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Biogeography and diversification of Rhegmatorhina (Aves: Thamnophilidae): Implications for the evolution of Amazonian landscapes during the Quaternary

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Original Article

Biogeography and diversification of *Rhegmatorhina* (Aves: Thamnophilidae): implications for the evolution of Amazonian landscapes during the Quaternary

Running title: Biogeography of *Rhegmatorhina*

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38 FAPESP (grant #2012/50260-6); PEER-USAID Cycle 5 to CCR, and NSF 1146423 to JC. **Abstract**

39 **Aim** To test the importance of alternative diversification drivers and biogeographical processes for the
40 evolution of Amazonian upland forest birds through a densely sampled analysis of diversification of the
41 endemic Amazonian genus *Rhegmatorhina* at multiple taxonomic and temporal scales.

42 **Location** Amazonia

43 **Taxon** Antbirds (Thamnophilidae)

44 **Methods** We sequenced four mtDNA and nuclear gene regions of 120 individuals from 50 localities
45 representing all recognized species and subspecies of the genus. We performed molecular phylogenetic
46 analyses using both gene tree and species tree methods, molecular dating analysis and estimated population
47 demographic history and gene flow.

48 **Results** Dense sampling throughout the distribution of *Rhegmatorhina* revealed that the main Amazonian
49 rivers delimit the geographic distribution of taxa as inferred from mtDNA lineages. Molecular phylogenetic
50 analyses resulted in a strongly supported phylogenetic hypothesis for the genus, with two main clades
51 currently separated by the Madeira River. Molecular dating analysis indicated diversification during the
52 Quaternary. Reconstruction of recent demographic history of populations revealed a trend for population
53 expansion in eastern Amazonia, and stability in the west. Estimates of gene flow corroborate the possibility
54 that migration after divergence had some influence on the current patterns of diversity.

55 **Main Conclusions** Based on broad-scale sampling, a clarification of taxonomic boundaries, and strongly
56 supported phylogenetic relationships, we confirm that, first, mitochondrial lineages within this upland forest
57 Amazonian bird genus agree with spatial patterns known for decades based on phenotypes, and second, that
58 most lineages are geographically delimited by the large Amazonian rivers. The association between past
59 demographic changes related to palaeoclimatic cycles and the historically varying strength and size of rivers
60 as barriers to dispersal may be the path to the answer to the long standing question of identifying the main
61 drivers of Amazonian diversification.

62 **Keywords:** Antbirds, Amazonia, diversification, molecular systematics, Neotropic, phylogeny,
63 phylogeography, taxonomy

64 **Introduction**

65 Amazonian diversity and its distribution are still poorly known, even in well-studied groups such as birds
66 (Whitney & Cohn-Haft, 2013). Numerous regions of this vast forest lack modern biotic inventories, and
67 many taxa remain to be described (Hopkins 2007, Barrowclough et al. 2016). Despite gaps in our taxonomic
68 and distributional knowledge, Amazonia harbours the highest species diversity of any biome on Earth (de
69 Groot *et al.*, 2012). However, our understanding of its evolutionary origins remains unclear because an
70 answer relies on fundamental knowledge about the region's constituent taxa, their distributions, and their
71 interrelationships, for which there are still many gaps. Multiple studies have shown that currently recognized
72 species significantly underestimate the number of evolutionary taxa that are relevant for analyses of
73 diversification and biogeography (e.g. Ribas *et al.*, 2006, 2012; Fernandes *et al.*, 2012; Thom & Aleixo,
74 2015; Harvey & Brumfield, 2015). Thus, how we think about species-level taxa and how well each group is

75 geographically sampled and taxonomically known is central to solving the question of the evolutionary
76 drivers of Amazonian diversity.

77 An important uncertainty is how the origin of this high diversity relates to landscape history, which
78 has been the subject of considerable debate over many decades (Haffer, 1969; Hoorn *et al.*, 2010; Ribas *et*
79 *al.*, 2012; Smith *et al.*, 2014). Our growing knowledge of the geological and palaeoclimatological history of
80 Amazonia (Hoorn *et al.*, 2010; Latrubesse *et al.*, 2010; Nogueira *et al.*, 2013; Cheng *et al.*, 2013; Wang *et al*
81 2017) is now allowing us to ask specific questions about patterns of biotic history (Baker *et al.*, 2014). In
82 particular, how past climate variation may have affected the demography and connectivity of forest-adapted
83 populations, and how landscape reorganization relating to drainage evolution may have contributed to the
84 origin of upland forest species. Traditionally, these candidate palaeoenvironmental drivers are supposed to
85 have operated at different time frames: glacial cycles occurred mostly during the Quaternary (Haffer, 1969),
86 whereas the current configuration of the Amazonian drainage system has been largely assumed to date to the
87 Miocene (Hoorn *et al.*, 2010), despite evidence pointing to more recent times (Latrubesse *et al* 2010,
88 Nogueira *et al* 2013). Due to this temporal difference, the two drivers are rarely integrated. Therefore, studies
89 have assumed that species may be old, with their origin related to Miocene drainage evolution (Hoorn *et al.*
90 2010), or young and their origin related to Quaternary glacial cycles, a very simplistic approach as pointed
91 out by Rull (2015).

92 Recent, more detailed phylogenetic and phylogeographic studies, associated systematic revisions,
93 and temporal diversification analyses of extant Amazonian taxa show constant diversification rates
94 throughout the Neogene/Quaternary (Patel *et al.* 2011, Brumfield, 2012, d'Horta *et al.*, 2013). Detailed
95 taxonomic sampling and phylogenetic analysis have also shown a Plio-Pleistocene origin of species of the
96 genus *Psophia*, whose ranges are clearly delimited today by the large Amazonian rivers (Ribas *et al.*, 2012),
97 indicating that the dynamic history of the Amazonian drainage system may be an important driver of
98 diversification during this period (Nogueira *et al.*, 2013). Population level sampling of *Psophia* species also
99 shows distinct demographic signatures for populations from different Amazonian areas of endemism,
100 suggesting differential effects of climatic cycles on forest bird populations (Ribas *et al.*, 2012, Cheng *et al*
101 2013, Wang *et al* 2017). Additional studies of other groups of Amazonian birds corroborate these findings,
102 supporting both drainage evolution and climatic-driven changes on vegetation cover as important drivers of
103 diversification during the Plio-Pleistocene (d'Horta *et al.*, 2013; Fernandes *et al.*, 2012, 2014; Thom &
104 Aleixo 2015, Ferreira *et al* 2017, Schultz *et al* 2017). However, incongruent patterns (Smith *et al* 2014),
105 suggest a complex history and a combination of biogeographic processes (including dispersal and vicariance)
106 acting across multiple time scales.

107 The genus *Rhegmatorhina* includes taxa specialized for army-ant following in the understory of tall
108 upland *Terra Firme* Amazonian forest (Willis 1969). There are five recognized species - *R. gymnops*, *R.*
109 *hoffmannsi*, *R. berlepschi*, *R. melanosticta* and *R. cristata* (Zimmer and Isler 2003). Large rivers define the
110 majority of ranges for these species, which occur in five Amazonian areas of endemism (*sensu* Cracraft
111 1985, Silva *et al* 2005, Borges and Silva 2011): Tapajós, Rondônia, Inambari, Napo and Jaú. The genus is
112 absent from the three easternmost areas: Guiana, Xingu and Belém. Two species (*R. hoffmannsi* and *R.*

113 *berlepschi*) occupy the Rondônia area, and one species (*R. melanosticta*) occupies two areas (Inambari and
114 Napo). Although species distributions within the genus are delimited by major Amazonian rivers,
115 hybridization has been documented between *R. gymnops* and *R. hoffmannsi* in the headwaters of the Tapajós
116 River, which is the limit between the Tapajós and Rondônia areas (Weir et al 2015). Here, we present
117 combined phylogeographic and phylogenetic multilocus analyses for the genus to test the importance of
118 alternative diversification drivers and biogeographical processes that have been suggested by previous
119 studies on Amazonian upland forest birds. With extensive sampling both in terms of range representation and
120 number of individuals, we address intraspecific genetic structure, refine taxonomic and lineage distribution
121 limits, and estimate the demographic history of populations. We integrate genetic analyses at both intra and
122 inter-population scales to understand spatially and temporally the history of this genus, comparing its
123 pattern with other Amazonian groups, and relate these results to the different hypotheses about Amazonian
124 landscape evolution.

125

126 **Materials and methods**

127 *Sampling*

128 We sampled 120 specimens representing a broad geographic coverage of the distribution of all seven named
129 taxa within *Rhegmatorhina*: the three subspecies of *R. melanosticta* plus the remaining four monotypic
130 species (Fig. 1, Table S1, Appendix S1 in Supporting Information). We also included sequences of the five
131 *Gymnopythys* species (*G. salvini*, *G. bicolor*, *G. leucaspis*, *G. lunulatus*, and *G. rufigula*) as this genus has
132 been shown to be paraphyletic, with *G. leucaspis* and *G. rufigula* forming a sister clade to *Rhegmatorhina*
133 (Isler et al., 2014). *Phlegopsis nigromaculata* was included as the outgroup.

134

135 *DNA extraction, amplification and data analysis*

136 Genomic DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN). Three mitochondrial genes
137 (cytochrome b, *cytb*; NADH dehydrogenase subunit 2, *ND2*; NADH dehydrogenase subunit 3, *ND3*) and one
138 nuclear intron (b-fibrinogen intron 5, *FIB5*) were sequenced (for the primers used see Table S2.1, Appendix
139 S2). Sequences were aligned, gametic phase was estimated, recombination tests were performed and
140 summary statistics were calculated (see Appendix S2 for details). Statistically significant differences in
141 genetic diversity indices (haplotype and nucleotide diversities) among species for each marker were tested
142 using a two-way ANOVA test in R 3.0.3 (R Core Team 2014).

143

144 *Phylogenetic analyses and haplotype networks*

145 All analyses were performed using a concatenated mtDNA matrix with partitions and models selected in
146 PARTITIONFINDER 1.1.1 (Lanfear et al., 2012). Bayesian phylogenetic inference (BI) was performed using
147 MRBAYES 3.2.2 (Ronquist et al., 2012), with two independent runs of 5×10^6 generations and four Markov
148 chain Monte Carlo (MCMC) chains. Convergence was assessed in TRACER 1.6 (Rambaut et al., 2014).
149 Maximum likelihood analyses (ML) were performed in PHYML 3.0 (Guindon et al., 2010), with 1,000
150 bootstrap replications. Genealogical relationships among haplotypes were estimated using median joining

151 networks (Bandelt *et al.*, 1999).

152

153 *Species tree and divergence time estimates*

154 A species tree was reconstructed using the multilocus dataset in *BEAST, as implemented in BEAST 1.8.1
155 (Heled & Drummond, 2010). The seven main mitochondrial lineages (Fig.1) were assigned as “species” in
156 the analysis. The lognormal distribution for the relaxed uncorrelated rates and Yule process were used for all
157 genes. The substitution rate of cytochrome b (2.1% divergence per million years, Weir & Schluter, 2008)
158 was used for time calibration, and rates for the remaining markers were estimated. We ran the analysis for
159 2×10^8 generations and sampled every 1,000 generations. Another *BEAST analysis was run including the
160 five *Gymnophis* species (see Fig. 1) to date the origin of the *Rhegmatorhina* lineage.

161 As there was extensive *Fib5* allele sharing among species (see results), and the *BEAST species tree
162 method does not account for gene flow after isolation (Heled & Drummond, 2010), divergence times were
163 also estimated in a gene tree framework, using only the mtDNA genes in BEAST 1.8.1 (Drummond &
164 Rambaut, 2007). The analysis was run for 5×10^7 generations and time calibrated as described above.

165 In addition, divergence times and migration rates were co-estimated under an isolation-with-
166 migration model (Hey & Nielsen, 2004), implemented by IMA2 (Hey, 2010). We performed the analysis
167 independently for pairs of taxa with adjacent distributions (total of 8 pairs, see Appendix S2 for details).

168

169 *Demographic analysis*

170 Significant deviations from neutral evolution and constant population sizes were tested for using summary
171 statistics (see Appendix S2 for details). Historical fluctuations of population sizes were estimated using
172 extended Bayesian skyline plots (EBSPs) in BEAST (Heled & Drummond, 2010) with fifty million
173 generations and an uncorrelated lognormal molecular clock with the *cytb* substitution rate of 2.1% per
174 million years (Weir and Schluter, 2008).

175

176 **Results**

177 *Sequence Data and Genetic diversity*

178 A total of 2945 bp were sequenced (Table S2.2 in Appendix 2). The nuclear locus showed no recombination
179 ($p=0.684$) and higher intraspecific nucleotide diversity (Table S2.3 in Appendix 2). *Rhegmatorhina*
180 *melanosticta* A and B had significantly higher genetic diversity for *cytb* and *ND2* (p -values of 0.042 and
181 0.035 respectively).

182

183 *Phylogenetic inferences*

184 The mitochondrial dataset strongly supported the monophyly of *Rhegmatorhina*, and a sister relationship to
185 the clade formed by (*G. bicolor*, (*G. leucaspis*, *G. rufigula*)) (Fig. 1). Within *Rhegmatorhina* there are two
186 well-supported clades separated by the Madeira River (Fig. 1). The western clade includes *R. cristata*, from
187 the Jaú area of endemism (Japurá-Negro interfluve, Borges and Silva 2011) and *R. melanosticta*, from
188 western Amazonia, which was subdivided into two main clades separated by the Solimões and Ucayali rivers

189 (Fig. 1). The eastern clade includes *R. gymnops* from the Tapajós area of endemism (Tapajós-Xingú
190 interfluve), and *R. hoffmannsi* and *R. berlepschi* from the Rondônia area of endemism (Madeira-Tapajós
191 interfluve). The recovered sister relationship between *R. hoffmannsi* and *R. berlepschi* had moderately high
192 support (0.98/72). Two strongly supported clades were also found within *R. hoffmannsi*, yet they currently
193 are not separated by any geographic barrier and co-occur in locality H5 (Cachoeira de Nazaré, on the West
194 bank of Rio Ji-Paraná, Fig. 1).

195

196 *Haplotype networks, coalescence, and phylogenetic information*

197 No shared haplotypes among the seven lineages were found in the mtDNA markers (Fig. 2 a-c). In contrast,
198 the nuclear gene revealed one most common haplotype found in all species but *R. cristata*, plus three shared
199 haplotypes between the two *R. hoffmannsi* lineages, one between *R. melanosticta* and *R. cristata*, and one
200 between *R. hoffmannsi* and *R. berlepschi* (Fig. 2d).

201 These results, and our dating analysis discussed below, indicate that diversification of crown
202 *Rhegmatorhina* is relatively young, dating to less than ~2.0 million years ago (Ma). The haplotype networks
203 show that taxonomic structure exists in mitochondrial DNA but not in the nuclear marker, consistent with
204 population genetic predictions of their relative coalescent times (Zink and Barrowclough 2008; Hung et al.
205 2016). Our results for *Rhegmatorhina* are consistent with those found in the Amazonian clade *Psophia*
206 (Ribas et al. 2012) in which a nuclear marker was only capable of resolving the oldest divergence (~2.0-2.7
207 Ma). Species of *Rhegmatorhina* are common monogamous understory birds, and would be expected to have
208 a larger effective population size (N_e) relative to species of *Psophia*, which have smaller census populations
209 and are co-operatively polyandrous, thus further suggesting that nuclear loci would not be predicted to
210 coalesce in *Rhegmatorhina* over this short time-period. Thus, for reasons of age and population size,
211 mitochondrial DNA is more appropriate than a single or a few nuclear markers for recovering evolutionary
212 relationships within the *Rhegmatorhina* species-complex. A genomic perspective on the evolution of the
213 genus will only be informative if it relies on thousands of independently segregating loci (Harvey and
214 Brumfield 2014).

215

216 *Species tree and divergence time estimates*

217 The species tree corroborated the presence of two well-supported clades, yet did not fully support the internal
218 relationships as recovered by the mitochondrial gene tree (Fig. S3.1 in Appendix 3). Dating analysis based
219 only on the mtDNA dataset using a gene tree approach in BEAST results in older dates when compared to
220 those estimated by the species tree (Fig. 3). The species tree analysis including outgroups suggests an
221 estimated date for the origin of the *Rhegmatorhina* lineage at about 5Ma (Fig. S3.2 in Appendix 3).

222 Migration rates and divergence times were estimated under a coalescent framework for seven pairs
223 of *Rhegmatorhina* species with adjacent distributions (Table S5). It was not possible to estimate parameters
224 between the two *R. hoffmannsi* lineages, probably due to their shallow divergence. Estimated divergence
225 times fall within the confidence intervals obtained in both the species tree and mtDNA dating analysis, but
226 are in general more congruent with the mtDNA dating. In the eastern clade, divergence between *R.*

227 *berlepschi* and *R. hoffmannsi* A (point estimate at 0.5Ma) was more recent, and migration rates were higher
228 than between *R. berlepschi* and *R. gymnops* (0.6Ma), agreeing with the mtDNA topology and supporting a
229 sister relationship between *R. berlepschi* and *R. hoffmannsi*. Within the western clade, the divergence
230 between *R. cristata* and *R. melanosticta* B was estimated at about 1 Ma, whereas the divergence between the
231 two *R. melanosticta* lineages was estimated at about 0.82 Ma; both dates are in agreement with the mtDNA
232 dating and older than the dates estimated by the species tree analysis (Fig. 3). The highest unidirectional
233 migration rates were from *R. melanosticta* A to *R. cristata* and from *R. melanosticta* A to B. However, all
234 HPDs for migration estimates included zero, thus not excluding the possibility of no migration after
235 divergence. The possible presence of migration between the two pairs of taxa analyzed within the western
236 clade and the fact that IMA dating is congruent with the mtDNA dating suggest that migration after
237 divergence may have affected the time estimates produced by the species tree analysis, as *BEAST does not
238 consider the possibility of migration after isolation, considering all similarities among species as ancestral
239 polymorphism (Heled & Drummond, 2010).

240

241 *Historical demographic analysis*

242 The EBSP analyses indicated increasing population sizes for most species, with the exception of *R.*
243 *cristata* and *R. melanosticta* A (Fig. 4). There is evidence of gradual population growth in the last 50,000-
244 150,000 years, with *R. berlepschi* exhibiting the sharpest increase among all species (Fig. 4). *Rhegmatorhina*
245 *cristata* and *R. melanosticta* A showed no significant deviations of the null hypothesis of neutrality and
246 constant population for any marker, while *R. berlepschi* had significant deviations for all tests for both cyt b
247 and ND2. The remaining species showed indications of expanding populations in some loci (Table S2.3 in
248 Appendix 2).

249

250 **Discussion**

251 *Distribution, diversity and taxonomy*

252 Dense sampling throughout the distribution of *Rhegmatorhina* confirmed that the main Amazonian rivers
253 delimit the geographical distributions of most taxa as inferred from mtDNA lineages. *Rhegmatorhina*
254 *gymnops* occupies a larger range than previously reported (Zimmer and Isler, 2003), with the Juruena river as
255 the western limit to its distribution (Fig. 1). However, recent genomic analyses of samples from the southern
256 limit of this distribution (at the Teles Pires river) have revealed a hybrid individual between *R. gymnops* and
257 *R. hoffmannsi* (Weir *et al.*, 2015). According to our mtDNA topology, *R. gymnops* and the eastern lineage of
258 *R. hoffmannsi* (A) are not sister taxa and IMA analyses showed migration estimates close to zero in both
259 directions (Table S2.4 in Appendix 2). In addition, no hybrid specimens or specimens outside of the expected
260 distribution ranges have been reported at the field or in scientific collections. This may indicate that although
261 hybridization between *R. gymnops* and *R. hoffmannsi* (A) occurs in the headwaters of the Tapajós River, it
262 has had little effect on the phenotypes of these two species.

263 Our sampling of *R. berlepschi* extends south within the Madeira-Tapajós interfluvium up to
264 Jacareacanga (B4). Our northernmost sample of *R. hoffmannsi* is from the left margin of the Aripuanã River

265 (H2), but Willis (1969) reported its occurrence as north as the Abacaxis river, a Madeira tributary, indicating
266 that the two taxa occur at localities less than 200km apart. More detailed sampling is needed to understand
267 this additional potential contact zone.

268 Most surprising, there are two distinct lineages of *R. hoffmannsi* that overlap, with individuals from
269 both lineages at the same locality (H5) and in two adjacent localities close to Porto Velho (H1 and H8).
270 Denser sampling is needed to better understand the origin of this mtDNA structure, which may be related to
271 major drainage capture events involving the Madeira River and its tributaries, as inferred previously for
272 several avian lineages (Fernandes *et al.*, 2012, 2014; Sousa-Neves *et al.*, 2013; Ferreira *et al.*, 2017; see also
273 below). Detailed morphological analyses may help clarify whether there is more than one diagnosable
274 phenotype involved, but visual inspection of *R. hoffmannsi* skins at the MPEG and INPA collections (about
275 70 specimens total) did not reveal any obvious difference in plumage patterns (AA, CCR and M. Cohn-Haft
276 personal observation).

277 Two divergent mtDNA lineages are also evident within *R. melanosticta*, with their distributional
278 limits clearly associated with the Solimões-Ucayali Rivers. The nuclear dataset reveals two shared
279 haplotypes between the two lineages and the dating analysis suggests they have been isolated for about
280 0.8Ma (Fig. 3 and Table S2.4). The south-eastern mtDNA lineage (*R. melanosticta* B, M7-M22, Fig. 1)
281 corresponds to *R. m. purusiana* (type locality Cachoeira, on the Purus River, nearest to sampling locality
282 M21; Peters, 1951). Another previously recognized subspecies (*R. m. badia*; type locality La Pampa, nearest
283 to M22) was recently merged with *R. m. purusiana* (Zimmer and Isler, 2003), in agreement with our results.
284 Within the range of the north-western mtDNA lineage (*R. melanosticta* A M1-M6, Fig.1) there are two
285 described subspecies, separated by the Marañon river (*R. m. melanosticta* and *R. m. brunneiceps*). We find
286 only moderate support for this as samples from localities M3 and M5, corresponding to *R. m. brunneiceps*
287 (type locality Moyobamba, nearest to M5; Peters, 1951), form a clade that also includes one individual from
288 M4, that has a phenotype typical of *R. m. melanosticta* (RB personal observation) and occurs within its range
289 (north of the Marañon, type locality Sarayacu, nearest to M6). This result may suggest gene flow between *R.*
290 *m. melanosticta* and *R. m. brunneiceps*, despite noticeable phenotypic differences (crown color, Zimmer and
291 Isler, 2003), as has been found for several other species pairs separated by the Marañon river (Winger and
292 Bates, 2015).

293 Based on the evidence presented above, there are two distinct reciprocally monophyletic lineages
294 within *R. melanosticta* corresponding to *R. m. melanosticta* and *R. m. purusiana* (the names with priority),
295 while the status of *R. m. brunneiceps* remains to be investigated in more detail. The fact that the
296 morphologically more divergent *R. m. brunneiceps* individuals group with *R. m. melanosticta* underscores a
297 conflict between phenotypic and genetic traits (not observed in other *Rhegmatorhina* taxa), and the
298 comparatively higher migration rate estimated from *R. melanosticta* A into *R. melanosticta* B suggests that
299 introgression could have originated this pattern. Therefore, given this conflict, we refrain from splitting *R.*
300 *melanosticta* at this point, despite the reciprocal monophyly between *R. melanosticta* A and *R. melanosticta*
301 B, but recommend that future studies with denser sampling in terms of specimens and markers further
302 investigate the issue, as there is clearly more than one evolutionary unit involved.

303 *Rhegmatorhina cristata* is a poorly known species that occurs in the recently described Jaú area of
304 endemism, and is sparsely represented in biological collections. Our results show that it has high haplotype
305 and nucleotide diversity and shares one nuclear haplotype with both *R. melanosticta* lineages. Recent field
306 data confirms that the Japurá river is the distributional limit between *R. melanosticta* and *R. cristata*, with
307 individuals of each species being captured at the southern (*R. melanosticta*) and northern (*R. cristata*)
308 margins of this river in Vila Bittencourt, close to the Brazilian/Colombian border (M. Cohn-Haft, personal
309 communication).

310

311 *Establishment of upland forests in western Amazonia*

312 *Rhegmatorhina* and *Gymnopithys* species (Fig. 1 and S3.2) occur across the entire Amazon basin, Chocó and
313 Central America. The complex (*G. leucaspis*, *G. bicolor*, *G. rufigula*) is sister to *Rhegmatorhina* with good
314 support and includes altogether 12 recognized subspecies distributed in northern Amazonia, Chocó and
315 Central America. Although *G. bicolor* has previously been considered a subspecies of *G. leucaspis*, the *G.*
316 *leucaspis* individual included in our analysis is sister to *G. rufigula* with high support, indicating an expected
317 closer relationship between taxa that occur east of the Andes. *Gymnopithys lunulata* and *G. salvini* form the
318 sister clade to the above group, and occur in western Amazonia (Napo and Inambari areas of endemism
319 respectively), probably separated by the same rivers (Solimões-Ucayali) that demarcate the distribution
320 limits between *R. melanosticta* and *R. purusiana*. The *Rhegmatorhina-Gymnopithys* clade includes at least
321 six taxa from the western Amazonian areas of endemism (Napo and Inambari). Both the distribution of
322 *Gymnopithys* species, mostly on the western portion of the Amazon basin, and the significantly higher
323 intraspecific diversity found in both *cytb* and *ND2* for *R. melanosticta* (Table S2.3) suggest that the ancestor
324 to *Rhegmatorhina* had a western Amazonian distribution, occupying upland *Terra Firme* forests on the
325 Andean slopes and possibly in western lowlands. The fact that there is no *Rhegmatorhina* taxon east of the
326 Negro and Xingu rivers is consistent with a western ancestral distribution of this genus, although extinction
327 could produce the same pattern. Taxonomic uncertainty and lack of sampling for *Gymnopithys* precludes a
328 biogeographic analysis of the whole group, but its initial diversification was probably affected by Pliocene
329 landscape evolution in western Amazonia. It is still debated in the literature when upland (*Terra Firme*)
330 forest was established in western Amazonia. Although some authors think this may have happened toward
331 the end of the Miocene (Hoorn *et al.*, 2010), the diversification history of this clade seems to support the
332 alternative scenario of a dynamic landscape during the Plio-Pleistocene with more recent establishment of
333 this habitat (Latrubesse *et al.* 2010; Nogueira *et al.*, 2013).

334

335 *Diversification across the Madeira River*

336 The first split within *Rhegmatorhina* separated Western Amazonian (*R. cristata* and *R. melanosticta*)
337 taxa from Brazilian shield taxa (*R. berlepschi*, *R. gymnops* and *R. hoffmannsi*). These clades are currently
338 separated by the Madeira River. The age of this split (about 1.6/1.8 Ma, Fig. 3 and S3.1) coincides with the
339 split associated with this same river within *Psophia* (*Psophia leucoptera* versus Brazilian shield clade), dated
340 at about 1.6 Ma (CI 1.1-2.1) (Ribas *et al.* 2012). Splits between at least nine other clades of Amazonian

341 *Terra Firme* understory birds (genera *Willisornis*, *Schiffornis* and *Hylophylax*, and complexes *Myrmeciza*
342 *hemimaelena*, *Automolus ochrolaemus*, *A. infuscatus*, *Sclerurus caudacutus*, *Xiphorhynchus*
343 *pardalotus/ocellatus* and *Thamnophilus aethiops*) with distributions delimited by the Madeira river also
344 diverged from about 0.2 to about 2 Ma (Fernandes *et al.*, 2012, 2014; Smith *et al.*, 2014; d'Horta *et al.*, 2013;
345 Souza-Neves *et al.* 2013; Thom & Aleixo, 2015; de Deus Schultz *et al.* 2017), or have substantial genetic
346 differentiation on opposite margins of the Madeira River (*Drymophila*, Bates *et al.* 1999). Despite the
347 variation in ages, it is clear that diversification within all these groups of birds from the same geographic
348 region and with distributions currently delimited by the same barrier (the Madeira river) occurred during the
349 last 2 Ma. This date corresponds quite well to the history of the Madeira River itself. The origin of the
350 Madeira River is probably related to the Pliocene orogeny of the Fitzcarrald arch, due to the subduction of
351 the Nazca plate (Espurt *et al.*, 2010). The subduction started at about 4 Ma, and the uplift of the arch is
352 subsequent to that. This uplift probably initiated a process that resulted in the connection of the lower
353 Madeira with the Amazon River in the upper Pliocene/Pleistocene. The differences among estimated ages for
354 the splits in different groups may have several causes, including differential dispersal capacity of each group
355 (Smith *et al.*, 2014), more recent changes in river course due to neotectonics (Latrubesse, 2002; Hayakawa &
356 Rossetti, 2015), and/or recent changes in river discharge due to changes in precipitation regimes through
357 time (Govin *et al.* 2014, Wang *et al.* 2017). Although all the understory birds cited above occupy similar
358 habitats, the few studies that have addressed their sensitivity to fragmentation have shown there are
359 differences among them (Ferraz *et al.*, 2007), which may have influenced the way each one responded to the
360 formation or change in position of geographic barriers.

361

362 *Contrasting effects of landscape evolution in western and eastern Amazonia*

363 The three lineages from the western clade have their distributions delimited by the Japurá and the
364 Solimões-Ucayali rivers. The origin of *R. cristata* at about 0.6Ma and the split between the two *R.*
365 *melanosticta* lineages at about 0.4 Ma probably occurred in the absence of considerable changes in climate,
366 considering that western Amazonia is probably less affected by drought in glacial periods (Cheng *et al.*,
367 2013) and that, consistent with this scenario, there is no signal of recent demographic change in *R. cristata*
368 and *R. melanosticta* A (Fig. 4, Table S4). This points to drainage evolution as one possible driver of
369 diversification and is in agreement with a Plio-Pleistocene establishment of upland forest in western
370 Amazonia due to the entrenchment of the drainage system (Latrubesse *et al.*, 2010, Nogueira *et al.*, 2013).

371 Diversification in eastern Amazonia may have occurred under a different environmental regime, with
372 marked periods of aridity in glacial times (Wang *et al.* 2017), reflected in the signal of recent population
373 expansion for all four lineages in the eastern clade during the last 50 thousand years (Fig. 4). This result
374 corroborates the suggestion of Weir *et al.* (2015) that a combination of climate change and riverine barriers
375 (River-Refuge "hypothesis", Ayres & Clutton-Bock, 1992) could be related to diversification on opposite
376 margins of the Tapajós, with repeated cycles of isolation in drier periods and contact in humid periods
377 ultimately leading to speciation. The river-refuge mechanism predicts that barriers would be stronger in dry
378 periods due to the association between physical (rivers) and ecological (open vegetation) factors. In addition,

379 during dry periods river discharge may have decreased (Govin et al 2014), lowering the strength of the lower
380 river courses as barriers to dispersal, but also originating less incised river channels and larger floodplains
381 that also constitute barriers for upland *Terra Firme* birds.

382 The existence of three different lineages within the Rondônia area of endemism (Madeira-Tapajós
383 interfluve) highlights the complexity of this region already noted in previous phylogenetic studies (Ribas *et*
384 *al.*, 2012; Fernandes *et al.*, 2014). Willis (1969) commented on this complexity based on the distribution of
385 *Rhegmatorhina* taxa within the interfluve, and attributed the parapatry of *R. berlepschi* and *R. hoffmannsi* to
386 occasional changes in river directionality within the Madeira and Tapajós basins. Here we show that the
387 pattern first described by Willis is even more complex, involving three instead of two lineages. Latrubesse
388 (2002) and Hayakawa & Rossetti (2015) report evidence for a recent establishment of the current course of
389 the Aripuanã river, describing palaeochannels that indicate that drainage was previously distributed by other
390 smaller rivers, such as the Manicoré. The river capture and consequent establishment of the Aripuanã is a
391 possible mechanism of recent isolation of understory bird populations. Our samples collected between the
392 Aripuanã and Manicoré rivers (from sites H2, H3 and H4) all group in the *R. hoffmannsi* A clade and are
393 identified as *R. hoffmannsi*. Interestingly, our dating analysis suggests that the split between *R. hoffmannsi*
394 and *R. berlepschi*, which corresponds spatially to this river capture, occurred during the Pleistocene (about
395 0.7 Ma, Fig. 3), as suggested by Latrubesse (2002).

396 The other river reported as being a barrier to understory bird distributions within the Madeira-
397 Tapajós interfluve is the Ji-Paraná River (Fernandes *et al.*, 2014). The two *R. hoffmannsi* lineages seem to be
398 sympatric in portions of their ranges, as *R. hoffmannsi* A occurs on both margins of the Ji-Paraná River,
399 while *R. hoffmannsi* B was not collected on the eastern margin. According to Hayakawa & Rossetti (2015)
400 the Ji-Paraná River drained northward and was located near 60km eastward from its modern position, with
401 several changes in its course in the recent past generating a radial palaeodrainage network. This dynamic
402 history, associated with changes in forest distributions during the Pleistocene, may have caused differential
403 isolation and contact among populations, and the scenario we describe here may be the result of the most
404 recent changes in drainage configuration and forest cover.

405 406 *Amazonian diversification in time and space*

407 It is becoming clear in the literature that the major problem precluding a better understanding of
408 diversification within Amazonia is trying to find single biogeographic explanations for the whole biome.
409 Palaeoclimate models indicate that Eastern and Western Amazonia follow different climatic patterns, with a
410 modest increase in precipitation in western Amazonia and a significant drying in eastern Amazonia during
411 the last glacial (Cheng *et al.*, 2013; Wang *et al.* 2017). The geological history of these regions is also very
412 distinct, with western Amazonia apparently being covered by a large wetland system for a large portion of
413 the recent past, and eastern Amazonia being dominated by drainage systems in the uplands of the Brazilian
414 and Guiana shields (Hoorn *et al.*, 2010; Latrubesse *et al.*, 2010; Nogueira *et al.*, 2013). The models of
415 drainage evolution suggest that the western and eastern drainages were isolated during a large portion of
416 their recent evolution (Figueiredo *et al.*, 2009; Nogueira *et al.*, 2013). In light of these differences, we should

417 not expect a single process to be the main driver of Amazonian diversification, and it is not realistic to accept
418 or reject "universal" hypotheses as the "Refugia Hypothesis" or the "Riverine Hypothesis".

419 Our analysis of *Rhegmatorhina* exemplifies another instance of Pleistocene diversification in an
420 Amazonian genus restricted to *Terra Firme* understory forest. Based on broad sampling, a well-resolved
421 taxonomy, and strongly supported phylogenetic relationships we show that mitochondrial lineages
422 corroborate the spatial patterns known for decades based on phenotypes of upland forest Amazonian birds in
423 that they are geographically delimited by the large Amazonian rivers (Haffer, 1969; Cracraft 1985). Our
424 results also corroborate palaeoclimatic records obtained so far for the Amazon basin, with a signal of recent
425 population expansion for all taxa, except for those from North-Western Amazonia (*R. cristata* and *R.*
426 *melanosticta*) (Fig. 4, Table S4), a region that was less affected by past climatic oscillations (Cheng *et al.*,
427 2013; Wang *et al.* 2017). These demographic changes are too recent to be related to taxic origination, but this
428 may indicate that southern and eastern Amazonian taxa associated with upland wet forests would also be
429 susceptible to previous cycles of drier climate. The association between past demographic changes related to
430 palaeoclimatic cycles and the historically varying strength of rivers as barriers to dispersal may be the path to
431 the answer to the long-standing question of which are the drivers of Amazonian diversification. Instead of
432 testing the "general hypotheses" of drainage evolution *versus* climate change, we should try to integrate both,
433 spatially and temporally, and explicitly relate them to biological diversification data.

434

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576 **Data Accessibility**

577 All DNA sequences and alignments generated for this study are available from GenBank
578 (<https://www.ncbi.nlm.nih.gov/genbank/>).

580 **Biosketch**

581 The authors are broadly interested in the biogeography of the Neotropics, with emphasis on Amazonian
582 birds. This work represents work started by CCR as a F.M. Chapman Post Doctoral Fellow at the American
583 Museum of Natural History. Author contributions: CCR and JC conceived the study; CCR, FMH, RB and
584 AA collected part of the samples; CCR, CG and FMH collected data and performed the analyses; CCR, JC
585 and CG wrote the paper with input from all other authors.

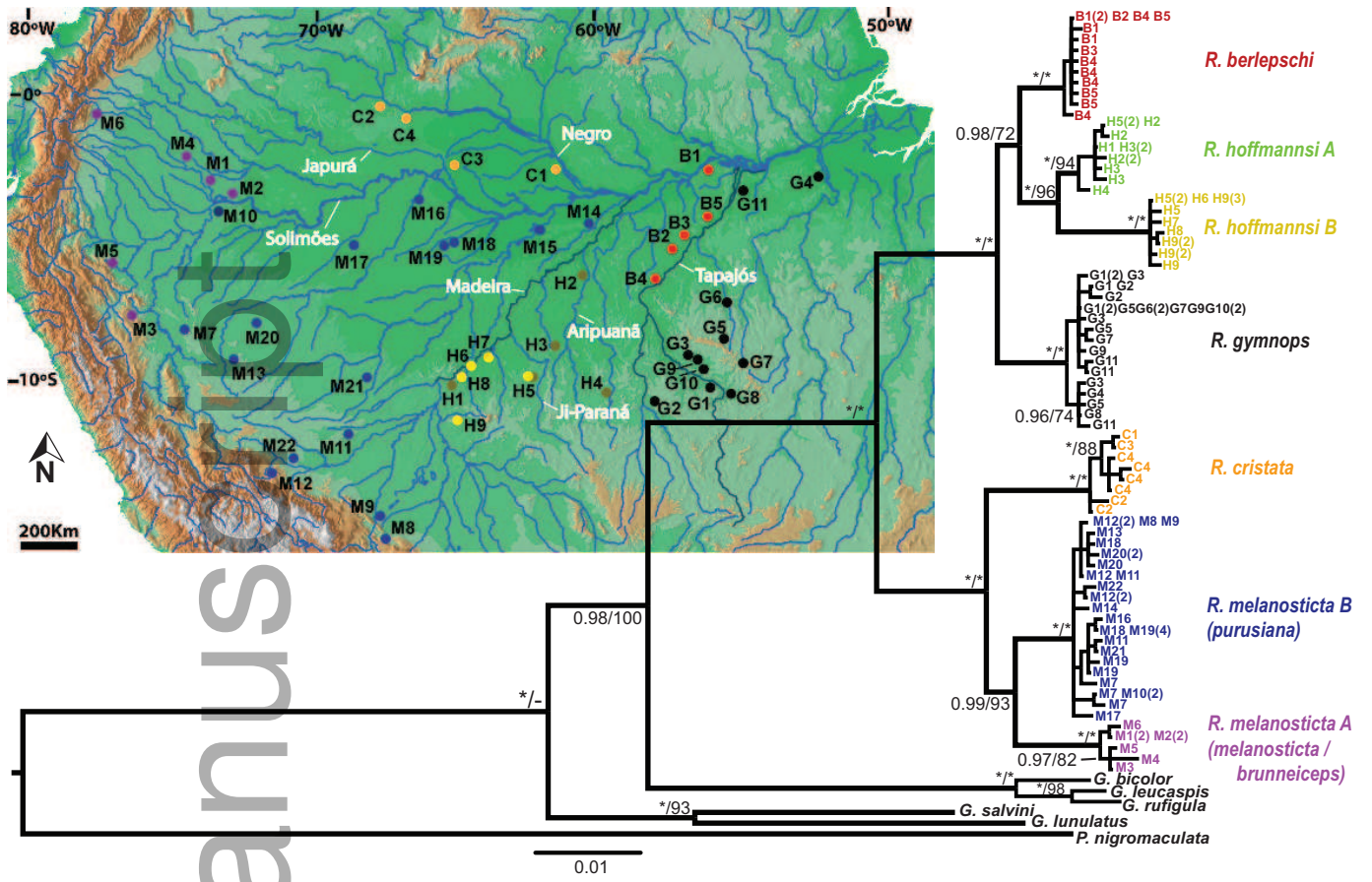
587 **Figure legends**

588 Figure 1. Sampling localities and molecular phylogeny of the genus *Rhegmatorhina* based on Bayesian
589 analysis of the mtDNA genes Cyt b, ND2 and ND3 (2440 bp). Maximum likelihood analysis yields the same
590 topology. Numbers on nodes are posterior probabilities / ML bootstrap. * indicates maximum support values.

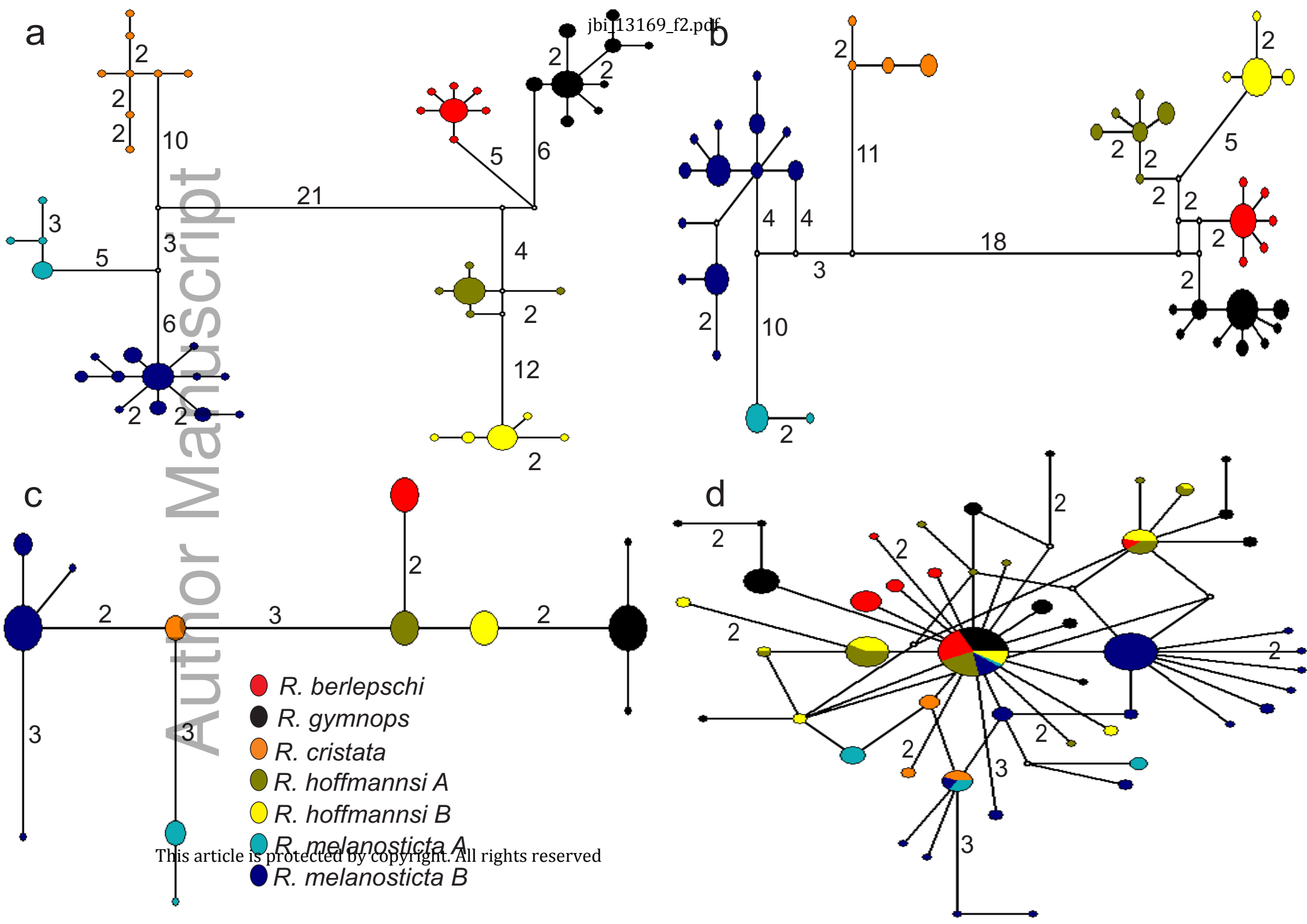
592 Figure 2. Median joining haplotype networks for (a) Cytb, 119 individuals, 1050 bp (b), ND2, 116
593 individuals, 1000 bp, (c) ND3, 120 individuals, 390 bp, and (d) Fib5, 107 individuals, 505 bp.

595 Figure 3. BEAST chronogram based on mtDNA. Values above branches are the mean of the node height
596 (age) based on a calibration rate of 2.1% divergence per M.Y. for cytochrome b. Mean values obtained in the
597 species tree analysis, when available, are shown below the values found on the BEAST analysis. Bars show
598 the 95% HPD. Values below branches are posterior probabilities, shown only when larger than 0.95.

600 Figure 4. Extended Bayesian skyline plots (EBSPs) estimated in BEAST based on mtDNA and nuclear
601 sequence data. Time scale in millions of years before present.



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