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Many neotropical species have a complex history of diversification as a result of the influence of geographical, ecological, climatic, and geological factors that determine the distribution of populations within a lineage. Phylogeography identifies such populations, determines their geographic distributions, and quantifies the degree of genetic divergenced. In this work we explored the genetic structure of *Habia rubica* populations, a polytypic taxon with 17 subspecies describes, in order to obtain hypotheses about their evolutionary history and processes of diversification. We undertook multilocus analyses using sequences of five molecular markers (ND2, ACOI-I9, MUSK, FGB-I5 and ODC), and sampling from across the species' distribution range, an area encompassing from Central Mexico throughout much of South America. With these data, we obtained a robust phylogenetic hypothesis, a species delimitation analysis, and estimates of divergence times for these lineages. The phylogenetic hypothesis of concatenated molecular markers shows that *Habia rubica* can be divided in three main clades, the first includes Mexican Pacific coast populations, the second is formed by population from east of Mexico to Panama and the third comprises the South American populations. Within these clades we recognize seven principal phylogroups whose limits have a clear correspondence with important geographical discontinuities including the Isthmus of Tehuantepec in southern Mexico, the Talamanca Cordillera, and the Isthmus of Panama in North America. In South America, we observed a marked separation of two phylogroups that include the populations that inhabit mesic forests in western and central South America (Amazon Forest) and those inhabiting the seasonal forest from the eastern and northern regions of the continent (Atlantic Forest). These areas are separated by an intervening dry vegetation "diagonal" (Chaco, Cerrado and Caatinga). The geographic and genetic structure of these phylogroups describes a history of diversification more active and complex in the northern

distribution of this species, producing at least seven well-supported lineages that could be considered species.

2 **Deep divergence of Red-crowned Ant Tanager (*Habia rubica*: Cardinalidae), a multilocus**

3 **phylogenetic analysis with emphasis in Mesoamerica**

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27 **ABSTRACT**

28 Many neotropical species have a complex history of diversification as a result of the influence of

29 geographical, ecological, climatic, and geological factors that determine the distribution of

30 populations within a lineage. Phylogeography identifies such populations, determines their

31 geographic distributions, and quantifies the degree of genetic divergenced. In this work we

32 explored the genetic structure of *Habia rubica* populations, a polytypic taxon with 17 subspecies

33 describes, in order to obtain hypotheses about their evolutionary history and processes of

34 diversification. We undertook multilocus analyses using sequences of five molecular markers

35 (ND2, ACOI-I9, MUSK, FGB-I5 and ODC), and sampling from across the species' distribution

36 range, an area encompassing from Central Mexico throughout much of South America. With

37 these data, we obtained a robust phylogenetic hypothesis, a species delimitation analysis, and

38 estimates of divergence times for these lineages. The phylogenetic hypothesis of concatenated

39 molecular markers shows that *Habia rubica* can be divided in three main clades, the first

40 includes Mexican Pacific coast populations, the second is formed by population from east of

41 Mexico to Panama and the third comprises the South American populations. Within these clades

42 we recognize seven principal phylogroups whose limits have a clear correspondence with

43 important geographical discontinuities including the Isthmus of Tehuantepec in southern

44 Mexico, the Talamanca Cordillera, and the Isthmus of Panama in North America. In South

45 America, we observed a marked separation of two phylogroups that include the populations that

46 inhabit mesic forests in western and central South America (Amazon Forest) and those

47 inhabiting the seasonal forest from the eastern and northern regions of the continent (Atlantic

48 Forest). These areas are separated by an intervening dry vegetation "diagonal" (Chaco, Cerrado

49 and Caatinga). The geographic and genetic structure of these phylogroups describes a history of

50 diversification more active and complex in the northern distribution of this species, producing at

51 least seven well-supported lineages that could be considered species.

52

53 **INTRODUCTION**

54 The origins and evolutionary drivers of neotropical diversity are one of the most studied and

55 hotly debated topics since the first biological explorations in the 19th century (Salvin &

56 Goldman, 1879-1904). Two main hypotheses have been proposed to explain the high levels of

57 neotropical biodiversity (Rull, 2008). On one hand, the glacial-interglacial cycles of the

58 Pleistocene (last ~2.6 Mya) have been suggested as the major drivers of divergence, during

59 which allopatric speciation took place in isolated rainforest refuges, particularly during the

60 cooler and drier conditions of the glacial maxima (Whitmore & Prance, 1987; Hooghiemstra &

61 van der Hammen, 1998; Hewitt, 2000; Bennett, 2004). On the other hand, the paleogeographic

62 changes that occurred throughout the Cenozoic (i.e. the last ~66 Mya), like the Andean orogeny

63 and the uprising of the Isthmus of Panama (Hewitt, 2000; Willis & Niklas, 2004; Nores, 2004),

64 have been postulated as major factors driving diversification, as new geographical barriers both

65 increased isolation and promoted divergence. Both hypotheses have however been criticized. For

66 instance, there is little palynological (Colinvaux et al., 2001; Bush & de Oliveira, 2006),

67 phylogenetic (e.g. Moritz et al., 2000; Glor, Vitt & Larson, 2001), or ecological-modeling

68 support (Cowling, Maslin & Sykes, 2001; Mayle et al., 2004) for a Pleistocene origin of tropical

69 diversity. Indeed, most divergence times estimated so far date back to the Neogene, supporting a

70   role for more ancient diversification events. However, differentiation patterns across taxa are

71   highly discordant and frequently cannot be linked to specific landscape or vicariant events

72   (Burns & Naoki, 2004; Burns & Racicot, 2009; Mauck & Burns, 2009) as this hypothesis

73   proposes; instead, they seem more related to each species ability to persist through

74   environmental changes and independently disperse across putative geographic barriers (e.g.

75   Smith et al., 2014).

76   The role of ecological divergence in producing neotropical diversity has taken force in

77   the last decade. For instance, alternating cycles of contracting and expanding tropical forests

78   could have resulted in multiple bouts of differentiation that produced assemblages of "old" and

79   "young" taxa exclusive to each habitat (e.g. Pennington et al., 2004; Smith et al., 2013). This is

80   not only important for species diverging in situ, but also for taxa that dispersed and differentiated

81   across newly available habitats, such as those involved in inter-continental exchange after the

82   uplifting of the Isthmus of Panama. It has been suggested that animals tolerant to a variety of

83   habitats and elevation zones quickly expanded through this corridor into North America, while

84   species restricted to more humid conditions could only disperse more recently into northern

85   latitudes (Zamudio-Beltrán & Hernández-Baños, 2015).

86   Another points that fuels this debate is the species concept itself. That is, evidence

87   supporting one or another hypothesis can be dismissed or not depending on whether the taxa

88   studied are considered (or not) independent species. Traditionally, species boundaries have been

89   drawn based on morphology, but the onset of high-throughput molecular techniques has allowed

90   inferring these boundaries from statistical models based on the coalescent framework (e.g.

91   Bayesian Phylogenetics and Phylogeography; BP&P; Yang & Rannala, 2010). As both views

92   have their own limitations (see for instance Sukumaran & Knowles 2017), an integrative

93   approach based on different types of data (morphological, ethological, ecological, molecular,

94   etc.) seems necessary to operationally determine whether lineages have been evolving separately

95   and therefore can be considered different species (De Queiroz 2007).

96   The Red-crowned Ant Tanager (*Habia rubica*, Cardinalidae) is a highly polytypic taxon

97   with marked geographical variation; it comprises up to 17 subspecies (Avibase, 2012) most of

98   which were described based on the variation in the hue and intensity of plumage coloration

99   (Hilty, 2011). Its current distribution ranges from central Mexico to north-eastern Argentina and

100   southeastern Brazil, and encompasses regions with very different ecological conditions and/or

101   separated by recognized biogeographical barriers (Hilty, 2011). A previous phylogeographic

102   survey (Lavinia et al., 2015) suggested that this species originated in South America, where it

103   bears at least two clearly differentiated clades, one in the Atlantic Forest of Brazil and another in

104   the rainforests of the Amazon basin. However, a limited sampling precluded the authors to

105   explore the colonization and diversification within Central America and Southern Mexico.

106   The objective of this paper is to evaluate the nature and geographic structuring of genetic

107   variation within and among populations of *Habia rubica,* using both nuclear and mitochondrial

108   genetic markers in order to provide a phylogenetic hypothesis about the evolutionary history of

109   the species. We used Bayesian and coalescent methods to generate a phylogeny and estimate

110   divergence times between clades, to assess the validity of the genetic groupings identified using

111   species delimitation methods (Rannala & Yang, 2003; Yang & Rannala, 2010). Emphasis was

112   made on the missing parts of the biogeographical history of the species.

113

114   **MATERIAL & METHODS**

115   **Taxon sampling and laboratory procedures**

116   We sequenced 125 individuals of *Habia rubica* species that covered the species distribution,

117   including samples from Mexico (Using field collecting permit from Instituto Nacional de

118   Ecología, SEMARNAT: FAUT-0169), Guatemala, El Salvador, Honduras, Nicaragua, Costa

119   Rica, Panama, Peru, Bolivia, Paraguay, Brazil, Argentina and Venezuela (Fig. 1A). We also

120   included 16 samples from the genus *Chlorothraupis* (*C. olivacea*, *C. carmioli* and *C.*

121   *stolzmanni*), the sister group of *H. rubica* (Klicka, Burns & Spellman, 2007), and three

122   congeneric samples that were used as outgroups (*H. fuscicauda*, *H. atrimaxillaris*, and *H.*

123   *gutturalis*; see Table S1).

124   We isolated genomic DNA from tissues using Qiagen DNAeasy kit (Qiagen, Valencia,

125   CA, USA) following the manufacturer's protocol. We amplified five molecular markers via the

126   polymerase chain reaction (PCR) in 12.5 µl volumens. The mitochondrial marker *ND*2 (1041

127   bp), was generated for all individuals (*n* = 144) using the primers L5219 and H6313 (Sorenson et

128   al., 1999). We then amplified four nuclear markers, for a subset of individuals with the highest

129   DNA quality (*n* = 37). These nuclear loci included two Z-linked introns, the aconitase I gene

130   (*ACOI*-I9, 852 bp) amplified using the primers ACOI-I9F and ACOI-I9R (Kimball et al., 2009),

131   and the receptor of the tyrosine kinase MUSK gene (*MUSK*, 576 bp) amplified using the primers

132   MUSK-I3F and MUSK-I3R (Kimball et al., 2009). We also amplified two autosomal loci, a

133   beta-fibrinogen gene (*FGB*-I5, 577), using the primers Fib5 and Fib6 (Kimball et al., 2009) plus

134   the Ornithine Decarboxylase ODC gene (*ODC*, 711 bp) using the primers ODCF and ODCR

135   (Primmer et al., 2002). We used the following annealing temperatures: 54°C (ND2), 56°C

136   (MUSK), 60°C (ACOI and FGB-I5) and 65°C (ODC). PCR success was verified on 1% agarose

137   gels.

138   DNA sequencing was performed in the High Throughput Genomic Unit Service of the

139   University of Washington. We edited and aligned chromatograms with Sequencher v4.8

140   (GeneCodes Corporation, Ann Arbor, MI, USA). We corroborated the origin of all of our

141   sequences by combining at least 2 of the following methods: amplifying overlapping gene

142   segments, amplifying or sequencing 1 region with different primer sets, sequencing both DNA

143   strands for all amplified fragments, or using multiple individuals of a single species. No evidence

144   of NUMT contamination was found (Zhang & Hewitt, 1996; Sorenson & Quinn, 1998;

145   Bensasson et al., 2001), and all nuclear sequences could be easily aligned with the corresponding

146   genes from other avian species. The overall length of the concatenated mitochondrial and nuclear

147   sequences was ~3,800 bp. All sequences were deposited in FigShare

148   (https://figshare.com/s/fe9f9f6fed1686782f62).

149

150   **Phylogenetic analyses**

151   Phylogenies were constructed from two different matrices; the first matrix included 144

152   sequences of the mitochondrial marker ND2 (hereafter referred to as the mtDNA dataset), and

153   the second matrix included 37 concatenated sequences of the mitochondrial marker ND2 plus the

154   four nuclear markers describe above (hereafter referred to as multilocus dataset). Phylogenetic

155   analyses were performed for each matrix using a Bayesian Inference method (BI), with

156   independent partitions and evolutionary rates defined for each gene region. The model

157   parameters of nucleotide evolution for each DNA region were obtained with jModeltest v3.8

158   (Posada, 2008) using the Akaike Information Criterion (Akaike, 1973). Analyses were conducted

159   in MrBayes v3.2 (Ronquist et al., 2012), and consisted of four MCMC chains of 50 million

160   generations each, starting from random trees and using a uniform prior distribution of

161   parameters. Trees were sampled every 250 generations, using Tracer v1.6.0 (Rambaut et al.,

162   2013), after discarding the initial 20% as burn-in. The remaining trees were used to construct a

163   majority rule consensus tree with a posterior probability distribution. The final tree was

164   visualized in FigTree v1.2.3 (http//tree.bio.ed.ac.uk/software/figtre/). Finally, we used MEGA v7

165   (Kumar et al., 2016) to estimate the pairwise genetic distances between lineages from

166   mitochondrial and nuclear markers, applying a bootstrap of 1000 iterations; this allowed us to

167   indirectly determine the relative amounts of information provided by the mitochondrial and

168   nuclear markers.

169

170   **Diversity and genetic structure**

171   A haplotype network was obtained for the mtDNA dataset with the Median-Joining algorithm

172   available in Network v4.6 (Bandelt, Foster & Röhl, 1999). We used DnaSP v5.0 (Librado &

173   Rozas, 2009) for estimating the number of haplotypes (H) and segregating sites (S), the

174   haplotype (Hd) and nucleotide (π) diversities, and the Tajima's D (Tajima, 1989) and Fu's F (Fu,

175   1997) values for the genetic clusters recovered in the BI mitochondrial phylogeny mentioned

176   above. Significance for the last two summary statistics was inferred from 1000 replicates of the

177   coalescent algorithm.

178

179   **Species delimitation**

180   The phylogeny obtained from the multilocus dataset was used to assess species delimitations

181   under the Bayesian framework implemented in BP&P (Yang & Rannala, 2010). This method

182   allows the estimation of posterior distributions of competing models with differing number of

183   species. It incorporates a reversible-jump Markov chain Monte Carlo (rjMCMC) algorithm and a

184   user-specified tree, where it assigns speciation probability values (Rannala & Yang, 2003; Yang

185   & Rannala, 2010). BP&P assumes constant population sizes, no gene flow among lineages, and

186   no population structure within lineages after speciation occurred. It furthers includes divergence

187   time (τ) and mutation-scaled effective population size (θ) parameters. We first performed several

188   preliminary runs with the two available algorithms (0: fixed tree, and 1: tree as guide tree) and

189   different seed numbers to confirm consistency and fine-tuning the priors (ϵ and τ). After

190   selecting algorithm 0, we conducted a first set of analyses allowing the program to make

191   automatic adjustments to the priors (finetune = 1). Because different values of τ and θ can affect

192   the model posterior probabilities for a same guide tree (Leaché & Fujita, 2010; McKay et al.,

193   2013), and to explore a wide range of speciation histories, we performed a series of simulations

194   using three different parameter combinations: 1) large population size/deep divergence [G (1, 10)

195   for both θ and τ], 2) small population size/ shallow divergence [G (2, 2000) for both θ and τ], and

196   3) large population size/shallow divergence [G (1, 10) for θ and G (2, 2000) for τ] (Leaché &

197   Fujita, 2010; Smith et al., 2013). We then performed a final set of analyses that incorporated the

198   finetune parameters determined in the previous analysis (finetune = 0) with the same three

199   different combinations for θ and τ parameters described above. Each set was composed of

200   100,000 generations, and samples were taken every 5 steps after discarding the initial 50,000

201   iterations as burn-in. All analyses were performed on the BPPX graphics user interface

202   (http://abacus.gene.ucl.ac.uk/software) using the command line.

203

204   **Tests of divergence times**

205   Divergence times were inferred from the multilocus dataset. Analyses were performed in Beast

206   v1.7 (Drummond et al., 2013) using the previously defined gene partitions with the

207   corresponding evolutionary rates and models as estimated above (Ellegren, 2007; Smith &

208   Klicka, 2010) and the seven phylogroups identified with the multilocus dataset. Clade divergence

209   was simulated as a Yule's first tree (Yule, 1924) with a lognormal relaxed molecular clock

210   (Drummond et al., 2006). Analyses included 100 million iterations, with samples taken every

211   1,000 generations after eliminating 25% of the trees as burn-in. Mixing and likelihood stability

212   were confirmed with TRACER v1.6 (Rambaut & Drummond, 2013) to make sure that the

213   appropriate effective sample size (ESS) was over 200 units for each parameter (Drummond &

214   Rambaut, 2007). The best supported tree was chosen with TreeAnnotator 1.8.0 (Drummond &

215   Rambaut, 2007) through Maximum Clade Credibility.

216   Given the absence of fossils for *H. rubica*, or any close relatives, two calibrations based

217   on geological data were used. First, the closing of the Isthmus of Panama, estimated between 3.1

218   and 4.0 Ma (Daza, Castoe & Parkinson, 2010; Smith & Klicka, 2010), was used for the

219   separation of South and Central American populations; and second, the uprising of the

220   Talamanca Cordillera near the Costa Rica – Panama border, estimated to have occurred between

221   2.5 to 3.9 Ma (Marshall & Liebherr, 2000; Daza, Castoe & Parkinson, 2010), was employed as

222   the time of divergence between populations in Panama and those occurring elsewhere throughout

223   Mesoamerica. In addition, as estimating divergence times based on external event that could be

224   unrelated to a species biology can result in wrong inferences and circular reasoning, we

225   estimated divergence dates a second time but using only the mtDNA dataset (which has a higher

226   sample size) and no calibration points. This could be seen as an independent test of confidence

227   for the estimates obtained for the multilocus analysis with geological constraints. We assumed a

228   mutation rate of 1.25×10^{-2} sustitutions/site/Ma (Smith & Klicka, 2010), other parameters and

229   priors were kept identical.

230

RESULTS

Phylogenetic analyses

We obtained mitochondrial (1041 bp) and concatenated (3,757 bp) datasets. The best-fit models

for each molecular markers were follows: ND2, TrN+I+G; ACOI-I9, TIM2+G; MUSK, TrN+G;

FGB-I5, HKY+G and ODC, TrN.

The phylogenies obtained with mitochondrial and multilocus dataset revealed well-

supported topologies, independently of the method employed. Both topologies are composed of

three main clades with seven and eight subclades (considered here as "phylogroups") for the

multilocus and mitochondrial datasets, respectively. . The first clade in the multilocus phylogeny

(I) is comprised of two phylogroups with an apparent separation between populations occupying

the western (A; Fig. 1B) and southern (B; Fig. 1B) Pacific coast of Mexico. The second clade (II)

was composed of three well-supported phylogroups distributed from eastern Mexico to Panama.

The first includes all individuals from the Mexican Gulf Coast, west of the Isthmus of

Tehuantepec (C; Fig. 1B); the second one comprised samples collected east of this Isthmus and

southward into Nicaragua (D; Fig. 1B); and the third one was composed by samples from

Panama (E; Fig. 1B). The last clade (III) was composed of (at least) two phylogroups, one with

individuals from the Amazon basin (F; Fig. 1B) and another one with birds from eastern South

America (G; Fig. 1B). A single sample from Venezuela appears distinctive and is sister to this

eastern clade. However, additional samples from Venezuela will be required for a proper

systematic assessment.

The mitochondrial phylogeny, although based on many more samples, was almost

identical to the one obtained with the multilocus dataset (Fig. S1); it did however, show that

253   phylogroup D was composed of two subgroups: one comprised of individuals from the Yucatan

254   Peninsula, Honduras, Nicaragua and Costa Rica, and the other one of individuals from

255   Guatemala and El Salvador.

256   The pairwise genetic distances showed significant genetic differentiation in the

257   mitochondrial but not in the nuclear markers (Table 1). That is, the genetic distances were

258   smaller for nuclear than for mitochondrial markers (Table 1). This implies that the mitochondrial

259   marker provides larger amounts of relative information than the nuclear gene-regions (Table 2).

260

261   **Diversity and Genetic structure**

262   The haplotype mtDNA network (Fig. 1C) revealed the same eight groups obtained with BI

263   phylogeny (Fig. S1). A high number of haplotypes had to be reconstructed to link some of these

264   groups. For instance, 30 mutational steps separated the two groups from the Mexican Pacific

265   coast from the other clusters, 45 more steps were needed to link the clades from South and

266   Central America, while the two groups from South America were 41 steps apart; an additional 29

267   mutations were necessary to join the lone sample from Venezuela to the southeastern South

268   America clade.

269   Overall, diversity estimates differed greatly between mtDNA phylogroups, with the South

270   American and Panamanian ones being the more diverse, and the groups from the Mexican

271   Pacific coast the least genetically rich. Tajimas D and Fu´s F values were negative in all

272   phylogroups, excepting phylogroup D (eastern Mexico to northern Central America). However,

273   these values were significant only for the two South American Clades (F & G); thus suggesting

274   recent population expansions (Table S2).

275

276   **Species delimitation**

277   Considering a hypothesis of seven species for this taxon, the Bayesian species delimitation

278   supported the guide tree displaying speciation probabilities of 1.0 in most nodes, independently

279   of the finetune parameter used (finetune = 1 or 0, Fig. 2A and 2B respectively). Seven species

280   were supported with high speciation probabilities (0.97 to1.0) on the tree guide. These species

281   corresponded to the lineages A, B, C, D, E, F and G. The different prior distributions for θ and

282   τ had no effect on most nodes, the only exception being that of lineages A and B which were

283   influenced by at least one of the prior combinations (from 0.37 to 0.97). However, while these

284   and other analyses with similar topologies and highly supported clades suggest a multi-species

285   complex within *H. rubica*, other types of data are still necessary to support this hypothesis

286   (Sukumaran & Knowles, 2017; see below).

287

288   **Tests of divergence times**

289   According the multilocus dataset, the divergence between *Habia rubica* and *Chlorothraupis*

290   dated back to the late Neogene, between 6.65 and 3.91 Myr ago (Fig. 3A), and the ensuing

291   diversification of *H. rubica* was rather rapid. The first split was estimated some 3.75 to 3.35

292   Myr, between the South American (clade III) and Central/Mesoamerican lineages (clades I and

293   II); these two groups split again around 3 Myr ago. In Central America, the first divergence event

294   (3.4 - 2.7 Myr) occurred between the lineages from the Mexican Pacific coast (Clade I) and the

295   other phylogroups (Clade II). Within clade II, there was a gradual and northward divergence of

296   lineages from Panama to eastern Mexico; these three diversification events during the last ~3Myr

297   or ~2Myr. An independent analysis performed on the mtDNA dataset and with no calibration

298   constraints, produced an identical topology with older mean divergence times (i.e. divergence

299 within *H. rubica* occurred during the last 5.6 Myr). However, as confidence intervals were much

300 wider (Fig. 3B) and overlapped with those obtained with the multilocus dataset, they provide

301 support for the dates estimated based on geological times.

302

DISCUSSION

304 Our results show that *Habia rubica* has considerable geographic genetic structure. We found

305 seven lineages through its distribution; five of them are in Mesoamerica, where the evolutionary

306 history has been more dynamic and complex, and two in South America (this last results is

307 similar with was found by Lavinia et al., 2015). Most lineages are delimited by recognized

308 geographic and ecological barriers, like the Rio Balsas depression in Mexico, the Central

309 American Volcanic Arc, the Isthmus of Panama in Central America, or the dry vegetation

310 diagonal in South America. We recovered a reciprocally monophyletic relationship between *H.*

311 *rubica* and the genus *Chlorothraupis*, which coincides with previous studies (Klicka, Burns &

312 Spellman 2007; Klicka, Johnson & Lanyon, 2000; Barker et al., 2015; Lavinia et al., 2015);

313 therefore the genus *Habia* must be considered paraphyletic.

314

Phylogeny, Diversity and Genetic structure

316 The mitochondrial DNA topology indicates that populations of Mexico, Central and South

317 America are profoundly differentiated. In this topology eight phylogroups were identified, six in

318 Mesomerica and two in South America (Fig. S1). The relationship between mitochondrial

319 haplotypes could be determined by the action of various historical processes that promoted a

320 deep genetic structure in Mesoamerica, resulting in a clear example of cryptic speciation. This

321   complicated phylogenetic structure in Mesoamerica is consistent with the complex geological

322   and biogeographic history of the region (Coates & Obando, 1996), and the emergence of

323   geographic features such as the Rio Balsas depression, the Isthmus of Tehuantepec in southern

324   Mexico, the Motagua-Polochic-Jocotán fault system in the North of Central America, and the

325   Talamanca Cordillera in Costa Rica, which presumably played roles in shaping the observed

326   geographic and genetic patterns (Daza, Castoe & Parkinson, 2010; Gutiérrez-García & Vázquez-

327   Domínguez, 2012, 2013; Suárez-Atilano, Burbrink & Vázquez-Domínguez, 2014). Our results

328   together with the study of Lavinia et al. (2015) provide a robust hypothesis of the evolutionary

329   history of *Habia rubica*. For instance, our study confirms the main results of Lavinia et al.

330   (2015) for South America, and improves the resolution for Mesoamerica given our larger sample

331   size and wider area covered.

332   In South America, our results, in combination with those of Lavinia et al. (2015), showed

333   two distinct phylogroups that reflected population divergence according to the habitats preferred

334   by *Habia rubica* (Pennington et al., 2004, 2009; Lavin, 2006; Smith et al., 2013, 2014;

335   Pennington & Lavin, 2016): one western phylogroup distributed in the mesic forest of the

336   Amazon basin, and another one in the seasonal forests of the eastern and northern parts of the

337   continent. This last lineage included the single sample from Venezuela, instead of the western

338   clade that is geographically more proximate (Prates et al., 2016; Prates et al., 2017). This could

339   be indicating that the deep evolutionary history of the seasonal forests in South America

340   (Pennington et al., 2004, 2009) are driving the divergence of other taxa. Additional sampling in

341   this region is thus necessary to test this hypothesis.

342   The multilocus DNA topology identified seven of the eight phylogroups found with the

343   mitochondrial DNA (Fig. 1B), in all cases the phylogroups are the same with the exception of an

344   additional separation of a Yucatan Peninsula and northern of Central America clade identified

345   using the more rapidly evolving mtDNA marker. All identified phylogroups (mitochondrial and

346   multilocus dataset) are consistent with clear geographical discontinuities and are supported with

347   high posterior probabilities.

348   Although, the haplotype network recovered the same groups identified in the

349   mitochondrial DNA phylogeny (Fig. 1C), it was necessary to invoke five hypothetical haplotypes

350   between Mexican and Central American groups. These results support the idea about the

351   complex geological history in Mesoamerica resulting in a high degree of genetic structure in the

352   northernmost distribution of this species. In South America, 12 hypothetical haplotypes were

353   required, but unlike the previous case, this could be explained by the lack of sampling in this

354   specific area.

355   The genetic distances between phylogroups were relatively high and supported the groups

356   identified in our phylogenetic and BPP analyses. The largest difference found was between the

357   group from the Western Pacific Coast of Mexico (group A) and the group from the Southeast of

358   South America (group G) (Table 1). This result is not surprising because the geographical

359   distance between them is also the largest and is congruent with the divergence date (Fig. 3A and

360   3B). On the other hand, the more similar phylogroups were the Western (A) and the Southern

361   Pacific Coast of Mexco (B) groups, which represent the most recent divergence event (Fig. 3A

362   and 3B). As expected the mitochondrial DNA provides more poymorphic sites because its rate of

363   evolution is faster (Table 2) (Brown, George & Wilson, 1979).

364

365   **Species delimitation and Divergence times**

366   The deep divergence times among lineages estimated herein (Fig. 3A and 3B), most of which

367   dated back to Pleistocene and Pliocene, support the criticisms raised regarding the allopatric

368   speciation hypothesis that invoked the Pleistocene glacial cycles as the major drivers of

369   neotropical bird diversity and closing of the Isthmus of Panama (e.g. Moritz et al., 2000;

370   Cowling, Maslin & Sykes, 2001; Glor, Vitt & Larson, 2001; Mayle et al., 2004). According to

371   the ND2 divergence analyses, the first split was during the Miocene (5.69 Myr), and this is the

372   main discordance with respect to the calibrated multilocus divergence analyses (3.55 Myr).

373   These dates are also consistent with the compelling evidence for speciation drawn from the

374   BP&P analyses, which suggest that enough differences have accumulated between most of the

375   *Habia rubica* lineages to consider them a complex of cryptic species (Fig. 2).

376   At present, intraspecific delimitation within *H. rubica* is based on geographic variation in

377   plumage color, with 17 subspecies currently recognized (Avibase, 2012). Many of these

378   subspecies are not supported by the lineages described herein (Fig. 2). For instance, the

379   subspecies *rosea* and *rubicoides* are paraphyletic (lineages A&B, and C&D, from BP&P

380   analysis, respectively). The first taxon is further intermingled with ssp. *affinis* individuals in

381   lineage B, while the second one is combined with individuals of ssp. *holobrunnea* and *nelsoni* in

382   the lineages C and D. Some subspecies also appear indistinguishable with the markers that we

383   used (e.g. ssp. *hesterna*, *peruviana* and *rhodinolaema* within lineage F). Overall, our results

384   support a taxonomic revision of *H. rubica*.

385   Our test for species delimitation suggests that *Habia rubica* should minimally be split

386   into the following species: 1. *H. rosea*, distributed along the Pacific coast of western Mexico

387   (Jalisco, Nayarit and Colima; lineage A); 2. *H. affinis*, distributed along the Pacific coast of

388   southern Mexico (Michoacan, Guerrero and Oaxaca; lineage B); 3. *H. holobrunnea*, distributed

389 over the slope of Gulf of Mexico (lineage C); 4.- *H. rubicoides*, distributed from eastern Mexico

390 to southern Nicaragua (lineage D); 5. *H. vinacea*, allopatric in Panama (lineage E); 6. *H.*

391 *rhodinolaema*, distributed in the Amazon basin (lineage F); and 7. *H. rubica*, distributed in

392 eastern Brazil, Argentina, and Paraguay (lineage G). Further tests and sampling should be

393 necessary for a more adequate description of the Venezuela's individuals, currently classified as

394 spp. *perijana* (Hilty, 2011).

395   There are criticisms to the BP&P analysis implemented herein. For instance, simulation

396 data have shown that it tends to misidentify population structure as species boundaries

397 (Sukurmaran & Knowles 2017). However, it must be noted that our delimitation not only

398 detected genetics lineages, but lineages whose distribution coincides with the geographical

399 barriers and ecological differences (habitat preference) of each taxon. That is, it highlights the

400 role of other factors, like vicariance, dispersal and ecology, under a framework of ecological

401 allopatric speciation, which is one the drivers proposed for generating bird species diversity in

402 the neotropics (Hooghiemstra & van der Hammen, 1998; Hewitt, 2000; Bennett, 2004; Smith et

403 al., 2013). It also shows that this bird diversity is underestimated, and that species cannot be

404 described or estimated based only on morphological criteria. From this point of view,

405 diversifying the techniques for species delimitation, like incorporating bioacoustics, high-

406 throughput sequencing, niche modelling and advanced statistical tools, will also help addressing

407 consequential questions in taxonomy and biogeography. For instance, such multifactorial data

408 should help determining operationally whether the *H. rubica* lineages inferred herein are indeed

409 evolving separately (see De Queiroz, 2007).

410

411 **CONCLUSIONS**

412 The phyogenies obtained independently with mitochondrial and multilocus datasets revealed

413 well-supported topologies for the polytypic taxon *Habia rubica*. Both topologies are composed

414 of three main clades with eight and seven phylogroups (lineages), respectively. The mtDNA

415 haplotype network produced the same eight groups obtained with the mitochondrial data. The

416 Bayesian species delimitation analysis found seven species highly supported: 1) *H. rosea*,

417 distributed along the Pacific coast of western Mexico, 2) *H. affinis*, distributed along the Pacific

418 coast of southern Mexico, 3) *H. holobrunnea*, distributed distributed over the slope of Gulf of

419 Mexico, 4) *H. rubicoides*, distributed from eastern Mexico to southern Nicaragua, 5) *H. vinacea*,

420 allopatric in Panama, 6) *H. rhodinolaema*, distributed in the Amazon basin and 7) *H. rubica*,

421 distributed in eastern Brazil, Argentina and Paraguay. Further tests and sampling should be

422 necessary for a more adequate description of the samples from Venezuela, currently classified as

423 spp. *perijana*. The species subdivision of *H. rubica* coincides with geographical barriers and

424 ecological differences between taxa, highlighting the role of vicariance, dispersal and ecology in

425 bird species diversification in the neotropics.

426

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434

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Figure 1

Geographical distribution, phylogenetic consensus tree and haplotypes network

(A) Geographical distribution (indicated by pink shading) and sampling points of the *Habia rubica* species; the mitochondrial DNA sampling is represented by dots color and the nuclear DNA sampling is highlighted with a black dot on the dots color. ArcGIS (ArcMAP 10.2.2). (B) Phylogenetic consensus tree representing the relationship among populations from *H. rubica*, based on Bayesian inference from a multilocus dataset. Values above branches denote posterior probabilities (PP). (C) Haplotypes network, where the phylogroup "D" corresponds with individuals from Chiapas-Yucatan peninsula to Costa Rica and "d" corresponds with individuals from Guatemala and El Salvador (the number inside of circles indicated the number of individuals that shared each haplotype).

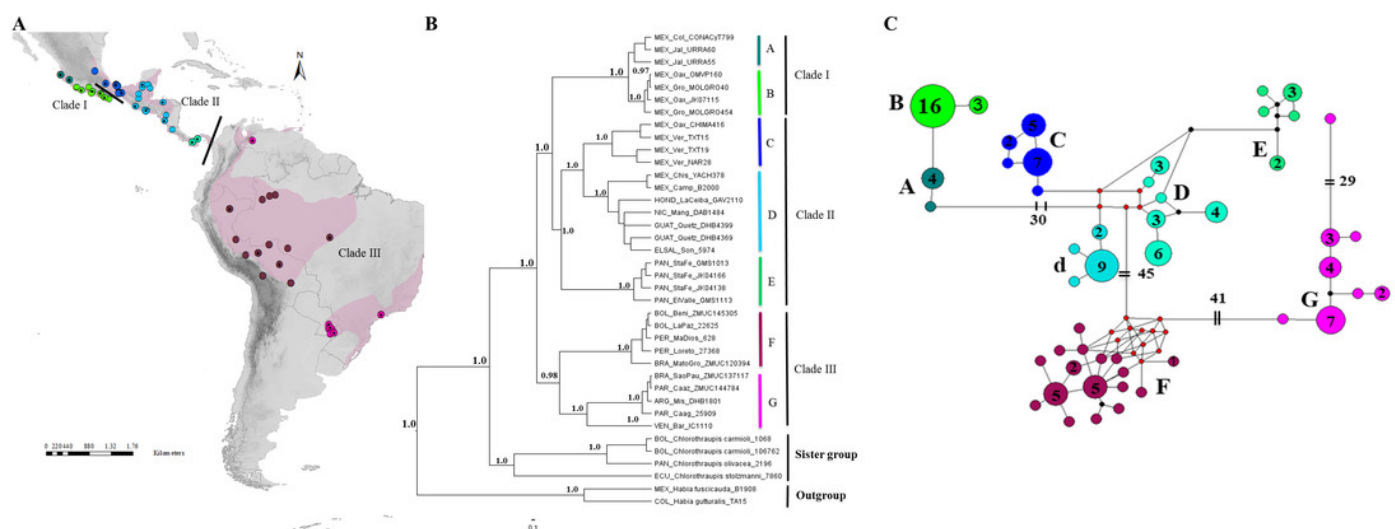



Figure 2

Bayesian species delimitation

(A) Bayesian species delimitation results assuming seven species (lineages) from multilocus phylogeny of Habia rubica (Finetune = 1). (B) Bayesian species delimitation results assuming seven species (lineages) from multilocus phylogeny of Habia rubica (Finetune = 0, parameters estimated previously). The speciation probabilities are provided for each node under each combination of priors for τ and θ: top, priors $\theta\sim G(1, 10)$ and $\tau0\sim G(1, 10)$; middle, priors $\theta\sim G(2, 2000)$ and $\tau0\sim G(2, 2000)$; bottom, priors $\theta\sim G(1,10)$ and $\tau0\sim G(2,2000)$. We consider speciation probability values >0.95 as strong support for a speciation event.

Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.

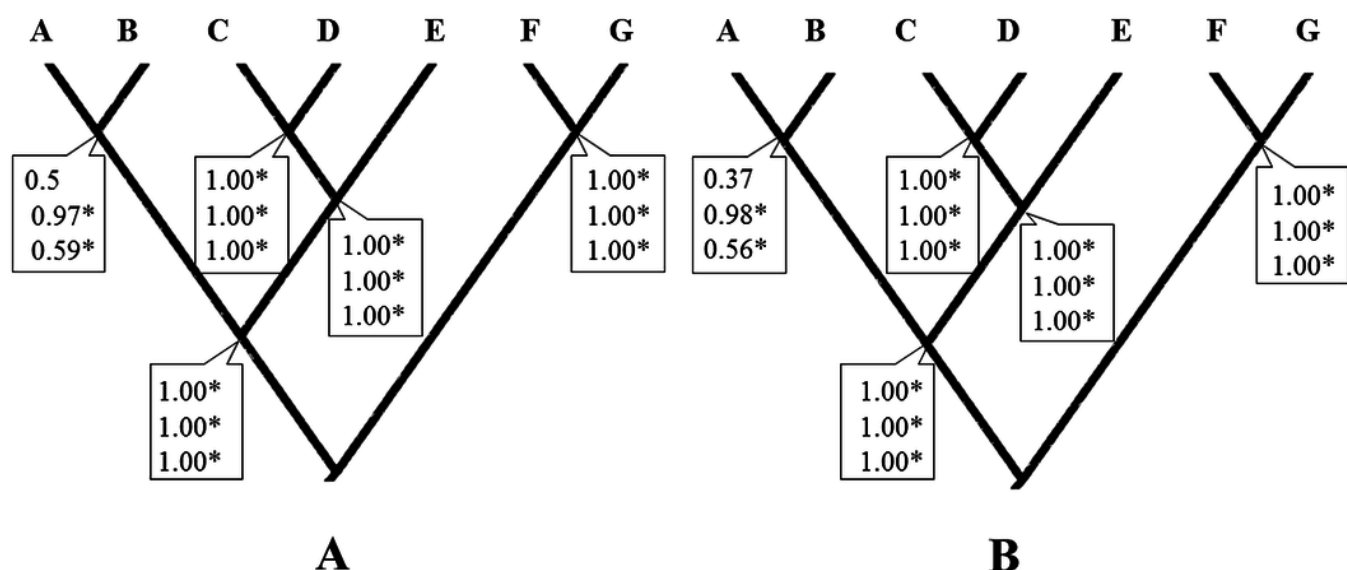
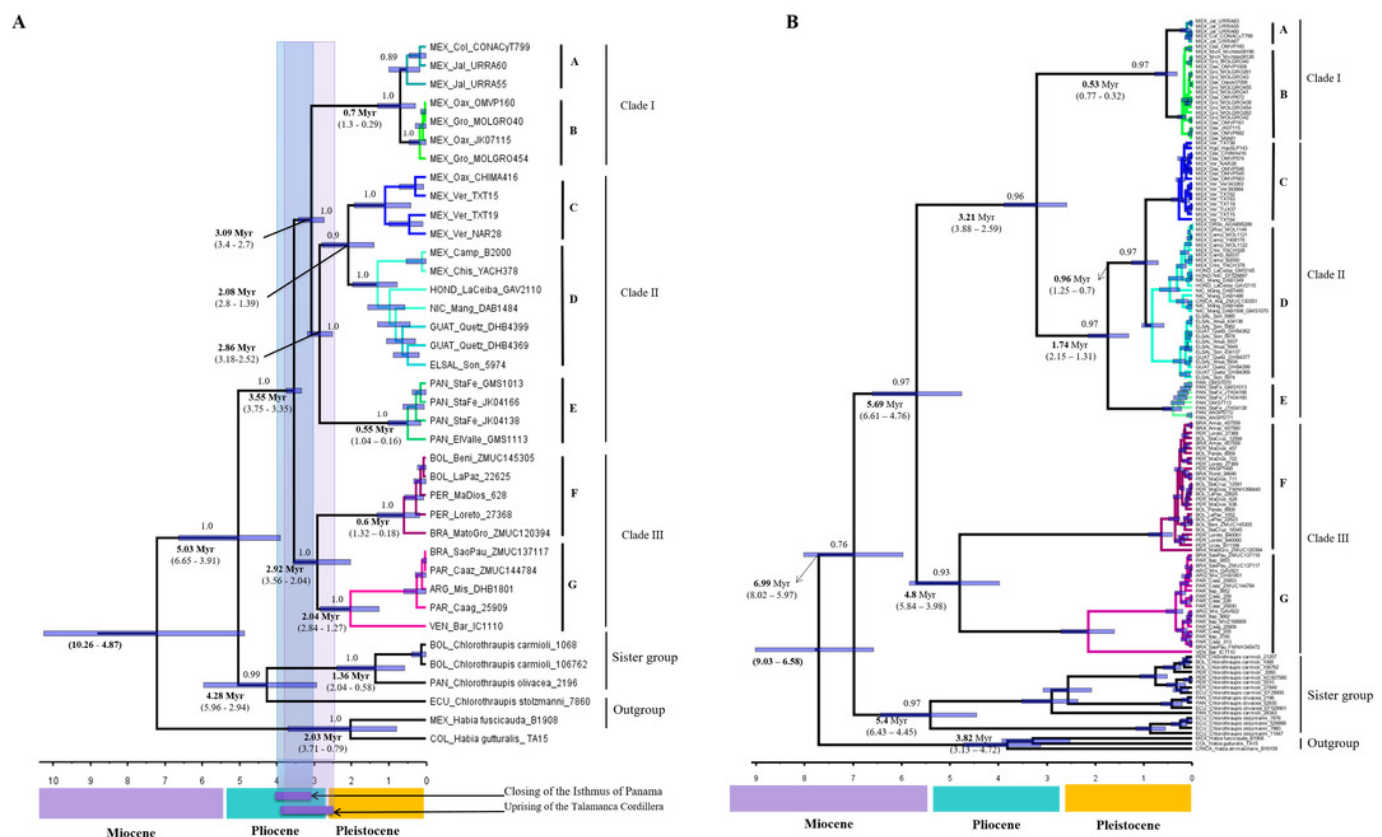



Figure 3

Divergence times trees

Divergence times trees. (A) For multilocus dataset (Confidence interval values are inside parentheses), calibration points are shown in the time bar. (B) For ND2 dataset, using a sustitution rates of 2.5 x 10^{-2} sustitutions/sites/lineages/Myr .

Pairwise genetic p-distances

Phylogroups	A	B	C	D	E	F	G
			mtDNA				
A							
B	0.7**						
C	4.1*	4.4*					
D	4.1*	4.5*	1.7**				
E	5.1*	5.5*	2.7**	2.4**			
F	6.4*	6.4*	5.7*	5.8*	6.6*		
G	6.8*	6.6*	5.7*	5.8*	6.7*	5.4*	
			nDNA				
A							
B	0.0**						
C	0.1**	0.1**					
D	0.1**	0.1**	0.1**				
E	0.1**	0.1**	0.1**	0.1**			
F	0.2**	0.2**	0.1**	0.2**	0.2**		
G	0.6**	0.6**	0.5**	0.6**	0.6**	0.4**	

3
4   * < 0.05
5   ** <0.005
6
7   Corrected pairwise genetic p-distances between lineages of *Habia rubica* based on mitochondrial and
8   nuclear markers.

Basic Information of mitochondrial and nuclear DNA

Molecular marker	Samples	Sequences	Bases pair	Monomorphic sites	Polymorphic sites	More than two variants	Gaps	Missing data
Mitochondrial DNA	125	125	1041	782	259	229	-	-
ACOI-I9	38	76	852	452	112	8	7	281
MUSK	39	78	576	181	54	2	4	337
FGB-I5	38	76	577	236	60	4	2	279
ODC	39	78	711	584	46	1	8	73
Nuclear DNA	37	74	2716	1733	268	17	20	695

3   Relative amounts of information in the mitochondrial and nuclear DNA.