

1 **Comparative phylogeography reveals widespread cryptic diversity driven by ecology in**
2 **Panamanian birds**

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50 We primarily used existing museum specimens, with additional material collected under permits
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52

53 *Author contributions-*

54 JFM and MJM conceived the study, based on DNA barcoding efforts initiated by MJM, KS,
55 LDL, and OLC and with input from SEL, RD, and JRL. JFM wrote the initial draft with input
56 from MJM. All authors then contributed to later drafts. Sequencing was carried out by CA and
57 WGN-G. Data processing and analysis was conducted by JFM, LCA, AA, BA, RC, AT, SJV,
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59

60 *Data depository-* Mitochondrial COI sequences are available in the Barcode of Life Database
61 (DS-BSPUSNM). Scripts used for analysis are available on Dryad. Raw sequencing reads used
62 to generate whole mitogenomes are available at on the NCBI Short Read Archive (BioProject
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64

65 **ABSTRACT**

66 Widespread species often harbor unrecognized genetic diversity, and investigating the factors
67 associated with such cryptic variation can help us better understand the forces driving
68 diversification. Here, we identify potential cryptic species based on a comprehensive dataset of
69 COI mitochondrial DNA barcodes from 2,333 individual Panamanian birds across 429 species,
70 representing 391 (59%) of the 659 resident landbird species of the country, as well as

71 opportunistically sampled waterbirds. We complement this dataset with additional publicly
72 available mitochondrial loci, such as ND2 and cytochrome *b*, obtained from whole mitochondrial
73 genomes from 20 taxa. Using barcode identification numbers (BINs), we find putative cryptic
74 species in 19% of landbird species, highlighting hidden diversity in the relatively well-described
75 avifauna of Panama. Whereas some of these mitochondrial divergence events corresponded with
76 recognized geographic features that likely isolated populations, such as the Cordillera Central
77 highlands, the majority (74%) of lowland splits were between eastern and western populations.
78 The timing of these splits are not temporally coincident across taxa, suggesting that historical
79 events, such as the formation of the Isthmus of Panama and Pleistocene climatic cycles, were not
80 the primary drivers of cryptic diversification. Rather, we observed that forest species, understory
81 species, insectivores, and strongly territorial species—all traits associated with lower dispersal
82 ability—were all more likely to have multiple BINs in Panama, suggesting strong ecological
83 associations with cryptic divergence. Additionally, hand-wing index, a proxy for dispersal
84 capability, was significantly lower in species with multiple BINs, indicating that dispersal ability
85 plays an important role in generating diversity in Neotropical birds. Together, these results
86 underscore the need for evolutionary studies of tropical bird communities to consider ecological
87 factors along with geographic explanations, and that even in areas with well-known avifauna,
88 avian diversity may be substantially underestimated.

89

90 *Keywords:* barcoding, biogeography, cryptic species, dispersal, tropical biodiversity

91

92 **LAY SUMMARY**

- 93 - What factors are common among bird species with cryptic diversity in Panama? What
94 role do geography, ecology, phylogeographic history, and other factors play in generating
95 bird diversity?
- 96 - 19% of widely-sampled bird species form two or more distinct DNA barcode clades,
97 suggesting widespread unrecognized diversity.
- 98 - Traits associated with reduced dispersal ability, such as use of forest understory, high
99 territoriality, low hand-wing index, and insectivory, were more common in taxa with
100 cryptic diversity.

101

102 **Filogeografía comparada revela amplia diversidad críptica causada por la ecología en las**
103 **aves de Panamá**

104

105 **RESUMEN**

106 Especies extendidas frecuentemente tiene diversidad genética no reconocida, y investigando los
107 factores asociados con esta variación críptica puede ayudarnos a entender las fuerzas que
108 impulsan la diversificación. Aquí, identificamos especies crípticas potenciales basadas en un
109 conjunto de datos de códigos de barras de ADN mitocondrial de 2,333 individuos de aves de
110 Panamá en 429 especies, representando 391 (59%) de las 659 especies de aves terrestres
111 residentes del país, además de algunas aves acuáticas muestreada de manera oportunista.
112 Adicionalmente, complementamos estos datos con secuencias mitocondriales disponibles
113 públicamente de otros loci, tal como ND2 o citocroma b, obtenidos de los genomas
114 mitocondriales completos de 20 taxones. Utilizando los números de identificación de código de
115 barras (en ingles: BINs), un sistema taxonómico numérico que proporcina una estimación

116 imparcial de la diversidad potencial a nivel de especie, encontramos especies crípticas putativas
117 en 19% de las especies de aves terrestres, lo que destaca la diversidad oculta en la avifauna bien
118 descrita de Panamá. Aunque algunos de estos eventos de divergencia conciden con
119 características geográficas que probablemente aislaron las poblaciones, la mayoría (74%) de la
120 divergencia en las tierras bajas se encuentra entre las poblaciones orientales y occidentales. El
121 tiempo de esta divergencia no coincidió entre los taxones, sugiriendo que eventos históricos tales
122 como la formación del Istmo de Panamá y los ciclos climáticos del pleistoceno, no fueron los
123 principales impulsores de la especiación. En cambio, observamos asociaciones fuertes entre las
124 características ecológicas y la divergencia mitocondrial: las especies del bosque, sotobosque,
125 con una dieta insectívora, y con territorialidad fuerte mostraron múltiples BINs probables.
126 Adicionalmente, el índice mano-ala, que está asociado a la capacidad de dispersión, fue
127 significativamente menor en las especies con BINs múltiples, sugiriendo que la capacidad de
128 dispersión tiene un rol importante en la generación de la diversidad de las aves neotropicales.
129 Estos resultados demuestran la necesidad de que estudios evolutivos de las comunidades de aves
130 tropicales consideren los factores ecológicos en conjunto con las explicaciones geográficas.

131

132 *Palabras clave:* biodiversidad tropical, biogeografía, códigos de barras, dispersión, especies
133 crípticas

134

135 **INTRODUCTION**

136

137 Identifying the number of species in a given region is key to understanding the evolution and
138 maintenance of biodiversity (Bickford *et al.* 2007; Allendorf *et al.* 2009; Pérez-Ponce de León

139 and Nadler 2010). Yet characterizing species diversity remains a crucial challenge in biodiversity
140 research (De León *et al.* 2023), and cryptic species often go unrecognized as distinct due to their
141 high similarity (Fouquet *et al.* 2007; Yan *et al.* 2018; Chenuil *et al.* 2019; Levy and Cox 2020).
142 Despite this difficulty, the identification of cryptic species may illuminate evolutionary processes
143 that generate biodiversity (Campbell, Braile, and Winker 2016; Bickford *et al.* 2007; Barreira,
144 Lijtmaer, and Tubaro 2016; Struck *et al.* 2018). The investigation of cryptic species is often
145 framed as an issue of not adequately describing and cataloging variation (de León and Nadler
146 2010; Korshunova *et al.* 2017), but studies of cryptic taxa present an opportunity to understand
147 why some species diversify while remaining morphologically conserved (Roux *et al.* 2016;
148 Pulido-Santacruz, Aleixo, and Weir 2018). By linking the diversity of cryptic variation with
149 geography, history, ecology, or a combination of those factors, researchers can better understand
150 what drives the generation of underestimated variation in cryptic species (Bickford *et al.* 2007;
151 Struck *et al.* 2018). Considered together, these sources of variation can provide valuable insights
152 into speciation and diversification, with implications for conservation decision-making (Bickford
153 *et al.* 2007; Saitoh *et al.* 2015; Campbell, Braile, and Winker 2016; Struck *et al.* 2018).

154 Cryptic diversity is of particular interest in regions with high levels of biodiversity. The
155 Neotropics are home to incredible avian species diversity, with around one in four global bird
156 species found in the region (Haffer 1985; Orme *et al.* 2005). However, this is likely an
157 underestimate, given that studies frequently find species-level genetic diversity, even within
158 recognized species (Tavares *et al.* 2011; Milá *et al.* 2012; Rheindt, Cuervo, and Brumfield 2013;
159 Mendoza *et al.* 2016). This is often the case in widely distributed species, which can harbor
160 extensive genetic variation characteristic of species complexes, such as in *Lepidothrix* (Cheviron,
161 Hackett, and Capparella 2005), *Manacus* (Brumfield *et al.* 2008), *Habia* (Lavinia *et al.* 2015;

162 Ramírez-Barrera *et al.* 2018, 2019), *Pachyramphus* (Musher and Cracraft 2018), *Malacoptila*
163 (Ferreira *et al.* 2017), *Arremon* (Cadena, Klicka, and Ricklefs 2007; Navarro-Sigüenza *et al.*
164 2008; Cadena and Cuervo 2010) and *Phaeothlypis* (Lovette 2004). This underestimation of avian
165 biodiversity across hampers our understanding of the processes that have made the Neotropics
166 such an important hotspot of species diversification (Bickford *et al.* 2007), and it has practical
167 implications for conservation (Bickford *et al.* 2007; Allendorf *et al.* 2009; Valentini, Pompanon,
168 and Taberlet 2009; Lohman *et al.* 2010; Funk, Caminer, and Ron 2012; Crawford *et al.* 2013;
169 Gonçalves *et al.* 2015; Mendoza *et al.* 2016). Although there is some risk that the search for
170 cryptic species may lead to oversplitting and taxonomic inflation (Chaitra, Vasudevan, and
171 Shanker 2004; Isaac, Mallet, and Mace 2004; Hundsdoerfer *et al.* 2019; Chan *et al.* 2020),
172 evidence in birds suggests that their diversity is underestimated (Sangster 2009; Barrowclough *et*
173 *al.* 2016). Moreover, even if such cryptic taxa may not represent distinct species, they are distinct
174 populations on their own evolutionary trajectories, and identifying such population-level
175 differences can allow us to understand different parts of the speciation continuum.

176 Mitochondrial barcoding provides us with a powerful tool to detect cryptic diversity
177 (Arnot, Roper, and Bayoumi 1993; Floyd *et al.* 2002; Hebert, Ratnasingham, and deWaard 2003;
178 Hebert and Gregory 2005; Clare *et al.* 2007; Vasconcelos *et al.* 2016; Imtiaz, Nor, and Naim
179 2017; Bernstein *et al.* 2021). Previous efforts using mitochondrial markers have documented
180 cryptic variation in multiple Panamanian birds (González *et al.* 2003; Miller *et al.* 2008; Miller *et*
181 *al.* 2011; Bryson *et al.* 2014; Loaiza *et al.* 2016; Lopez *et al.* 2016). However, as useful as
182 single-taxon studies are, they provide only one example of potential widespread patterns of
183 phylogeographic diversity. Yet, mitochondrial barcoding, with its relative ease of locus
184 acquisition and low cost, provides a simple but powerful tool to build large scale comparative

185 datasets across a wide range of taxa (Kerr *et al.* 2007, 2009; Pereira *et al.* 2013; Mendoza *et al.*
186 2016). Mitochondrial barcoding is particularly helpful due to the same locus having been
187 sequenced in a wide range of taxa (Bronstein, Kroh, and Haring 2018). With comparative
188 datasets, we can better estimate the occurrence of cryptic species, which in turn allows us to
189 better understand the origin of biodiversity in a given region.

190 Barcoding many species exhibiting cryptic variation can detect patterns in where species
191 turnovers occur geographically (e.g., such as corresponding with known suture zones in other
192 taxa) or what ecological factors are more common in cryptically diverse taxa (Bickford *et al.*
193 2007; Struck *et al.* 2018). Addressing the vast biodiversity of the Neotropics necessitates large
194 comparative phylogeographic datasets (Kerr *et al.* 2009; Tavares *et al.* 2011; Miller *et al.* 2021).
195 Hypotheses on the origin of Neotropical diversity tend to fall into a few broad groups. First,
196 biogeographic explanations explain how the geologic and environmental history of a region has
197 created isolated populations that lead to diversification (Sick 1967; Haffer 1969, 1985, 1997;
198 Bush 1994; Sedano and Burns 2010; Smith *et al.* 2014; Ferreira *et al.* 2017). In Panama, these
199 are further grouped into those emphasizing the process of the formation of the Isthmus of
200 Panama (DaCosta and Klicka 2008; Smith and Klicka 2010; Leigh, O’Dea, and Vermeij 2014a),
201 and those emphasizing paleoclimatic fluctuations driving a shifting mosaic of forest and
202 savannah across the landscape (Smith, Amei, and Klicka 2012). Secondly, there are ecological
203 explanations which focus on the role of competition and diversification, with the profusion of
204 niches driving the diversification of species to fill them (Klopfer and MacArthur 1960, 1961;
205 Emerson and Kolm 2005; Brown 2014; Moles and Ollerton 2016). While both explanations
206 likely play roles in generating Panamanian biodiversity (Bush 1994; Smith *et al.* 2014), the

207 question of which prevails in a given region and at finer spatial and temporal scales remains
208 unclear.

209 By DNA barcoding multiple taxa across wide geographic ranges, we can test hypotheses
210 of both biogeographic and ecological divergence. The Panamanian region is a topographically
211 and ecologically diverse region, especially considering its small size (Ridgely and Gwynne 1992;
212 Siegel and Olson 2008; Angehr and Dean 2010). We expect that Panama’s many islands and
213 disjunct highlands (Figure 1A) will harbor a disproportionate number of cryptic species, as has
214 been found elsewhere (Saitoh *et al.* 2015; Campbell, Braile, and Winker 2016). Both the
215 communities of these discrete regions and the more continuously distributed lowland taxa may
216 have been subject to historic isolation, especially prior to the final closure of the Isthmus of
217 Panama approximately 2.7 to 4.2 million years ago (Leigh, O’Dea, and Vermeij 2014b; O’Dea *et al.*
218 *al.* 2016; Jaramillo *et al.* 2017), or by the possible expansion of savannah habitats and formation
219 of forest refugia during the Pleistocene (Smith, Amei, and Klicka 2012). In particular, lowland
220 Panama has been recognized as a hotspot for species turnover—replacement of a given taxa with
221 other taxa—in birds (Miller, Bermingham, and Ricklefs 2007; Miller *et al.* 2011; Loaiza *et al.*
222 2016; Lopez *et al.* 2016; McLaughlin, Garzón, *et al.* 2020), as well as in freshwater fish
223 (Bermingham and Martin 1998; Martin and Bermingham 2000; Perdices *et al.* 2002; Smith and
224 Bermingham 2005; Bagley and Johnson 2014), mammals (Cortés-Ortiz *et al.* 2003),
225 herpetofauna (Crawford 2003; Crawford and Smith 2005; Bagley and Johnson 2014), insects
226 (Bagley and Johnson 2014; Eskildsen *et al.* 2018), and plants (Dick, Abdul-Salim, and
227 Bermingham 2003). Thus, using barcoding, we can investigate if biogeography is an important
228 factor in generating avian diversity, by testing if divergence events are broadly coincident in time
229 (Naka and Brumfield 2018). Beyond biogeographic explanations, our diverse sampling (Figure

230 2) allows us to investigate whether specific ecological traits, such as habitat preference (De León
231 *et al.* 2010; Zhang *et al.* 2012; Harvey *et al.* 2017), dispersal ability (Claramunt *et al.* 2012;
232 Weeks and Claramunt 2014; Crouch *et al.* 2019), territoriality (Tobias *et al.* 2016), and diet
233 (Sheard *et al.* 2020; Miller *et al.* 2021) are associated with cryptic divergence.

234 We set out to investigate the connections between geography, ecology, and the
235 occurrence of cryptic phylogeographic variation indicative of species with a multifaceted
236 mitochondrial dataset. We barcoded 429 species of birds, including 391 landbird species (59%)
237 of the 659 documented resident landbirds in the country. We found that specific ecological traits,
238 including dispersal ability, territoriality, diet, and habitat, were significantly over-represented in
239 taxa with mitochondrial breaks, suggesting these may strongly contribute to the diversification of
240 Neotropical birds.

241

242 **METHODS**

243

244 **COI Barcode Survey of Panamanian Birds**

245 We developed a COI barcode dataset of 2,333 birds from across Panama as part of sequencing
246 for the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007, 2013). Our dataset
247 includes 429 species as defined in the original taxon dataset from BOLD; however, Angehr and
248 Dean 2010 define 484 by splitting several taxa that are not in the taxonomy used in BOLD. The
249 majority of these birds were sequenced through the Smithsonian Institution's (SI) Barcode of
250 Life initiative (Schindel *et al.* 2011) and the Smithsonian Tropical Research Institute (STRI;
251 original data presented here). With a few exceptions, every sequence in the SI and STRI
252 barcoding datasets is represented by a museum voucher specimen (Table A1). We determined

253 whether a species had mitochondrial splits—i.e., had 2 or more barcode clusters defined in
254 BOLD—by using the barcode index number (BIN; Ratnasingham and Hebert 2007, 2013) as
255 implemented in BOLD via alignment and clustering of COI sequences. In this method,
256 individuals that are more than twice the distance of divergence within a cluster being taken as the
257 start of a new cluster, followed by use of a Markovian analysis to refine clusters (Ratnasingham
258 and Hebert 2013). Key benefits of this method are in the ease and low-cost of the method
259 (Tavares *et al.* 2011; Milá *et al.* 2012), and the relatively high reliability in assigning individuals
260 to species in past studies (Yoo *et al.* 2006; Kerr *et al.* 2007). It does carry the standard limitations
261 of any single-marker method of evaluating diversity, namely that a single locus may not be
262 reflective of the total evolutionary history of a taxon. However, mitochondrial studies are still
263 valuable where large-scale nuclear sequencing is not feasible, and are useful in determining
264 where to focus with more in-depth sequencing efforts.

265

266 **Improving Geographic Coverage Through Mitogenomic Haplotyping**

267 Though covering over 2000 birds, our COI database does not fully capture available data on the
268 distribution of mitochondrial diversity and structure in Panamanian birds that is available either
269 as part of previously published studies, (e.g. Miller *et al.* 2010; Smith *et al.* 2014; Miller *et al.*
270 2021). Because mitochondrial DNA is non-recombining, whole mitochondrial genomes can
271 connect disparate mtDNA datasets into congruent haplogroups, functioning as a “Rosetta Stone”
272 to leverage multiple mitochondrial loci into a large common dataset. As part of several long-term
273 projects on the comparative genomics of Panamanian lowlands, we filtered mtDNA reads from
274 whole genome shotgun sequencing (e.g. do Amaral *et al.* 2015) for 20 taxa identified with
275 distinct COI BINs in Panama of resident lowland birds sampled in western (Bocas del Toro) and

276 eastern (Darién) Panama (Table 1). We sequenced two individuals from each of those
277 populations, preparing genomic libraries with the NEB Ultra II protocol and sequencing them on
278 an Illumina NovaSeq. We then used bbdduk, a utility within the bbmap program (Bushnell 2014),
279 to trim and perform initial quality control on reads. We then downloaded 215 additional
280 mitochondrial sequences, including ND2, cytB, ND3, ATPase 8, and ATPase6, from NCBI
281 (Table 1; details by individual in Table A1), increasing sampling density across Panama.

282 Assembly of one individual per population was performed using NovoPlasty v. 3.4
283 (Dierckxsens, Mardulyn, and Smits 2017), using either COI or ND2 as the seed, depending on
284 availability. In some individuals with a high number of reads, we subsampled the initial reads
285 with BBSplit, a part of the BBMap package (Bushnell 2014), to increase computational
286 efficiency by only including putative mitochondrial reads in our assembly inputs. Mitogenomes
287 were then aligned and annotated with MitoAnnotator (Iwasaki *et al.* 2013), from which we
288 calculated the pairwise K2P genetic distance for each of the protein-coding genes between
289 eastern and western populations.

290

291 **Quantifying Mitochondrial Splits**

292 To estimate where mitochondrial splits (i.e., multiple BINs as defined by COI barcoding) occur,
293 we generated locus-specific sequence alignments of available mitochondrial sequences for the 34
294 taxa above. Sequences were downloaded from BOLD and NCBI's Genbank (Table A1). We then
295 generated MUSCLE alignments (Edgar 2004) in MEGA7 (Kumar, Stecher, and Tamura 2016),
296 using these to build neighbor-joining trees in PAUP* (Swofford 2001) or MEGA (Kumar,
297 Stecher, and Tamura 2016) and ML trees in RaxML (Stamatakis 2014) using the GTR
298 substitution model with Lewis ascertainment bias correction for 100 bootstrap replicates. For the

299 21 taxa with multiple mitochondrial loci available (Table 1), the BOLD barcodes were used to
300 define groups, and then additional individual sequences were aligned to the whole mitochondrial
301 genomes for that taxon, which had already been assigned to BINs, and haplotyped accordingly.

302

303 **Testing Predictors of Mitochondrial Divergence**

304 We examined potential drivers of mitochondrial divergence using geographic, morphological,
305 and ecological data. To test how ecological factors such as habitat openness, forest stratum, diet,
306 and elevational distribution influenced the distribution of mitochondrial splits, we compiled and
307 scored these data for all 659 resident, breeding landbirds of Panama, using species accounts from
308 The Handbook of the Birds of the World Online (Billerman *et al.* 2020), supplementing
309 individual species accounts as needed from Angehr and Dean (2010), Ridgely and Gwynne
310 (1992), Stotz *et al.* (1996), and Wetmore (1965, 1968, 1972; 1984). Habitat was scored as forest,
311 edge, or open (Table A2). Stratum was scored as ground (primarily terrestrial foraging, and/or
312 prefers walking to flying), understory (forages primarily in undergrowth or directly above
313 ground), midstory (primarily found in middle strata of forest, up to subcanopy), canopy
314 (primarily found in the subcanopy and above), and aerial (forages above the forest canopy;
315 almost exclusively swifts and swallows). We also included data on territoriality, hand-wing
316 index (HWI), body size, and annual precipitation in range sourced from Sheard *et al.* (2020).
317 Finally, we identified whether each species had been sampled across multiple geographic regions
318 of Panama (Figure 1A), to identify the presence of geographic-based variation across the region.
319 We tested for sampling biases in these three categories by comparing the total list and sampled
320 subset by chi-squared tests to check if our 429 sampled taxa reflected the distribution of the
321 above traits within the total Panamanian avifauna. Then, we tested whether those taxa which had

322 been sampled across multiple geographic regions of Panama (Figure 1A) were representative of
323 the total Panamanian avifauna. Taxa were considered widespread enough for inclusion in these
324 tests if they occurred in two or more of the defined geographic regions of Panama (Figure 1A),
325 which yielded a total of 181 species.

326 Of the 181 species in our study which had been widely sampled across Panama based on
327 the above criteria, we then tested whether certain geographic, ecological, and morphological
328 traits were disproportionately observed in species with two or more mitochondrial BINs. For
329 each of the above ecological traits (stratum, territoriality, diet, habitat, HWI, body size, and mean
330 annual precipitation), we tested using either a chi-squared test or student's *t*-test whether there
331 were significant differences in the representation of traits between split and non-split taxa.

332

333 **Testing for Coincident Timing of Splits**

334 To assess if splits were coincident in time, and thus likely to have been driven by the same
335 biogeographic events, we estimated divergence times in BEAST v. 2.6.2 (Bouckaert *et al.* 2014)
336 as implemented on CIPRES (Miller, Pfeiffer, and Schwartz 2011). We generated an alignment of
337 COI barcodes in MUSCLE, and trimmed ends to have no missing data. We used a strict clock
338 rate of 1.8 % divergence per million years (Lavinia *et al.* 2016), gamma site model with JC69
339 substitution model (Naka and Brumfield 2018), and five fossil calibration points (Table A3).
340 Fossil calibrations and the tree were given a log normal prior distribution. We ran this model for
341 1 billion MCMC generations, sampling every 10,000 generations, and assessed convergence (all
342 ESS values > 200) in Tracer v. 1.7.1 (Rambaut *et al.* 2018). To generate a maximum clade
343 credibility tree, we generated the MCC tree in LogCombiner with the first 20% as burn-in using
344 median heights. We calculated the mean divergence time for each taxon using the rate of 1.8%

345 divergence per million years previously found for avian COI (Lavinia *et al.* 2016), and compared
346 this with the BEAST estimated means. With the 95% confidence intervals constructed in the
347 latter, we then used these estimates to establish whether divergence times were broadly
348 coincident.

349

350 **RESULTS**

351

352 **Barcoding and Data Collection**

353 We successfully barcoded 2,333 individuals from 429 species across Panama, 391 of which were
354 resident landbird species (Table A1). This sampling was representative of Panamanian avifauna,
355 as similar proportions of highland and lowland birds were present in the whole population of
356 landbirds in Panama as in our sampled subset of species ($\chi^2 = 0$, $df = 1$, $p = 1.0$) as well as similar
357 proportions by diet ($\chi^2 = 13.34$, $df = 9$, $p = 0.14$) and habitat ($\chi^2 = 6.18$, $df = 3$, $p = 0.10$; Table 2). Of
358 these 429 species, 181 (42.2%) were sampled across two or more geographic regions (Figure
359 1A), and were likewise representative of the whole population of resident landbirds (Table 2).

360 Thirty-four of these 181 taxa—18.8%—had more than 1 mitochondrial BIN, represented
361 by a total of 419 individuals barcoded in BOLD. We increased this to a total of 634 individuals
362 by adding 215 additional sequences from NCBI (Table A1). Twenty-one species were
363 supplemented by this method, but the remainder did not have the required samples of each BIN's
364 whole mitochondrial genomes to allow the building of a multi-locus dataset.

365

366 **Mitochondrial Splits**

367 Among landbirds, splits were observed in 20 of the 37 widely sampled families (Figure 2),
368 accounting for 33% of the 61 resident landbird families documented in Panama. We
369 characterized the geography of the 34 taxa with multiple BINs, plus two waterbirds (*Laterallus*
370 *albigularis* and *Jacana spinosa*) not included in the prediction testing due to overall low
371 sampling of waterbirds. Seven taxa, all lowland, had three BINs in Panama (Figure 3). Overall,
372 we found 41 splits across 34 landbird species, out of the 181 taxa with sufficient sampling across
373 multiple geographic regions.

374 Geographically, we observed two primary patterns in the distribution of splits (Figure 3).
375 The first, observed in six species, was a break between southwest Panama, in particular the
376 Burica peninsula (Chiriquí province; Figure 3B), and the rest of Panama. This pattern was
377 restricted to lowland taxa. The second pattern was one split between eastern and western
378 Panama, observed in 35 splits (Figure 3D-G). This included both highland taxa (4 splits; Figure
379 3A) and lowland (31 splits; Figure 3D-F). In highland taxa, these splits were between the
380 Cordillera Central and the highland areas of the east. However, in lowland taxa, there were two
381 general clusters of regions of rapid geographic replacement of BINs across multiple taxa. The
382 first, involving 15 splits (48% of lowland taxa with east-west splits), was along the Caribbean
383 versant in Veraguas province, extending into Colón province in some cases (Figure 1B, 3E). The
384 second, involving seven splits, was roughly located along the border of Darién and Panama
385 provinces (Figure 1B, 3D). Three lowland splits were in central Panama (Figure 3F), and an
386 additional three, representing distinct BINs in extreme eastern and western Panama, lacked
387 samples which prevented us from locating the precise area of turnover (Figure 3G). One
388 predicted geographic pattern that was not observed was differentiation of island and mainland

389 taxa, despite island samples being included in most species and preferentially including taxa
390 thought to represent distinct island groups (Table A1).

391

392 **Prediction Testing**

393 While our overall dataset of 181 species may have been ecologically and geographically
394 representative of resident landbird taxa (Table 2), the observed mitochondrial splits were
395 associated with different factors. While insectivores made up 47% of non-split species, they
396 accounted for significantly more (68%) of species with splits ($\chi^2=17.27$, $df= 7$, $p=0.02$; Figure
397 4B). Forest birds, while comprising the majority of non-split species, at 62%, had even greater
398 representation among the split species, at 85% ($\chi^2=6.49$, $df= 2$, $p=0.04$; Figure 4C). When
399 considering habitat stratum, we found that while non-split species were evenly distributed
400 throughout strata, with only 40% being classed as understory residents, in split taxa understory
401 birds were the overwhelming majority, at 74% of species ($\chi^2=14.04$, $df= 4$, $p=0.0072$; Figure
402 4C). Hand-wing index (HWI) was significantly ($t= -5.52$, $df= 154.29$, $p<0.001$) lower in split
403 species (Figure 4A). Finally, split taxa were far more likely to be strongly territorial, with 62% of
404 split taxa versus 35% of non-split ($\chi^2=12.04$, $df= 2$, $p=0.0024$; Figure 4D).

405 Some traits, however, were represented at similar proportions in both split and non-split
406 species (Table A2). Highland taxa were a minority of species in both cases, at 11% of non-split
407 and 12% of split species ($\chi^2=4.10 \times 10^{-29}$, $df= 1$, $p=1$). Habitat mean annual precipitation was
408 similar for both, at 2209 mm/yr in split and 2179 mm/yr in non-split ($t= 0.28$, $df= 48.22$,
409 $p=0.78$). Finally, scaled body size was largely similar, with non-split species being very slightly
410 larger, but not significantly so ($t= 0$, $df= 292$, $p=1$).

411

412 **Timing of Splits**

413 Depths of splits varied considerably. Pairwise differences in COI ranged from 1.24% to 8.49%
414 for those taxa defined as having multiple BINs by BOLD. Median pairwise divergence was
415 3.31%. These are equivalent to between 689 kya and 4.71 mya (Figure 5), with a median of 1.84
416 mya. However, estimates from BEAST were substantially older than from pairwise estimates, as
417 expected with dates from coalescent methods, and our confidence intervals were frequently very
418 wide (Figure 5) and do not coincide temporally.

419

420 **DISCUSSION**

421 Using mitochondrial barcoding, we both identified substantial cryptic diversity in Panamanian
422 birds and found support for ecological drivers of diversification in the region. We found that the
423 frequency of cryptic diversity in Panamanian resident landbirds is 18.8% (Table 2). This is
424 higher than estimates from similar barcoding efforts in other geographic regions, including the
425 2.7% of North American birds (Kerr *et al.* 2007), 11% of Korean birds (Yoo *et al.* 2006), 7.5%
426 of Palearctic birds (Kerr *et al.* 2009), 3.3% of Argentinian birds (Kerr *et al.* 2009), and 3.6% of
427 South American birds more generally (Tavares *et al.* 2011). Due to variations in study designs,
428 however, some caution is needed in directly comparing these without controlling for differences
429 in sample size, taxonomic biases, and other potential sources of variation. While other studies
430 have found higher incidence of potential cryptic diversity, these tended to focus on narrow
431 subsets of birds that may be particularly predisposed towards splits. For example, Milá *et al.*
432 (2012) found evidence of interspecific-level variation within 33 of 40 forest understory birds in
433 the Amazon, a habitat profile that we find to be significantly overrepresented in lineages with
434 potential cryptic species in our study. In general, many barcoding studies have tended to focus on

435 large-scale questions of overall divergence, rather than explicitly examining whether specific
436 ecological traits were over- or under-represented in taxa with potential splits (Yoo *et al.* 2006;
437 Kerr *et al.* 2007, 2009; Kerr *et al.* 2009; Tavares *et al.* 2011). Even in light of these differences in
438 study design and underlying questions, we find a notably high rate of cryptic diversity in
439 Panamanian birds compared with other studies.

440

441 **Geographical Patterns of Cryptic Diversity**

442 We sampled widely across Panama and evaluated several previously proposed
443 biogeographic patterns in cryptic diversity (Wetmore 1959; Summers *et al.* 1997; Anderson and
444 Handley 2001; Miller *et al.* 2011; Kaviar, Shockey, and Sundberg 2012; Miller *et al.* 2015). The
445 most common geographic pattern that we observed was differentiation between eastern and
446 western Panama. These splits could be further subdivided into three general patterns: disjunct
447 highland populations from the Cordillera Central and the highlands of eastern Panama, a suture
448 zone in the Caribbean versant of Veraguas and Colón, and a second suture zone in Darién
449 (Figure 1B).

450 Many of the taxa included samples from the various islands of Panama, including Isla
451 Coiba in the Pacific and the Caribbean islands of San Cristobal, Bastimentos, Cayo Agua, and
452 Escudo de Veraguas. Islands are a natural focus of investigation for undescribed biodiversity,
453 and previous studies have found evidence for island endemics in both birds and other taxa in
454 Panama (Summers *et al.* 1997; Anderson and Handley 2001; Kaviar, Shockey, and Sundberg
455 2012). Escudo, for example, has had four of its eight to ten resident breeding birds described as
456 endemic subspecies (Wetmore 1959). The Escudo hummingbird (*Amazilia (tzacatl) handleyi*) is
457 both phenotypically and genetically distinct from mainland populations (Miller *et al.* 2011) and

458 is sometimes treated as a separate species (Wetmore 1968; Angehr and Dean 2010). However,
459 overall, we found low mitochondrial divergence between mainland and island taxa. This is likely
460 due to their close proximity to the mainland, such as in the case of the islands of Bocas del Toro
461 (approximately 15 km for the furthest islands of Bocas del Toro, and 20 km to Isla Escudo) and
462 Coiba (24 km to mainland) which makes it likely that they were intermittently connected to the
463 mainland during Pleistocene sea-level fluctuations (Miller *et al.* 2011). Escudo, the furthest of
464 the Caribbean islands, is estimated to have become isolated only around 9000 years ago
465 (Summers *et al.* 1997; Miller *et al.* 2011). This lowers the probability of the development of
466 distinctive island endemics (MacArthur and Wilson 1963; Mayr 1965). Furthermore, the
467 majority of island avifauna are also found on the mainland (Wetmore 1965, 1968, 1972;
468 Wetmore, Pasquier, and Olson 1984; Ridgely and Gwynne 1989; Angher and Dean 2010),
469 suggesting that there has been a high degree of connectivity over time. Indeed, *Setophaga*
470 *petechia* was the only taxon with a distinctive BIN on Isla Coiba, but we suspect this was likely
471 an individual of the migratory subspecies (included unintentionally, as we overall excluded
472 migratory taxa) rather than the resident, as both occur in Panama. While island populations may
473 be a source of haplotype diversity in some taxa (González *et al.* 2003), they may not be
474 sufficiently diverged to be split under the BIN system.

475 An area where we expected to find unrecognized diversity is the Pacific coast of Chiriquí,
476 particularly the Burica Peninsula (Figure 1A). This region, separated from most of the rest of
477 Panama by the Cordillera Central, receives markedly less precipitation than the rest of the
478 country (Wetmore 1965), and is thus home to much more xeric ecosystems than on the rainy
479 Caribbean side (Blanco *et al.* 2013). We found support for this region being a hotspot of

480 unrecognized avian diversity, with six of the 41 splits located in this relatively small area (Figure
481 3).

482 The four highland splits are all between the two main highland regions of Panama (Figure
483 4). The predominant east-west split is consistent with the numerous species-pairs previously
484 observed to follow this pattern (Wetmore 1965, 1968, 1972; Wetmore, Pasquier, and Olson
485 1984; Ridgely and Gwynne 1992; Angehr and Dean 2010). Likewise, the Azuero Peninsula has
486 been previously noted for several potential endemic species (Miller *et al.* 2015). However, across
487 our overall dataset, highland splits are very much in the minority of those observed, making up
488 only 14.7% of splits.

489

490 **The Lowlands are a Hotspot for Cryptic Diversity**

491 We found a disproportionate number of lowland taxa with cryptic species-level variation. This is
492 likely due to the fact that previous efforts have focused on identifying potential cryptic species in
493 taxa with disjunct ranges such as highland species. Indeed, highland species showed splits of
494 equivalent depth, but have likely already been defined as separate species (e.g., *Basileuturus*
495 *ignotus*, Todd 1929; *Scytalopus*, Krabbe and Schulenberg 1997, Cadena *et al.* 2020; *Tangara*
496 *dowii* and *T. fucosa*, Burns and Naoki 2004). Thus, few highland taxa met our geographic
497 sampling criteria of being present in two or more regions (Figure 1A), and they represented only
498 15.1% of widely distributed species and 11.5% of species barcoded across multiple geographic
499 regions, despite making up more than 30% of the total avifauna (Table 2). This discrepancy in
500 the rates of splits in lowland and highland taxa is potentially due to bias from prior taxonomic
501 description of variation. While this bias may partially explain the discrepancy, it does not explain
502 what may generate the lowland variation we observed.

503 While many of Panama's lowland bird species are continuously distributed (Wetmore
504 1965, 1968, 1972; Wetmore, Pasquier, and Olson 1984; Ridgely and Gwynne 1992; Angehr and
505 Dean 2010), this may not have historically been the case, as Pleistocene climate variation may
506 have caused ranges to contract (Haffer 1969; Moritz *et al.* 2000; Weir, Bermingham, and
507 Schluter 2009; Smith, Amei, and Klicka 2012; Lavinia *et al.* 2015). Most of the split bird species
508 (85%) are forest birds (Table 2), and the increased aridity during the Pleistocene is thought to
509 have caused widespread advancement of savannah into formerly forested areas throughout the
510 tropics (Haffer 1985; Webb 1991). Thus, currently continuously-distributed species might not
511 have always been connected, and this climatic history may have driven the diversification of taxa
512 across Panama. However, it is unclear the extent of forest contraction in Panama throughout the
513 Pleistocene (Bush and Colinvaux 1990; Colinvaux, De Oliveira, and Bush 2000), and we find
514 little support for the observed divergence dates coinciding to even roughly similar time periods
515 (Table 1).

516 Clearly, while Panama's diverse landscape has played a role in generating its rich avian
517 diversity, this temporal incongruence of divergences (Figure 5) suggests that biogeographic
518 models centering geological history are not the only drivers of cryptic diversification in the
519 region. Previous work in Panama and lower Central America (LCA) more broadly has focused
520 on the timing of the emergence of the isthmus (Smith and Klicka 2010), and in the resulting
521 biotic interchange between North and South America (DaCosta and Klicka 2008; Weir,
522 Bermingham, and Schluter 2009). However, it is unlikely that dispersal following the formation
523 of the isthmus is responsible for the patterns of turnovers we see in our taxa. Our oldest splits (~4
524 mya) predate the 3 mya date generally accepted for the formation of the isthmus (Leigh, O'Dea,
525 and Vermeij 2014a), while the youngest (< 1 mya) are well after this time. However, the gradual

526 emergence of the isthmus would possibly better explain some of the splits (O’Dea *et al.* 2016). In
527 particular, we find a group of splits in the Veraguas/Colón suture zone around the Pleistocene-
528 Pliocene boundary (Figure 5), which line up with a potential episode of seawater breaching the
529 newly formed isthmus approximately 2.45 mya (Groeneveld *et al.* 2014; O’Dea *et al.* 2016).
530 Though overall our dates do not support a scenario of dispersal prior to the formation of the
531 isthmus, followed by secondary contact in most lowland cases, a more gradual process in which
532 a changing landscape of newly emerged land acted as a tenuous bridge for dispersal over a
533 longer period may be possible.

534

535 **Ecological predictors of mitochondrial turnover**

536 If biogeographic factors alone cannot explain the generation of diversity in lowland Panamanian
537 birds, what other factors may be important? Our results highlight how ecological traits alone
538 have the potential to drive divergence, even in a landscape that has historically lacked obvious
539 barriers to dispersal (Bush and Colinvaux 1990). Specifically, we found that traits such as
540 habitat use, diet, and morphology can be effective in generating diversity, with the majority of
541 taxa with mitochondrial splits sharing specific ecological traits. Insectivores (68% of sampled
542 taxa with splits vs. 47% non-split taxa), forest birds (85% vs 62%), understory foragers (74% vs
543 40%), and strongly territorial species (62% vs. 35%) were all overrepresented in lineages with
544 mitochondrial turnover (Figure 4).

545 A potential explanation for at least some of the role of these ecological factors is
546 detectability. Detectability can have large ramifications for studying groups in difficult-to-reach
547 areas, particularly in the context of conservation (Clark and May 2002; Ducatez and Lefebvre
548 2014; McKenzie and Robertson 2015; Smith *et al.* 2020). This can also extend to taxonomic

549 considerations, as more easily observed and “charismatic” organisms are more likely to be
550 oversplit (Pillon and Chase 2007). In particular, some habitats and strata are more likely to
551 harbor cryptic species not because they are inherently more likely to create diversity, but simply
552 because they are more difficult to observe. Therefore, the apparent overabundance of cryptic
553 types in understory forest birds may be a relative scarcity of human observations. However, the
554 overall well-described Panamanian avifauna make a lack of observation less likely than
555 elsewhere in the Neotropics (Angehr and Dean 2010; Ridgely and Gwynne 1992; Wetmore
556 1965, 1968, 1972; Wetmore, Pasquier, and Olson 1984), and many splits within inconspicuous
557 understory birds have been previously described (e.g. Saucier, Sánchez, and Carling 2015).

558 The traits observed to have a strong link to mitochondrial divergence are all associated
559 with low dispersal capability. This is most obvious in the much lower HWI of birds with
560 mitochondrial splits. HWI is a well-recognized proxy of dispersal ability (Claramunt *et al.* 2012;
561 Claramunt and Wright 2017; Sheard *et al.* 2020), as it describes wing shape and thus the ability
562 for sustained flight (Kipp 1959; Lockwood, Swaddle, and Rayner 1998; White 2016; Claramunt
563 and Wright 2017; Sheard *et al.* 2020), and the lower HWI of split species shows that lower
564 dispersal ability is significantly associated with mitochondrial turnover. The association of
565 ecological factors linked to lower dispersal ability with mitochondrial turnover holds through
566 other tested traits. Forest birds have much lower dispersal abilities than edge or open area species
567 (Moore *et al.* 2008; Burney and Brumfield 2009; Weir, Bermingham, and Schluter 2009),
568 especially those which primarily use the understory (Burney and Brumfield 2009; Woltmann and
569 Sherry 2011). Likewise, strongly territorial species were overrepresented in the split taxa. As
570 these species are less likely to disperse once they have established a territory (Greenwood 1980),
571 this provides further weight to the role of dispersal.

572 Our finding of insectivores having greater frequency of splits may also be tied to
573 differences in dispersal ability. Previous studies have found strong evidence that diet type, and
574 especially the extent to which a given species relies on plant-based food sources, can shape
575 dispersal and demography (Westcott and Graham 2000; Moore *et al.* 2008; Burney and
576 Brumfield 2009; Miller *et al.* 2021). While both plant and animal food sources are typically
577 available year-round in the tropics, seed, nectar, and fruit tend to be spatially and temporally
578 clustered (Morton 1973; Levey and Stiles 1994). While insectivores may reliably find arthropods
579 in a given home range (Levey and Stiles 1992; Burney and Brumfield 2009), birds feeding
580 primarily on fruit, seeds, and nectar may need to travel more widely to seek out food sources
581 throughout and between years (Westcott and Graham 2000). Furthermore, the relative
582 availability of these resources varies between years to different extents. While arthropods are
583 certainly subject to population cycles, they are usually less extreme (Jahn *et al.* 2010) than the
584 fluctuations between mast years and lean years in fruit and seed-bearing species typically relied
585 on for food by frugivorous and granivorous birds (Faaborg, Arendt, and Kaiser 1984;
586 Wheelwright 1986; Levey, Moermond, and Denslow 1994; Brawn, Karr, and Nichols 1995;
587 Ryder and Sillett 2016; Macario *et al.* 2017). As a result, birds which primarily feed on plants are
588 more subject to boom-and-bust population dynamics (Faaborg, Arendt, and Kaiser 1984;
589 Greenberg and Gradwohl 1986; Şekercioğlu *et al.* 2002; Woltmann and Sherry 2011; Sherry *et*
590 *al.* 2020), and during boom years will experience increased dispersal, potentially connecting
591 populations more regularly and slowing the accumulation of divergence between them.

592 The importance of traits directly or indirectly tied to dispersal ability may be to be
593 driving a simple isolation-by-distance (IBD) effect. Poor dispersers will develop greater
594 divergence across a given space than better dispersers, so that further populations will be

595 increasingly genetically differentiated (Wright 1943, 1946; Slatkin 1993). However, while that
596 may play a part for some of the taxa in our study, it is unlikely for all the observed splits. While
597 some taxa, such as *Mionectes oleagineus* and *Baryphthengus martii*, have repeated mitochondrial
598 breaks with increasing divergence across Panama (Figure 4), others have sharp turnovers within
599 Panama with equal or greater divergence estimates, yet these haplotypes are still found hundreds
600 of kilometers away in Nicaragua (*Arremon aurantirostris*, *Cyanocompsa cyanooides*), Honduras
601 (*Arremon aurantirostris*), Belize (*Cyanocompsa cyanooides*), and Ecuador (*Cantorchilus*
602 *nigricapillus*). Thus, it is likely that dispersal is the driver of divergence in concert with other
603 ecological factors in many cases.

604 Despite its small size, Panama is home to remarkable avian diversity, with 1000 currently
605 recognized species occurring in the region (Wetmore 1965, 1968, 1972; Wetmore, Pasquier, and
606 Olson 1984; Ridgely and Gwynne 1992; Angehr and Dean 2010). While some of this is likely
607 due to the region's position as a literal bridge between North and South America (DaCosta and
608 Klicka 2008; Smith and Klicka 2010; Leigh, O'Dea, and Vermeij 2014b), the region itself plays
609 an important role in generating biodiversity. The diverse elevational and climatic range of
610 habitats across the isthmus provide opportunity for biogeographic scenarios leading to endemism
611 (Stiles 1983; Barrantes 2009; Chavarría-Pizarro *et al.* 2010; Batista *et al.* 2020), but this alone
612 may not be the only driving force behind avian diversity. Consistent with other recent work in
613 the region (Miller *et al.* 2021), we find that ecological factors are strongly associated with cryptic
614 diversity, particularly in lowland Panama, emphasizing the need to look beyond physical barriers
615 as drivers of Neotropical biodiversity.

616

617 **CONCLUSIONS**

618

619 Panama is widely recognized as an area of high biodiversity; however, we find that avian
620 diversity is substantially underestimated. Potential cryptic species are in some cases associated
621 with landscape and geography, such as highland taxa and those in southwestern Chiriquí, but the
622 bulk of the observed splits are in lowland taxa in the absence of geographic barriers. The varying
623 ages of the observed splits, from approximately 0.75–4.2 mya, makes it unlikely that all the
624 observed variation is driven by a single historical factor. We find instead strong correlations
625 between dispersal ability, both directly (HWI) and indirectly (through ecological traits such as
626 habitat, diet, and territoriality), and the occurrence of mitochondrial turnover. This suggests that
627 intrinsic ecological and life history traits can be a major factor in driving species turnover and the
628 accumulation of biodiversity in the tropics. This also illustrates how examining cryptic species
629 can provide insights into the ecological and evolutionary processes that shape this diversity. The
630 potential cryptic species we identify are good candidates for further sequencing across the
631 nuclear genome, allowing us to more deeply explore the evolutionary processes in play. Overall,
632 we demonstrate that barcode data, when carefully designed and deployed, are useful both for
633 identifying drivers of divergence and in directing the focus of future genomic studies.

634

FIGURES

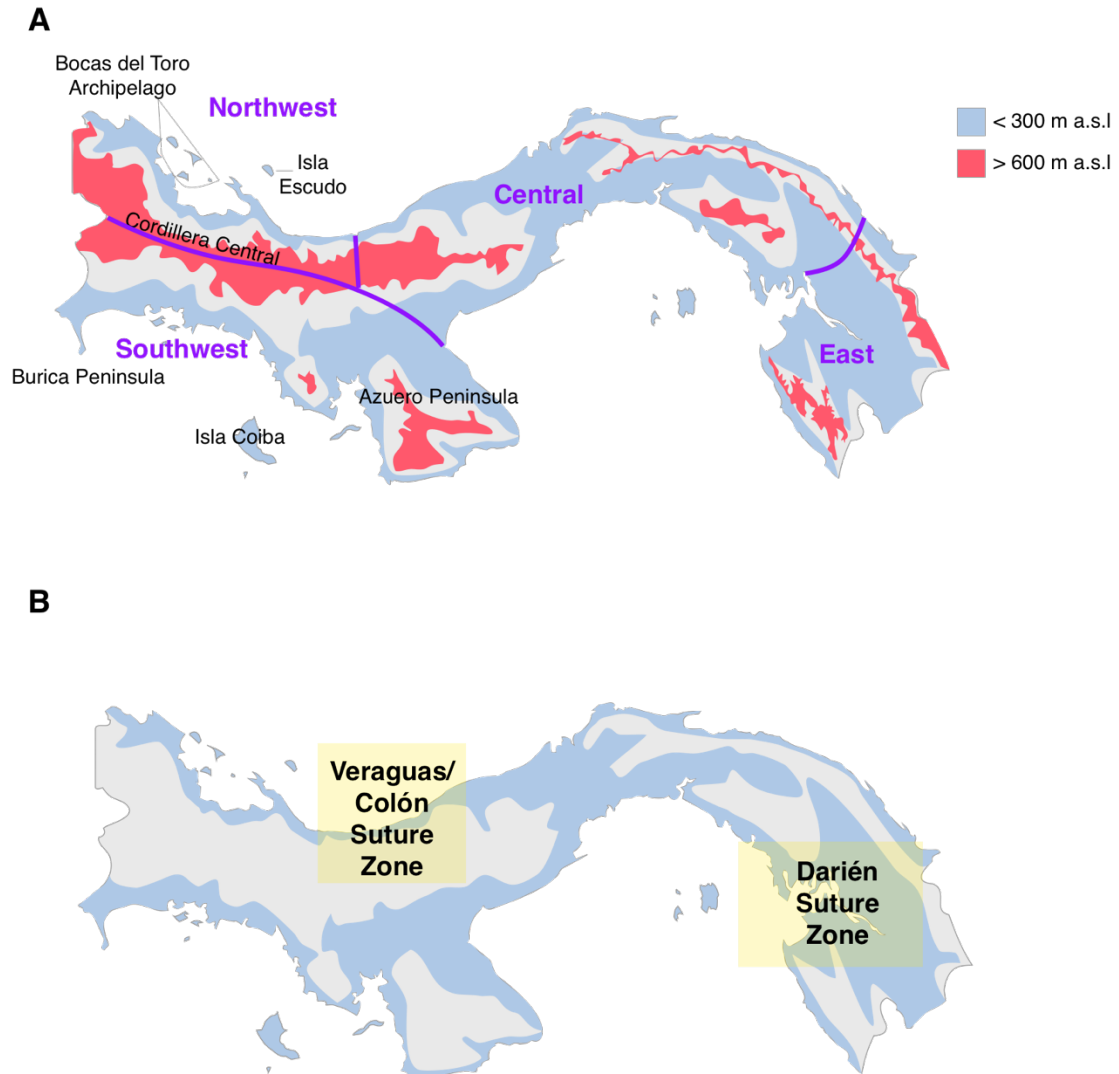


Figure 1: Map of Panama indicating major geographic features considered in this study. A) Pink areas are highland regions with greater than 600 meters above sea levels (m.a.s.l) elevation, while light blue show those with less than 300 m.a.s.l. Purple lines indicate the broad geographic areas used to define sampling regions, with names in purple. B) Lowland suture zones. Light yellow shading indicates general locations of suture zones described in this study.

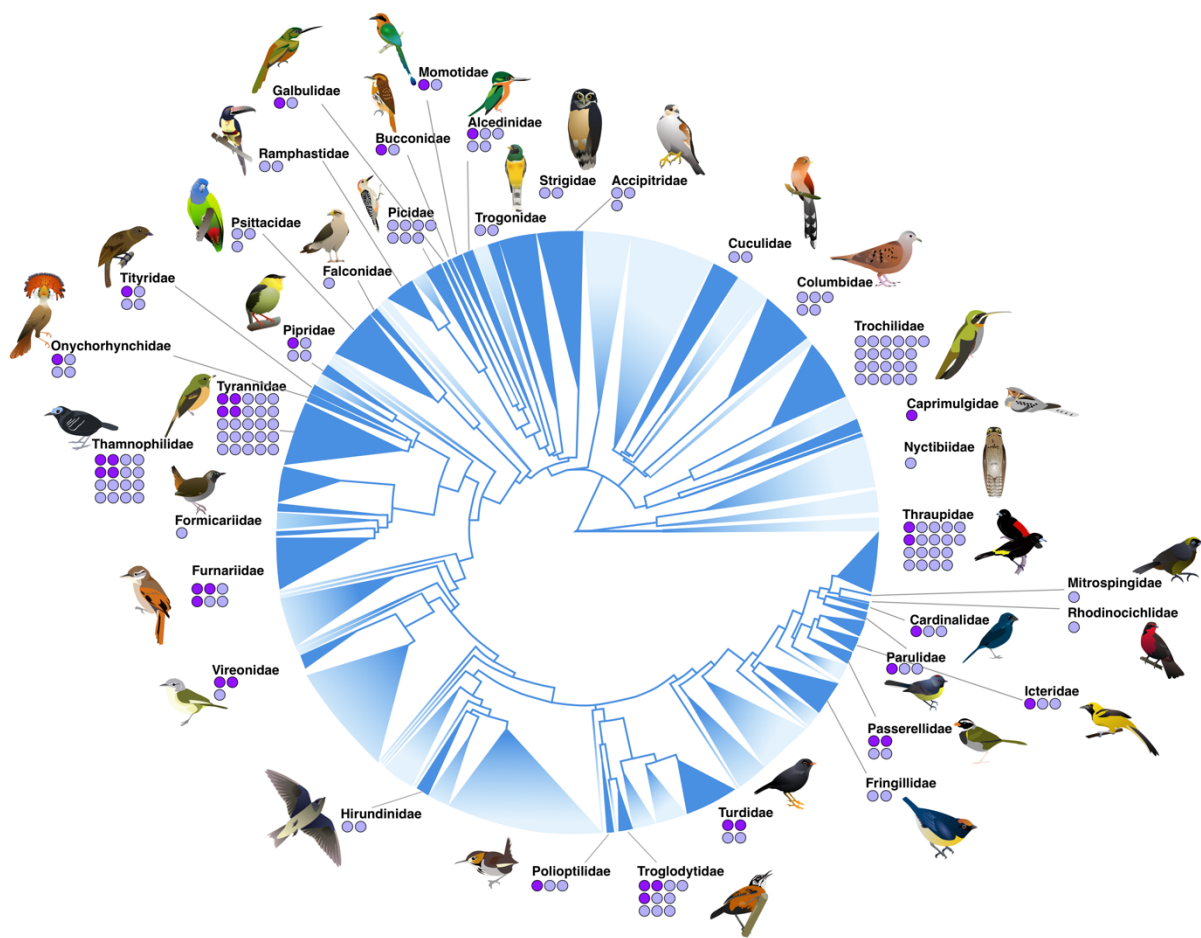


Figure 2: Phylogenetic sampling of Panamanian birds. Overall avian tree of life modified from Jetz *et al* (2012). Sampled families are shown in dark blue, with unsampled lineages shown with topology faded out; size of clade scaled by number of species. For each sampled family, dots

indicate the number of taxa sampled across multiple regions of Panama (one dot per sampled species). Light purple dots indicate lineages with a single mitochondrial BIN; dark purple indicate those with two or more.

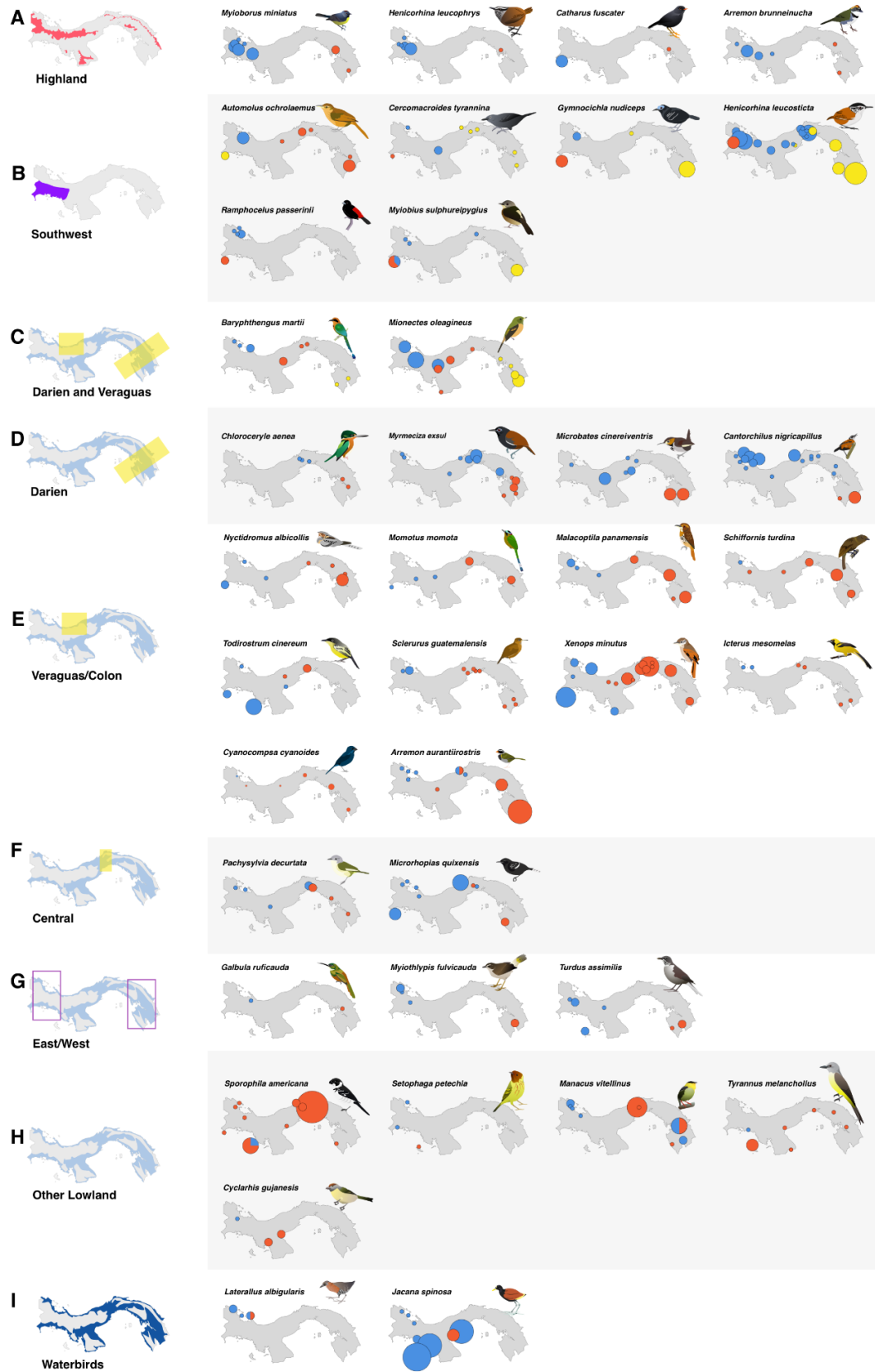


Figure 3: Geographic distribution of haplotypes in Panamanian birds. Haplotypes were defined initially by BIN for COI data, and then by sequence for additional markers for all taxa with observed mitochondrial breaks, grouped by geographic region of splits. Highland species (A) are separated from lowland birds, and lowland species are displayed by (B) southwest vs rest of Panama, with or without additional splits; (C) splits in both the Veraguas and Darién suture zones; (D) Darién suture zone splits; (E) Veraguas/Colón splits; (F) splits in central Panama, typically around Cerro Azul; (G) lowland taxa which have distinctive haplotypes in east and west, but lack sufficient sampling across the transect to determine the precise locality of the turnover; (H) taxa with idiosyncratic patterns that fit none of the above; and (I) waterbirds, which were generally excluded from analyses due to less consistent sampling. Dot colors indicate distinct BINs, size scaled by the number of samples from a given locality.

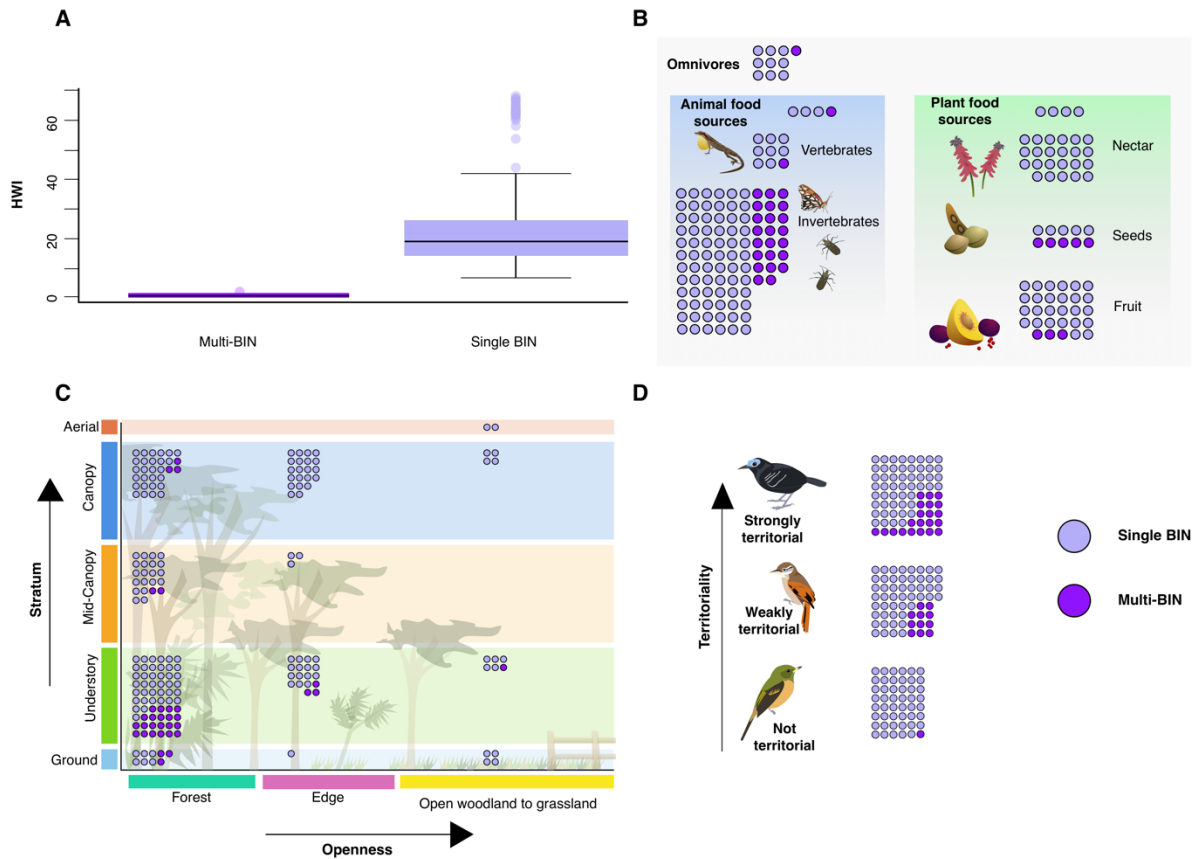
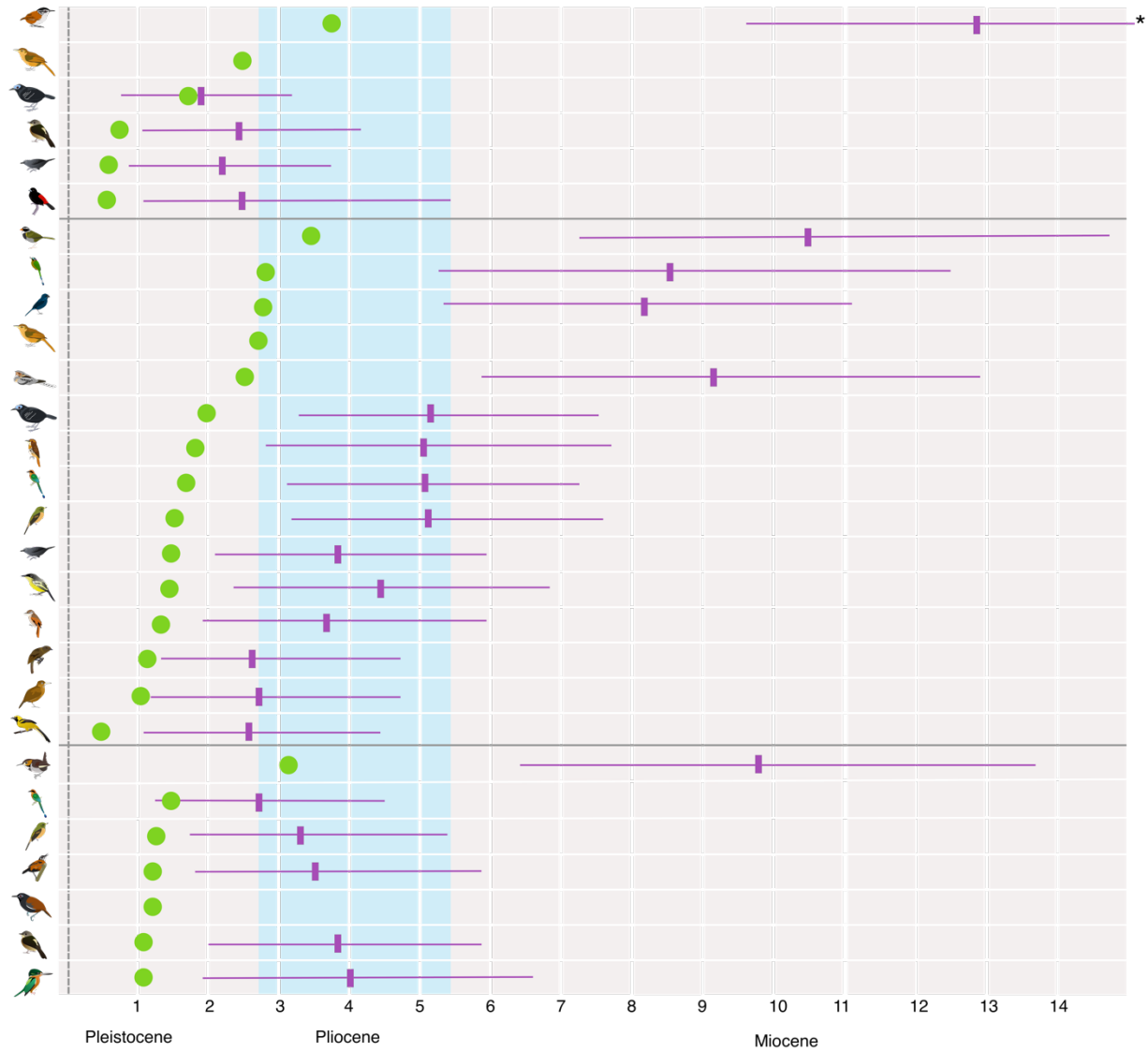
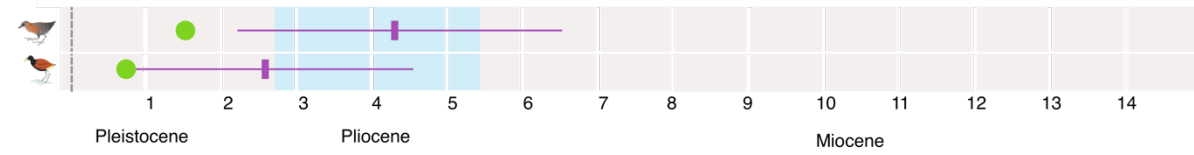
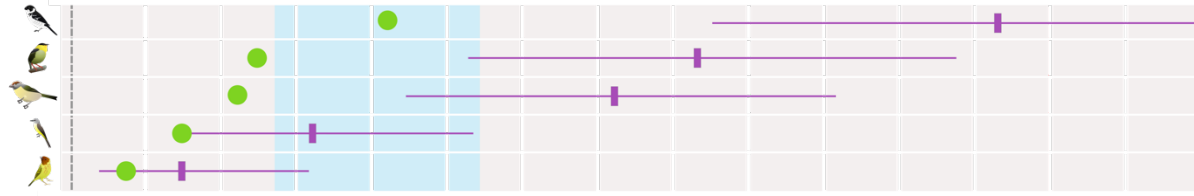
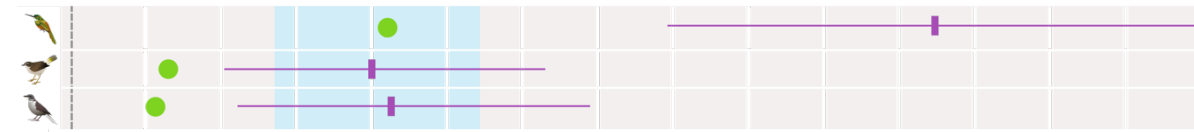
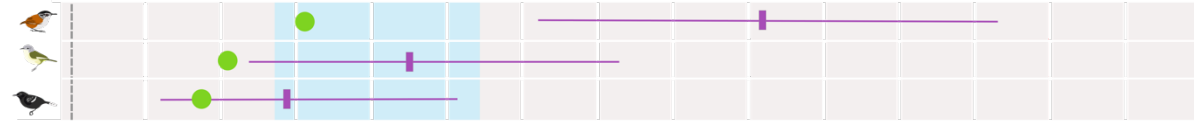


Figure 4: Associations of ecological traits with mitochondrial splits. Throughout the figure, dark purple circles represent taxa with two or more BINs, while light purple indicate those with only one. A) Hand wing index (HWI) is significantly lower in taxa with splits ($t = -5.52$, $df = 154.29$, $p < 0.001$), indicating lower physical dispersal capability is associated with mitochondrial turnover. B) Primary diet, showing that insectivores are represented significantly more than other dietary items ($\chi^2 = 17.27$, $df = 7$, $p = 0.02$) in taxa with splits. C) A visual representation of habitat use, showing that habitat type as measured by openness ($\chi^2 = 6.488$, $df = 2$, $p = 0.039$) and stratum ($\chi^2 = 14.04$, $df = 4$, $p = 0.007$) are associated with mitochondrial turnover, with it becoming increasingly likely in the closed forest understory. D) Despite the relatively even distribution of

territoriality across our sample, strongly territorial taxa were overrepresented among those with mitochondrial turnover ($\chi^2=12.04$, $df= 2$, $p=0.002$).





1 2 3 4 5 6 7 8 9 10 11 12 13 14
 Pleistocene Pliocene Miocene

Figure 5: Timing of mitochondrial splits for Panamanian birds in millions of years. Time was calculated from pairwise COI divergence (green) and in BEAST2 (purple, shown with median and 95% confidence intervals [CIs]). Splits are grouped by region as in Figure 3, with those taxa with multiple splits being shown for each. Some CIs are truncated due to space (indicated with asterisk); see Table 3 for full details.

TABLES

Table 1: Evidence for cryptic divergence in Panamanian birds, organized by taxonomy. Results show species with COI splits defined as multiple BINs, with additional mitochondrial markers indicated. Distances calculated on BOLD-aligned sequences with K2P method. Species with more than two unique BIN assignments have pairwise COI divergence for all inter-BIN comparisons listed.

Species	Family	Pairwise COI divergence	Other included markers
<i>Nyctidromus albicollis</i>	Caprimulgidae	5.16%	cytB, ND2, whole mitogenome
<i>Laterallus albigularis</i>	Rallidae	3.20%	
<i>Jacana spinosa</i>	Jacanidae	1.72%	
<i>Chloroceryle aenea</i>	Alcedinidae	2.19%	ND2, whole mitogenome
<i>Baryphthengus martii</i>	Momotidae	3.48% 3.31% 3.35%	Whole mitogenome
<i>Momotus momota</i>	Momotidae	5.64%	Whole mitogenome

Species	Family	Pairwise COI divergence	Other included markers
<i>Malacoptila panamensis</i>	Bucconidae	3.84%	ND3, whole mitogenome
<i>Galbula ruficauda</i>	Galbulidae	8.25%	cytB, whole mitogenome
<i>Manacus vitellinus</i>	Pipridae	5.08%	
<i>Schiffornis turdina</i>	Tityridae	2.20%	Whole mitogenome
<i>Myiobius sulphureipygius</i>	Onychorhynchidae	2.19%	
<i>Tyrannus melancholicus</i>	Tyrannidae	3.00%	
<i>Cercomacroides tyrannina</i>	Thamnophilidae	3.00%	
<i>Todirostrum cinereum</i>	Tyrannidae	2.87%	
<i>Microrhopias quixensis</i>	Thamnophilidae	2.51%	ND2, cytB, whole mitogenome
<i>Gymnocichla nudiceps</i>	Thamnophilidae	3.99%	ND2, cytB, whole mitogenome

Species	Family	Pairwise COI divergence	Other included markers
<i>Myrmeciza exsul</i>	Thamnophilidae		ND2, cytB, whole mitogenome
<i>Automolus ochralaemus</i>	Furnariidae	5.38%	cytB, whole mitogenome
<i>Sclerurus guatemalensis</i>	Furnariidae	2.08%	ND2, whole mitogenome
<i>Xenops minutus</i>	Furnariidae	2.77%	ND2, cytB, whole mitogenomes
<i>Cyclarhis gujanensis</i>	Vireonidae	4.66%	
<i>Pachysylvia decurtata</i>	Vireonidae	4.20%	ND2, whole mitogenome
<i>Microbates cinereiventris</i>	Poliophtilidae	6.12%	ND2, whole mitogenome
<i>Cantorchilus nigricapillus</i>	Troglodytidae	2.51%	cytB, ATPase 8 and 6, whole mitogenome
<i>Henicorhina leucosticta</i>	Troglodytidae	6.13% 6.89%	cytB, ATPase 8 and 6, ND2, whole mitogenome

Species	Family	Pairwise COI divergence	Other included markers
		7.61%	
<i>Henicorhina leucophrys</i>	Troglodytidae	4.63%	
<i>Turdus assimilis</i>	Turdidae	2.52%	
<i>Catharus fuscater</i>	Turdidae	5.67%	
<i>Arremon aurantirostris</i>	Passerellidae	6.92%	ND2, whole mitogenome
<i>Arremon brunneinucha</i>	Passerellidae	3.49%	
<i>Myiothlypis fulvicauda</i>	Parulidae	2.86%	
<i>Setophaga petechia</i>	Parulidae	1.24%	
<i>Myioborus miniatus</i>	Parulidae	3.18%	
<i>Icterus mesomelas</i>	Icteridae	1.24%	
<i>Cyanocompsa cyanoides</i>	Cardinalidae	5.60%	ND2, cytB, whole mitogenome

Species	Family	Pairwise COI divergence	Other included markers
<i>Ramphocelus passerini</i>	Thraupidae	1.65%	Whole mitogenomes
<i>Sporophila americana</i>	Thraupidae	8.49%	Whole mitogenomes

Table 2: Correlates of cryptic divergence in Panamanian birds. Distribution of traits across species, showing across all 658 resident landbirds, those distributed across multiple regions, those barcoded (including both widespread and regional species), those resident landbirds with widespread distributions that were barcoded, and those within that last group found to have more than one BIN.

	All species	Widely distributed	Barcoded (landbirds)	Sampled	Split
Total	658	338	388	181	34
Highland	199 (30.2%)	55 (15.1%)	105 (27.1%)	20 (11.5%)	4 (11.8%)
Habitat: Forest Edge	446 (67.8%)	224 (66.3%)	275 (70.9%)	121 (66.8%)	29 (85.3%)

	All species	Widely distributed	Barcoded (landbirds)	Sampled	Split
Open	132 (20.1%)	70 (20.7%)	74 (19.1%)	44 (24.3%)	4 (11.8%)
	76 (11.5%)	44 (13.0%)	38 (9.80%)	16 (8.84%)	1 (2.94%)
Stratum:					
Aerial	25 (3.80%)	17 (5.03%)	6 (1.55%)	2 (1.10%)	0
Canopy	274 (41.6%)	134 (39.6%)	146 (37.6%)	56 (30.9%)	4 (11.8%)
Mid-canopy	82 (12.5%)	47 (13.9%)	47 (12.1%)	25 (13.8%)	2 (5.89%)
Undergrowth	207 (31.5%)	102 (30.2%)	150 (38.6%)	84 (46.4%)	25 (73.5%)
Ground	69 (10.5%)	38 (11.2%)	38 (9.80%)	14 (7.73%)	3 (8.82%)
Diet:					
Omnivore	49 (7.45%)	18 (5.32%)	36 (9.28%)	10 (5.52%)	1 (2.94%)
Plant-based:	240 (36.5%)	106 (31.4%)	141 (36.3%)	66 (36.5%)	8 (23.5%)
General					
plant-based	26 (3.95%)	11 (3.25%)	15 (3.87%)	4 (2.21%)	0
Nectarivore	60 (9.12%)	22 (6.51%)	42 (10.3%)	23 (12.7%)	0
Granivore	41 (6.23%)	18 (5.32%)	20 (5.15%)	10 (5.52%)	5 (14.7%)
Frugivore	113 (17.2%)	55 (16.3%)	64 (16.5%)	29 (16.0%)	3 (8.82%)
Animal-based:	357 (54.3%)	207 (61.2%)	207 (53.3%)	105 (58.0%)	25 (73.5%)

	All species	Widely distributed	Barcoded (landbirds)	Sampled	Split
General					
animal-based	32 (4.86%)	25 (7.40%)	13 (3.35%)	4 (2.21%)	1 (2.94%)
Insectivore	296 (45.0%)	154 (45.6%)	181 (46.6%)	92 (50.8%)	23 (67.6%)
Vertebrates	29 (4.41%)	28 (8.28%)	13 (3.35%)	9 (4.97%)	1 (2.94%)

Table 3: Estimated time to the most recent common ancestor in Panamanian birds. Estimates were calculated in BEAST2 for the 34 taxa with multiple BINs. For taxa with three BINs, estimates for both split events are provided.

Taxon	Median divergence time (My), fossil calibrated	95% confidence interval (My), fossil calibrated
<i>Nyctidromus albicollis</i>	9.10	5.72 – 12.97
<i>Laterallus albigularis</i>	4.27	2.25 – 6.59
<i>Jacana spinosa</i>	2.48	0.91 – 4.35
<i>Chloroceryle aenea</i>	3.99	1.93 – 6.55

Taxon	Median divergence time (My), fossil calibrated	95% confidence interval (My), fossil calibrated
<i>Baryphthengus</i>	2.76	1.36 – 4.44
<i>martii</i>	5.05	3.03 – 7.39
<i>Momotus momota</i>	9.44	6.01 – 13.40
<i>Malacoptila</i>	5.07	2.77 – 7.82
<i>panamensis</i>		
<i>Galbula ruficauda</i>	11.66	7.95 – 15.89
<i>Manacus vitellinus</i>	8.28	5.20 – 11.71
<i>Schiffornis turdina</i>	2.76	1.26 – 4.73
<i>Myiobius</i>	2.43	1.02 – 4.12
<i>sulphureipygius</i>	3.87	2.06 – 5.96
<i>Mionectes</i>	3.42	1.84 – 5.39
<i>oleagineus</i>	5.16	3.13 – 7.58
<i>Tyrannus</i>	3.22	1.50 – 5.37
<i>melancholicus</i>		
<i>Cercomacroides</i>	2.15	0.86 – 3.72
<i>tyrannina</i>	3.88	2.07 – 6.11
<i>Todirostrum</i>	4.44	2.37 – 6.93
<i>cinereum</i>		
<i>Microrhopias</i>	2.92	1.25 – 5.11
<i>quixensis</i>		

Taxon	Median divergence time (My), fossil calibrated	95% confidence interval (My), fossil calibrated
<i>Gymnocichla nudiceps</i>	1.97 5.14	0.82 – 3.37 3.15 – 7.59
<i>Automolus ochralaemus</i>	8.58	5.44 – 12.24
<i>Sclerurus guatemalensis</i>	2.78	1.14 – 4.80
<i>Xenops minutus</i>	3.72	1.89 – 5.99
<i>Cyclarhis gujanensis</i>	7.14	4.44 – 10.33
<i>Pachysylvia decurtata</i>	4.66	2.48 – 7.25
<i>Microbates cinereiventris</i>	9.82	6.40 – 13.52
<i>Cantorchilus nigricapillus</i>	3.58	1.79 – 5.93
<i>Henicorhina leucosticta</i>	9.06 12.93	6.22 – 12.38 9.66 – 16.64
<i>Henicorhina leucophrys</i>	7.09	4.42 – 10.13
<i>Turdus assimilis</i>	4.32	2.14 – 6.90
<i>Catharus fuscater</i>	7.27	4.43 – 10.46

Taxon	Median divergence time (My), fossil calibrated	95% confidence interval (My), fossil calibrated
<i>Arremon aurantirostris</i>	10.49	7.31 – 13.94
<i>Arremon brunneinucha</i>	5.09	2.64 – 7.96
<i>Myiothlypis fulvicauda</i>	3.99	2.01 – 6.33
<i>Setophaga petechia</i>	1.64	0.53 – 3.15
<i>Myioborus miniatus</i>	4.19	2.20 – 6.52
<i>Icterus mesomelas</i>	2.61	1.10 – 4.49
<i>Cyanocompsa cyanooides</i>	8.23	5.31 – 11.52
<i>Ramphocelus passerini</i>	2.52	1.10 – 4.39
<i>Sporophila americana</i>	12.21	8.52 – 16.20

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