated mitochondrial dataset (mtDNA: COI + cyt b) showing four major clades recovered within *Habia rubica*. Subspecies are indicated to the right. “” indicates maximum node support of Bayesian posterior probability (PP) of 1.0 and maximum parsimony bootstrap (MP) of 100%; “” denotes PP ≥ 0.95 and MP ≥ 95%; other values are indicated with numbers. Support values from most of the internal nodes were omitted for clarity. The tree was rooted with *Ramphocelus carbo* (not shown for simplicity). The map on the upper left shows the distribution of *H. rubica* and the collection sites. The colors of the symbols represent the major phylogroups. Distribution was based on BirdLife International and NatureServe (2013). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

considered to represent significant color discrimination by the birds in all light conditions (Eaton, 2005). We calculated average ΔS values per patch for pairwise comparisons among genetic lineages and for comparisons performed within them. Even though the use of ΔS values is the most appropriate way to describe the perception of color differences by birds, this value does not measure the specific aspects of plumage color that generate such distance. Therefore, in addition to the ΔS values we calculated five spectral variables for each plumage patch which are typically used to describe plumage color (more details in Appendix B). These variables were analyzed with a PCA as described above. Since the species is sexually dichromatic, males and females were analyzed separately. All statistical analyses were performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA) and were two-tailed.

3. Results

3.1. Phylogenetic analyses

Bayesian and MP analyses of the mtDNA, nuDNA and multi-gene datasets recovered *H. rubica* as a monophyletic species with high support [posterior probability (PP) of 1.0 and MP bootstrap support from 86% to 99%; Fig. 1, Appendix Figs. C1–C3]. The Bayesian analysis of the mtDNA suggested also that the species is more closely related to *Chlorothraupis* species than to its congeners (Fig. 1), as previously noted by Klicka et al. (2007). Paraphyly of *Habia* was supported with moderate to high PP in the Bayesian tree (Fig. 1), whereas relationships among *Habia* and *Chlorothraupis* species remained unsolved in the MP analysis (Appendix Fig. C3).

Four highly supported and deeply divergent mitochondrial phylogroups were recovered within *H. rubica* matching the main regions of the species distribution (Fig. 1): two northern sister clades, one distributed throughout southeastern Mexico and Middle America (hereafter EM&MA clade) and the other one with individuals sampled in southwestern Mexico (WM clade); and two southern sister clades, one that comprised all individuals sampled in the Atlantic Forest (AF clade), and the other one that grouped all samples from the Yungas and Amazonia complex (Y&AC clade). These phylogroups were also recovered in the nuDNA and multi-gene phylogenies (Appendix Figs. C1 and C2), with some differences in the former: (1) the WM and EM&MA lineages were not differentiated and appeared as a single northern lineage, and (2) this northern lineage was placed as sister to the Y&AC clade.

No subspecies is shared among these major clades but more than one subspecies is comprised within some of them (Fig. 1). The Y&AC clade represents the subspecies *peruviana* (western Amazonia south of the Amazon River), *hesterna* (eastern Amazonia south of the Amazon River) and *rhodinolaema* (western Amazonia north of the Amazon River), while the nominal race *rubica* (southern and central Atlantic Forest) and the subspecies *bahiae* (north of the Atlantic Forest) are clustered within the AF phylogroup. The EM&MA and WM lineages represent the subspecies *rubicoides* and *affinis*, respectively. Three distinct *rubicoides* groups were recovered within the EM&MA clade based on the mtDNA and multi-gene datasets (Fig. 1, Appendix Fig. C2): *rubicoides* 1 from Nicaragua, *rubicoides* 2 from Nicaragua and Guatemala, and *rubicoides* 3 from Guatemala and Mexico. These *rubicoides* groups were consistently recovered across almost all topologies other than those obtained from nuclear markers (Appendix Fig. C1), with the sole exception of one sample (UWBM 56206) from Granada (Nicaragua) which relationship with the other groups was observed to vary across datasets. However, this individual appeared more closely related to the *rubicoides* 1 group in the statistical parsimony analyses (see below) and in

the multi-gene tree, and was therefore considered a member of this group for further analyses.

Mean uncorrected genetic distances among major phylogroups ranged from 5.8% to 8.0% for the mtDNA, from 0.6% to 1.0% for FIB5, and from 0.3% to 0.8% for VLDL9R (Table 1). As expected, genetic distances were higher among major clades than within them (Table 1; Appendix Fig. C4), which resulted in the clustering pattern observed in the PCoA (Fig. 2). Except for one almost significant comparison ($p = 0.052$), all pairwise Φ_{st} values among major phylogroups were significant and ranged from 0.77 to 0.96 for the mtDNA, from 0.29 to 0.96 for FIB5, and from 0.28 to 1 for VLDL9R (Table 1). In general, only a small proportion of the total variation was explained by differentiation among subspecies or sampling localities within major phylogroups, being most of the variation attributable to differences among the main clades (Table 2).

3.2. Genetic diversity and population structure

Genetic diversity was similar between the two South American lineages and higher in the EM&MA clade with nucleotide diversity (π) up to one order of magnitude higher (Table 3). The number of haplotypes (k) and the haplotype diversity (h) of the mtDNA were similar among these three phylogroups, but they were higher in the EM&MA clade for the nuclear loci. Genetic distances within the EM&MA phylogroup were also higher than in the other clades (Table 1; Appendix Fig. C4).

The statistical parsimony analysis of the EM&MA clade found three clearly distinct mitochondrial haplogroups (*rubicoides* 1, 2, and 3; Fig. 3) that matched the lineages found within this clade in the mtDNA and multi-gene trees. Differentiation among these groups accounted for a significant proportion (88.7%) of the total variation for the mtDNA dataset but less than 30% for the nuclear loci (nevertheless, this proportion was significant). Consistently, these groups were not distinguishable in the phylogenetic analyses of the nuDNA dataset (Appendix Fig. C2). Mean uncorrected genetic distances and pairwise Φ_{st} values among these haplogroups were higher for the mtDNA than for the nuclear loci (Appendix Table A4). Similarly, differentiation among localities explained a high and significant proportion of the total variation at the mtDNA (74.25%), but only a small but yet significant percentage at the nuclear loci (less than 40%; Appendix Table A5; Appendix Fig. C5a). Finally, we found a high and statistically significant signal of isolation-by-distance (Appendix Fig. C5b).

We observed a star-like mtDNA network (a common sign of recent population expansion) within the Y&AC phylogroup, in which only the subspecies *hesterna* appears to be differentiated (Fig. 3). Consistently, genetic divergence at the mtDNA was higher between *hesterna* and the other two subspecies, *peruviana* and *rhodinolaema*, than between these two (Appendix Table A6). In contrast, no clear differentiation was found at the nuclear loci (Appendix Table A6; Appendix Fig. C2). We found no signs of geographic structure (Appendix Table A7; Appendix Fig. C6a) other than a low but statistically significant signal of isolation-by-distance (Appendix Fig. C6b). In fact, only a small but significant percentage of the variation was explained by differentiation among sampling localities (7.6% for the mtDNA and 12.2% for FIB5; no variation was found at VLDL9R).

The network obtained for the AF clade showed relatively low variability (Fig. 3) and no signs of geographic structure (Appendix Table A8; Appendix Fig. C7a). Genetic variation within sampling localities (70.1% for the mtDNA, 95.4% for FIB5, and 70.9% for VLDL9R) was much higher than among them (even though the latter was statistically significant for all loci other than FIB5). Consistently, we found only a markedly weak but still significant sign of isolation-by-distance (Appendix Fig. C7b). No clear

Table 1

Pairwise comparisons among the four major lineages based on the concatenated mtDNA (COI + cyt *b*) and the nuclear loci (FIB5 and VLDL9R). Above the diagonal: pairwise Φ_{st} values; significant values ($p < 0.05$) in bold. Diagonal: mean uncorrected genetic distances within each lineage. Below the diagonal: mean uncorrected genetic distances between pairs of lineages. Genetic distances are expressed in percentage values.

Lineages	mtDNA				FIB5				VLDL9R			
	AF	Y&AC	EM&MA	WM	AF	Y&AC	EM&MA	WM	AF	Y&AC	EM&MA	WM
Atlantic Forest (AF)	0.30	0.95	0.90	0.96	0.02	0.74	0.65	0.96	0.10	0.93	0.61	0.95
Yungas and Amazonia Complex (Y&AC)	6.50	0.40	0.91	0.96	0.60	0.30	0.42	0.63	0.60	0.00	0.67	1.00
Eastern Mexico and Middle America (EM&MA)	8.00	8.00	1.50	0.77	1.00	0.80	0.70	0.29	0.70	0.70	0.40	0.28**
Western Mexico (WM)	7.90	7.90	5.80	0.10	1.00	0.80	0.90 ^a	0.30	0.80	0.80	0.30 ^a	0.00

^a Range of uncorrected genetic distances includes 0 (identical sequences).
 ** $p = 0.052$.

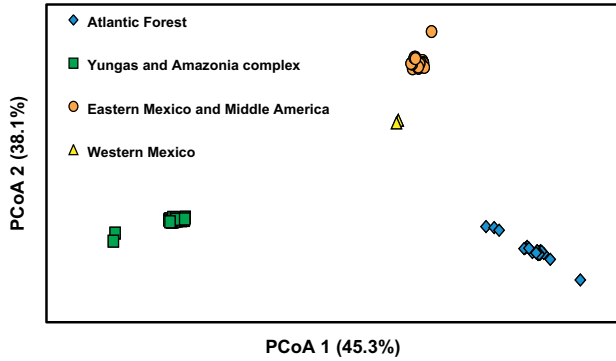


Fig. 2. PCoA derived from pairwise uncorrected genetic distances based on the concatenated mitochondrial and nuclear loci (COI + cyt *b* + FIB5 + VLDL9R). The proportion of the total variation explained by each principal coordinate is indicated in parenthesis.

differentiation was observed between the nominal race *rubica* and *bahiae* (Fig. 3; Appendix Table A9). Despite some significant pairwise Φ_{st} values between these subspecies for VLDL9R and the mtDNA, the variation within subspecies (80.7% for the mtDNA, 53.0% for VLDL9R, and 91.2% for FIB5) was higher than between them.

3.3. Demographic history

We found strong evidence of population expansion in the two South American clades (Table 3). In the Y&AC clade, Fu's F_s and Tajima's D values were negative and statistically significant for both FIB5 and the mtDNA (VLDL9R could not be evaluated because there were no polymorphic sites). In the case of the AF phylogroup, we found negative and significant F_s values and negative and almost significant ($p = 0.07$) D values for the mtDNA (F_s and D for nuclear loci were negative but non-significant). Additionally, the exponential growth parameter g was positive and statistically significant for both lineages, and the combination of high haplotype diversity and low nucleotide diversity observed in the mtDNA constitutes another sign of recent population expansion. On the

contrary, the EM&MA clade showed clear signs of population stability, with the sole exception of a negative and statistically significant F_s value for FIB5 (Table 3).

3.4. Species tree and molecular dating

The species tree analysis resulted in a fully resolved and time-calibrated ultrametric phylogeny (Fig. 4) that was coincident with the mtDNA and multi-gene trees. The cloudogram (Fig. 4) showed that most of the uncertainties in the tree were located close to the root, with the rest of the phylogeny having overall congruence.

The deepest split within *H. rubica* that separated the South American lineage from the Mexican and Middle American lineage was dated to the Miocene–Pliocene boundary c. 5.0 Ma (95% HPD: 3.4–6.2 Ma). Subsequently, the two South American clades diverged at c. 3.6 Ma (95% HPD: 2.3–4.9 Ma), while the two northern lineages diverged at c. 1.8 Ma (95% HPD: 0.5–3.1 Ma). The time to the most recent common ancestor of major phylogroups and divergence events within them were dated to the last million years for the EM&MA clade, and mostly to the last 500,000 years for the two South American clades (Fig. 4).

3.5. Song analyses

We were not able to obtain recordings from southwestern Mexico (WM clade), so comparisons were made among the other three major lineages. We extracted four PCs that explained almost 80% of the total variation in the song variables (Appendix Table A10). The Kruskal–Wallis performed on the PC scores showed the existence of statistically significant differences among major phylogroups for PC1 ($H = 9.42$, d.f. = 2, $p < 0.01$), PC3 ($H = 20.38$, d.f. = 2, $p < 0.001$) and PC4 ($H = 9.92$, d.f. = 2, $p < 0.01$); while no differences were found for PC2 ($H = 3.03$, d.f. = 2, $p = 0.22$). PC1 was significantly lower for the EM&MA clade than for the Y&AC clade ($U = 159$, $p < 0.01$). PC3 was significantly lower for the AF clade than for the Y&AC ($U = 157$, $p < 0.001$) and EM&MA ($U = 128$, $p < 0.001$) clades. PC4 was higher for the AF clade than for the Y&AC ($U = 260$, $p < 0.05$) and EM&MA ($U = 151$, $p < 0.01$) clades.

Table 2

Proportion of the total genetic variation explained by each hierarchical level specified in the AMOVA analyses using the concatenated mtDNA (COI + cyt *b*) and the nuclear loci (FIB5 and VLDL9R). Only sampling localities and subspecies with two or more individuals were considered in the analysis. Statistically significant ($p < 0.05$) values in bold. Negative values should be interpreted as zero.

Marker	Proportion of the variation (%)					
	Individuals grouped by sampling localities			Individuals grouped by subspecies		
	Among lineages	Among localities (within lineages)	Within localities	Among lineages	Among subspecies (within lineages)	Within subspecies
mtDNA	91.68	4.49	3.84	89.50	3.00	7.49
FIB5	59.38	4.73	35.89	63.14	-2.91	39.77
VLDL9R	72.25	10.56	17.19	71.90	2.37	25.73

Table 3
Summary statistics of the concatenated mtDNA (COI + cyt *b*) and the nuclear loci (FIB5 and VLDL9R) describing genetic diversity and demographic signature of three of the major lineages. The sample size (*n*), number of haplotypes (*k*), haplotype (*h*) and nucleotide (π) diversities, Tajima's *D*, Fu's F_s , and the global (mtDNA + FIB5 + VLDL9R) population growth factor (*g*) are indicated. Statistically significant ($p < 0.05$) *D* and F_s values in bold. Significance of *g* was based on confidence intervals.

	Lineages								
	Atlantic forest			Yungas and Amazonia complex			Eastern Mexico and Middle America		
	mtDNA	FIB5	VLDL9R	mtDNA	FIB5	VLDL9R	mtDNA	FIB5	VLDL9R
<i>n</i>	32	72	51	39	76	59	22	42	33
<i>k</i>	19	3	2	29	9	1	12	16	4
<i>h</i>	0.94	0.11	0.21	0.97	0.33	0	0.92	0.91	0.64
π	0.0032	0.00022	0.0059	0.0034	0.0009	0	0.0146	0.0060	0.0041
<i>D</i>	-1.35 ^a	-1.21	-0.06	-2.30	-2.06	-	0.82	0.78	1.32
F_s	-8.11	-2.31	0.41	-28.14	-7.60	-	2	-6.12	1.23
<i>g</i> (global)		5570.83 ^c			914.61 ^b			167.56 ns	

ns, not significant.

^a $p = 0.07$.

^b 95% confidence interval did not include 0.

^c 99% confidence interval did not include 0.

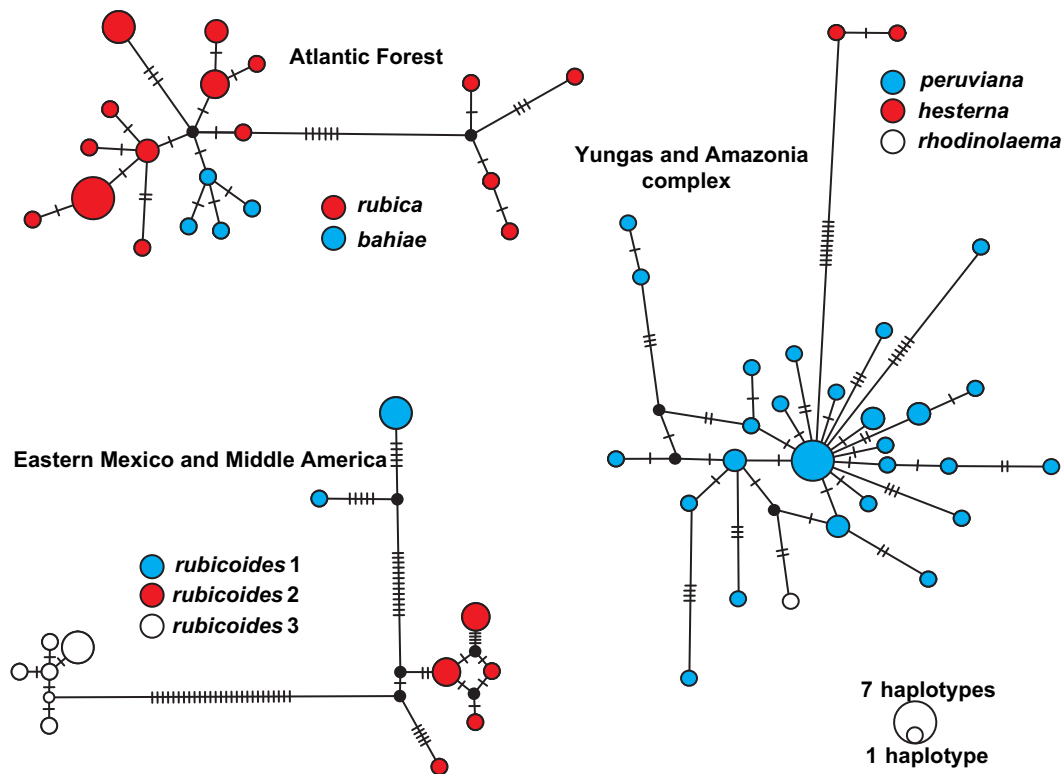


Fig. 3. Unrooted statistical parsimony networks of each of three major phylogroups of *Habia rubica* based on the concatenated mitochondrial dataset (mtDNA: COI + cyt *b*). The clade from southwestern Mexico was omitted because it has only two haplotypes that differ by a single mutational step. Colors of the haplotypes correspond to the subspecies found within major clades. For the EM&MA clade, the three *rubicoides* haplogroups are the same as in Fig. 1. Dashes represent the mutational changes and the length of the branches is proportional to the number of changes within each network. Circle sizes are proportional to the number of identical haplotypes and the small black dots indicate unobserved haplotypes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In this manner, significant differences were found between all pairs of phylogroups, with the vocalizations of the individuals from the AF clade being the most distinguishable, especially in their temporal aspects: they have longer songs with a higher number of notes that are separated by longer inter-note intervals, which is translated into a lower note rate. On the other hand, maximum frequencies of the songs of the individuals from the EM&MA clade are significantly lower than those from the Y&AC clade.

3.6. Coloration analyses

We were not able to analyze museum skins from southwestern Mexico (WM clade), so comparisons were made among the three

other major clades. We found almost all pairwise comparisons among lineages to have average ΔS values above 2.0 *jnd*, but coloration distances were in some cases higher within clades than among them (Appendix Tables A11 and A12). The throat in males and the chest in females, however, clearly showed more differentiation among lineages than within them (Appendix Tables A11 and A12).

We extracted three PCs that explained almost 70% of the total variation in the spectral variables of *H. rubica* males (Appendix Table A13). Due to the small sample size of the EM&MA clade, statistical tests were performed only between the two South American lineages. No significant differences between clades were detected in PC1 ($U = 21$, $p = 1.0$), but PC2 ($U = 3$, $p < 0.05$) and PC3

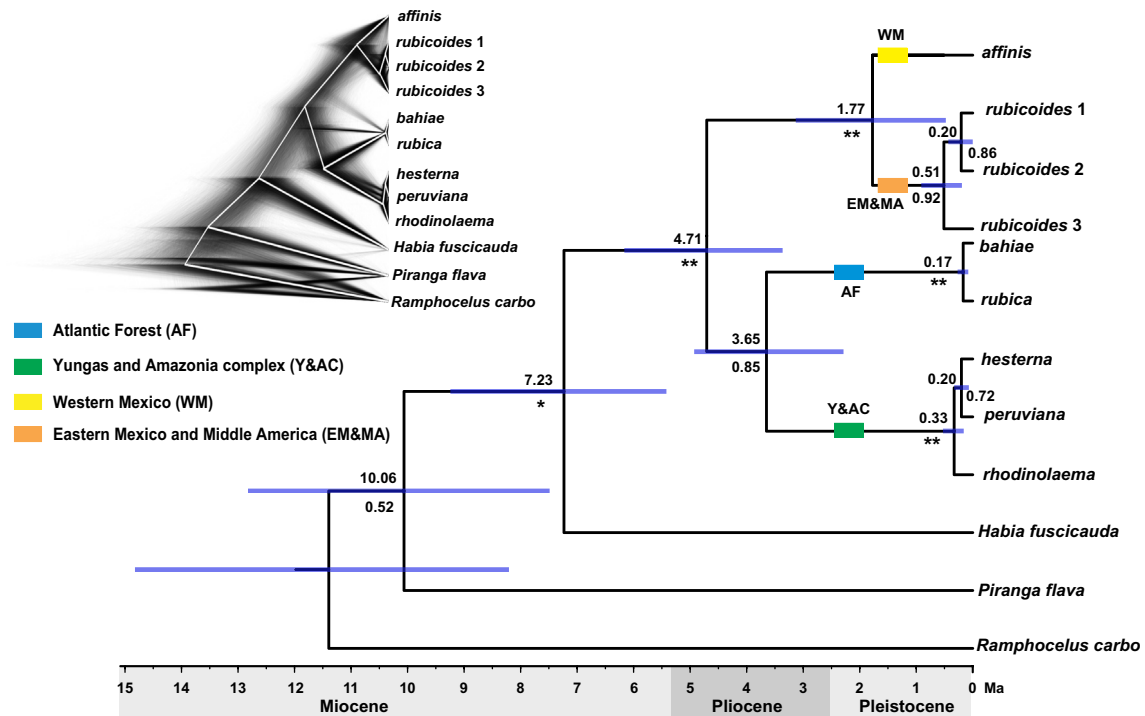


Fig. 4. Species tree reconstructed from the combined analyses of a subset of mitochondrial (concatenated COI + cyt *b*) and nuclear (FIB5 and VLDL9R inferred haplotypes) sequences (54 individuals). The tree shows the relationships among major clades within *Habia rubica*, which are represented by the subspecies that they include. The numbers above the nodes indicate the mean divergence time estimates and the bars correspond to the 95% highest posterior density intervals of those estimates. Divergence times and the scale bar are in millions of years ago (Ma). Posterior probability (PP) values are shown below the branches: ** indicates maximum support (PP = 1.0); * denotes PP \geq 0.95; other values are indicated with numbers. The inset (upper left) shows the cloudogram obtained from the superimposition of 18,000 post-burn-in gene trees, with the consensus topology shown in white and all other trees in gray (darker areas denote higher level of overlap).

($U = 2$, $p < 0.05$) were significantly higher for the Y&AC clade than for the AF phylogroup, meaning that there is a higher hue and a more saturated reddish coloration in the chest of the males of the former, and that their throats are brighter and also have a higher hue (Appendix Fig. C8). In the case of females two PCs accounted for 60% of the variation in the spectral variables (Appendix Table A14). PC1 was significantly higher ($U = 2$, $p < 0.05$) for the AF clade than for the Y&AC lineage, which means that females of the former have lower brightness in the throat and that the coloration of their chest is less bright with less UV and long wavelength chroma but with a higher hue and overall saturation (resulting in a more greenish color in the females of the AF clade; Appendix Fig. C9). PC2 did not show significant differences between groups ($U = 14$, $p = 0.5$).

4. Discussion

We investigated the phylogeographic history of *H. rubica* with a dense intraspecific sampling throughout its widespread distribution. Our results suggest the onset of a diversification process at the Miocene–Pliocene boundary that resulted in at least four deeply divergent lineages. We discuss below the different aspects of our findings in the context of the main factors that are thought to promote diversification in the Neotropics and debate their taxonomic implications.

4.1. Geographical origin of *H. rubica* and the split between the southern and northern lineages

The deepest split within *H. rubica* was dated to the Miocene–Pliocene boundary in the Neogene *c.* 5.0 Ma, being temporally coincident with the rapid uplift experienced by the Northern Andes

(Gregory-Wodzicki, 2000; Hoorn et al., 2010; Mora et al., 2008). We believe that the orogeny of this mountain range could have disrupted the distribution of *H. rubica* in South America and promoted vicariant diversification between the *cis*-Andean and *trans*-Andean populations. The *trans*-Andean population could have subsequently dispersed into Middle America and expanded northwards following the closure of the Isthmus of Panama at 4–2.7 Ma (Coates and Obando, 1996; Coates and Stallard, 2013; Leigh et al., 2014). Even though other studies suggest that the land bridge may have reached its current continuous form at some point in the Miocene (Montes et al., 2012; but see Carrillo et al., 2015 and references therein), most of the south to north dispersion events of birds across the Isthmus were associated with forest specialists like *H. rubica* and were accelerated around 3 Ma, continuing throughout the Pleistocene. On the other hand, interchange events before that time were mostly north to south and related with habitat generalists or dry habitat species (Smith and Klicka, 2010; Smith et al., 2012; Weir et al., 2009). This contrast in the temporal patterns of dispersion between habitat specialist and generalists, which could be related to the past dynamics of humid and dry environments across the Panamanian bridge (Smith and Klicka, 2010; Smith et al., 2012), supports the dispersion of *H. rubica* into Middle America after the basal split associated with the Northern Andes irrespective of the age of the Isthmus. Consistently, the orogeny of the Andes in concert with the crossing of the Isthmus of Panama have been proposed as the main diversification events in other groups of birds (Miller et al., 2008; Sweet and Johnson, 2015; Weir and Price, 2011). The South American origin of the species posited in this scenario is supported by the fact that most of its closest relatives from the genera *Habia* and *Chlorothraupis* are found in the Northern Andes area in northwestern South America.

Contrasting with the mtDNA and the multi-gene trees, the nDNA topology showed a sister relationship between the

Mexican and Middle American lineage (WM + EM&MA) and the Y&AC clade. This could be the result of lower resolution of the nuDNA, which is consistent with its relatively low node support values. Alternatively, this could indicate the existence of gene flow between these phylogroups. Considering the presumed limited dispersal ability of understory forest birds like *H. rubica* (Burney and Brumfield, 2009; Moore et al., 2008; Smith et al., 2014) and the fact that no mtDNA or nuclear haplotypes were shared among these phylogroups, this relationship could be indicating not current gene flow but instead past events of dispersal across or around the Eastern Cordillera and its bifurcations in Colombia and Venezuela (Miller et al., 2008; Weir and Price, 2011). The lack of a comprehensive sampling of the Northern Andes does not allow us to investigate further this possibility.

4.2. Diversification within South America

The split between the Y&AC and AF clades was dated to the Pliocene (c. 3.5 Ma), suggesting that the consolidation at some point in the Tertiary of the open vegetation corridor (the Caatinga, Cerrado and Chaco regions) that currently separates the Amazon and Atlantic forests could have been the vicariant event that resulted in the isolation of the two South American lineages. Alternatively, the Atlantic Forest could have been colonized by dispersion from the Amazon population after the establishment of this corridor (Costa, 2003; Harvey and Brumfield, 2015; Romo and Morrone, 2011).

After the establishment of the open vegetation corridor, the Amazonian and Atlantic forests have experienced cycles of connection and disconnection associated with Neogene and Quaternary geotectonic events and climatic oscillations. However, the exact spatio-temporal way in which these two forests have been in contact is still in debate: it could have been either through the Cerrado in Brazil (Remsen et al., 1991; Silva, 1994), the Chaco Region in Argentina and Paraguay (Nores, 1992), the Caatinga in extreme northeastern Brazil (Oliveira et al., 1999), or through a combination of these routes (Batalha-Filho et al., 2013a). Irrespective of which of these possibilities is correct, our results suggest that these cycles of connection and disconnection did not promote further dispersion of this species between these forests after their separation. Consistently, pre-Quaternary splits between the Amazonian and Atlantic forests have been found for other lineages of birds (Batalha-Filho et al., 2013a; Cabanne et al., 2008; Weir and Price, 2011).

On the other hand, Pleistocene climatic fluctuations and habitat shifts may have played a role in the diversification within these lineages following their split: both phylogroups lacked geographic structure and showed strong signature of recent population expansion. In the case of the Y&AC lineage, we did not find differences between the subspecies that occur to the north (*rhodinolaema*, Napo region of endemism) and south (*peruviana*, Inambari and Rondonia regions) of the Amazon River, but did find some degree of differentiation (in the mtDNA) between the subspecies to the east (*hesterna*, Pará region) and the west (*rhodinolaema* and *peruviana*) of the Amazon basin, a result consistent with those of previous studies with other bird species (Fernandes et al., 2014).

Regarding the Atlantic Forest, it has been hypothesized that two main forest refuges existed in the central and northern regions during the last glacial maximum (LGM; Carnaval and Moritz, 2008). Even though we were not able to include samples from the northern part of this region, our results do not support an effect of the putative central refuge on *H. rubica* since: (1) no phylogeographic breaks were observed; (2) genetic diversity within southern Atlantic Forest was similar or even higher than that of the central region (Minas Gerais and Bahia); and (3) we did not find higher levels of geographic structure in the central region

(Appendix Fig. C7a; Appendix Tables A15 and A16), contrary to what would be expected under the refuge model (Amaral et al., 2013; Batalha-Filho et al., 2013b; Cabanne et al., 2008). However, the absence of geographic structure and the signs of population expansion observed within this region suggest a potential effect of Quaternary climatic oscillations and geotectonic activity on the Atlantic Forest population. These results are highly congruent with those reported by Batalha-Filho et al. (2012) for the endemic warbler *Basileuterus leucoblepharus* and support the idea that Pleistocene glacial cycles have not affected all species in the same manner.

4.3. Diversification within Mexico and Middle America

The WM and EM&MA clades are currently isolated by the Mexican highlands and the arid high-elevation central Mexican Plateau in central and southern Mexico (Escalante et al., 1993; Weir, 2009). The divergence between these clades was deep for the mtDNA, but no clear differentiation was found in the nuDNA tree. This lack of differentiation could be explained by the low mutational rate and higher effective sample size of nuclear loci. If these phylogroups diverged recently, as suggested by our time estimates, shallow or absent differentiation in nuclear loci is expected, even in the absence of gene flow. Our results are consistent with a recurrent pattern of east–west deep divergence found among unrelated taxa in this region (e.g. Bryson et al., 2013; Chaves et al., 2013; McCormack et al., 2008). However, the possibility of a contact zone in southern Oaxaca and Chiapas remains to be explored and therefore the presence of gene flow cannot be fully discarded.

The split between the WM and EM&MA phylogroups is too recent to be explained by the orogeny of the Mexican Sierras (Ferrari et al., 1999). One possibility is that, subsequent to the dispersion across the Panamanian land bridge, the dispersing population split into east and west populations that continued to move northwards while isolated by the central Middle American mountain chains (Miller et al., 2011). This is supported by the fact that the three haplogroups found along the Atlantic Coast from Mexico to Nicaragua are more closely related to each other than to the WM clade.

The three haplogroups found within the EM&MA clade showed substantial differentiation at their mtDNA, which was dated to the Pleistocene. However, their structure correlated partially with geography, suggesting the presence of current or recent gene flow among them. Diversification within this clade is congruent with previous studies that have emphasized the role of Pleistocene cyclic retractions and expansions of humid lowland and lower montane forests throughout the intricate topography of this region (Escalante et al., 1993; Smith et al., 2012; Weir, 2009).

Finally, the elevated genetic diversity and the signs of population stability found in the EM&MA phylogroup contrast with the lower diversity observed within South American lineages. This could at first sight seem contradictory with the species tree and the recent diversification scenario proposed for the EM&MA clade. However, we believe that this is most likely a result of a higher degree of diversification across eastern Mexico and Middle America due to its complex array of highland systems interdigitated with a mosaic of humid and dry habitats (Escalante et al., 1993; Smith et al., 2012). In fact, divergence levels (i.e. genetic distances) within each of the three *rubicoides* haplogroups were comparable to those observed within the South American phylogroups, suggesting that each *rubicoides* group may have independently undergone demographic changes in the past too. A better sampling throughout Mexico and Middle America is needed, though, to analyze these haplogroups separately in order to further investigate

their demographic history and the existence of current or recent gene flow among them.

4.4. Differentiation in behavior and coloration

We found significant differences in song among the three phylogroups that we were able to study. The vocalizations of the individuals from the AF clade were the most distinguishable based on temporal characters, whereas the individuals from EM&MA clade had lower-pitched songs. Because there are no obvious differences in the habitats that are occupied by *H. rubica* across its distribution and no differences in size or bill shape among subspecies have been reported, we believe that differences in vocalizations are not related to the adaptation of song to the environment or to morphological differences between birds, but are instead a consequence of neutral differentiation among these phylogroups.

Regarding coloration, we found significant differences in the throat in males and in the chest in females between the two South American clades. Unfortunately, we could only analyze one male and one female representing the EM&MA clade and therefore we were not able to statistically compare this phylogroup with the other ones. Our results support the genetic evidence that indicates that the Y&AC and the AF clades are clearly differentiated.

4.5. Taxonomic implications

We found *H. rubica* to be monophyletic within our dataset with high support in spite of the deep divergences among its main lineages. The Crested Ant Tanager (*H. cristata*), a species that has not yet been included in any molecular studies and that based on morphological and behavioral evidence seems to be closely related to *H. rubica* (Willis, 1972), should be included in future analyses to confirm this result. In addition, and as evidenced by Klicka et al. (2007) and Barker et al. (2015), *H. rubica* appeared more closely related to species of the genus *Chlorothraupis* than to its congeneric species, making the genus *Habia* paraphyletic as currently defined. Since further analyses including *H. cristata* are needed to definitely establish the phylogenetic affinities among the species of these two genera, we are anyway keeping the genus name *Habia* for the new species suggested below.

Based on consistent genetic, morphological and behavioral evidence, we believe that splitting *H. rubica* into three species is the most reasonable and conservative alternative for the moment. One of the species should be the lineage present in the Atlantic Forest, which should keep the name *H. rubica*. Despite the fact that our genetic analyses do not distinguish between the subspecies *rubica* and *bahiae* from the Atlantic Forest, we believe that further morphological and behavioral objective analyses (like the ones performed here among main lineages) are needed to fully address their validity. Therefore, we propose that this species should keep the subspecies *rubica* and *bahiae* for the moment. Although we lack representatives of some races distributed north of the Amazon River, we recommend that all South American subspecies other than the ones from the Atlantic Forest are included in a new species called *H. rubra* (given its taxonomic priority). Until a more comprehensive sampling of the subspecies from northern South America allows further genotypic and phenotypic analyses of this group, *H. rubra* would include the subspecies *rubra*, *peruviana*, *rhodinolaema*, *hesterna*, *perijana*, *coccinea*, *crissalis*, and *mesopotamia*. Finally, we suggest considering the Mexican and Middle American lineage a single species named *H. rubicoides*. This species would temporally comprise the subspecies *rubicoides* and *affinis*, as treated by Navarro-Sigüenza and Peterson (2004), and *alfaroana* and *vinacea*, until further sampling in western Mexico and southern Middle America allows a deeper analysis of these subspecies.

5. Conclusions

The evolutionary history of *H. rubica* through the last 5.0 Myr seems to have been shaped by the uplift of the Northern Andes, the crossing of the Isthmus of Panama, the establishment of the open vegetation corridor, and Quaternary climatic fluctuations. This resulted in levels of genetic, morphological and behavioral differentiation that justify considering at least three different species within this diversified lineage. Further investigation on the origin and diversification of this taxon, as well as the assessment of the degree of differentiation and isolation among subspecies, would require a more comprehensive sampling, particularly in the Northern Andes, southern Middle America and western Mexico.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.04.018>.

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