

1       **Title:** Speciation and gene flow across an elevational gradient in New Guinea kingfishers

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23

24           **Abstract:** Closely related species with parapatric elevational ranges are ubiquitous in  
25 tropical mountains worldwide. The gradient speciation hypothesis proposes that these series are  
26 the result of *in situ* ecological speciation driven by divergent selection across elevation. Direct  
27 tests of this scenario have been hampered by the difficulty inferring the geographic arrangement  
28 of populations at the time of divergence. In cichlids, sticklebacks, and *Timema* stick insects,  
29 support for ecological speciation driven by other selective pressures has come from  
30 demonstrating parallel speciation, where divergence proceeds independently across replicated  
31 environmental gradients. Here, we take advantage of the unique geography of the island of New  
32 Guinea to test for parallel gradient speciation in replicated populations of *Syma* kingfishers that  
33 show extremely subtle differentiation across elevation and between historically isolated  
34 mountain ranges. We find that currently described high elevation and low elevation species have  
35 reciprocally monophyletic gene trees and form nuclear DNA clusters, rejecting this hypothesis.  
36 However, demographic modeling suggests selection has likely maintained species boundaries in  
37 the face of gene flow following secondary contact. We compile evidence from the published  
38 literature to show that while *in situ* gradient speciation in labile organisms such as birds appears  
39 rare, divergent selection and post-speciation gene flow may be an underappreciated force in the  
40 origin of elevational series and tropical beta diversity along mountain slopes.

41

42 *Key words:* gradient speciation, parapatric speciation, ecological speciation, *Syma*

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46           **Introduction:** Series of closely related species with parapatric elevational ranges are  
47 ubiquitous in tropical mountains worldwide, contributing to their globally high beta diversity  
48 (Diamond, 1972; Jankowski, Ciecka, Meyer, & Rabenold, 2009; Cadena et al., 2012; Terborgh  
49 & Weske, 1975). This striking biogeographic pattern is consistent with alternate hypotheses of  
50 how speciation proceeds in tropical forest faunas (Moritz, Patton, Schneider, & Smith, 2000).  
51 Under the gradient speciation hypothesis, elevational series are the result of local adaptation to  
52 divergent elevational niches that leads to the evolution of reproductive isolation *in situ* (i.e.,  
53 ecological speciation without geographic isolation; Smith, Wayne, Girman, & Bruford, 1997;  
54 Moritz, Patton, Schneider, & Smith, 2000; Nosil, 2012; Caro, Caycedo-Rosales, Bowie, &  
55 Cadena, 2013; Beheregaray, Cooke, Chao, & Landguth, 2015). Alternatively, allopatric  
56 speciation followed by secondary contact and elevational range displacement through  
57 competition could lead to identical distributional patterns (Mayr, 1942; Endler, 1982; Cadena et  
58 al., 2012; Freeman, 2015).

59           Despite significant research attention, the origin of elevational series eludes easy  
60 synthesis. This is partly due to the difficulty of inferring the geographic mode of speciation and  
61 its evolutionary mechanisms from biogeographic and phylogenetic data alone (Endler, 1982;  
62 Losos & Glor, 2003). While the gradient speciation hypothesis predicts that lineages with  
63 parapatric elevational ranges will be sister to one another—a relationship found in *Ithioma*  
64 butterflies (Elias et al., 2009), *Leptopogon* flycatchers (Bates & Zink, 1994) and some Andean  
65 amphibians and reptiles (Arteaga et al., 2016; Guayasamin et al., 2017), but not in many other  
66 elevational series of Andean birds and mammals (e.g., Patton & Smith, 1992; Dingle, Lovette,  
67 Canaday, & Smith, 2006; Cadena et al., 2012; Caro, Caycedo-Rosales, Bowie, & Cadena, 2013;  
68 Cadena & Céspedes, 2019)—this pattern alone cannot distinguish divergence with geographic

69 isolation from divergence without geographic isolation. Furthermore, both the lability of species  
70 ranges over evolutionary time and extinction can quickly obscure any phylogenetic signal of the  
71 geography of speciation (Losos & Glor, 2003). In contrast, population genomic data can provide  
72 information on rates of gene flow through time, and by proxy, the geographic mode of  
73 divergence (Moyle et al., 2017; Chapman, Hiscock, & Filatov, 2013; Chapman, Hiscock, &  
74 Filatov, 2016), but can only reveal the loci underpinning reproductive isolation (and by  
75 extension, its selective drivers) under particular circumstances (e.g., with exceptional sampling  
76 of hybrid zones).

77         To date, the strongest evidence for ecological speciation of any type has come from  
78 studies of parallel divergence on replicated selective gradients (Schluter & Nagle, 1995;  
79 Johannesson, 2002). The independent evolution of similar morphs of cichlids (Schliewen, Tautz,  
80 & Pääbo, 1994) and sticklebacks (Rundle, Nagel, Boughman, & Schluter, 2000) in response to  
81 similar ecological pressures convincingly demonstrates a link between local adaptation and the  
82 evolution of *in situ* reproductive isolation. In *Timema* stick insects, parallel adaptation to  
83 different host plants in allopatric populations provides a terrestrial analogue at small spatial  
84 scales (Nosil, Crespi, & Sandoval, 2002). While this approach evokes studies of phylogenetic  
85 relationships in clades with taxa that segregate by both latitude and elevation (e.g., Cadena &  
86 Céspedes, 2019), it differs in true geographic independence among replicates, putatively shallow  
87 timescales that reduce the probability that extinction and range shifts have obscured evolutionary  
88 signal, and phenotypic variance that is shared across similar environments. To our knowledge,  
89 only one study has explicitly tested predictions of parallel gradient speciation across elevation  
90 (Fuchs, Fjeldså, & Bowie, 2011).

91           Here, we take advantage of the unique geography of the island of New Guinea to perform  
92 such a test in replicated populations of interior forest kingfishers in the genus *Syma* that show  
93 extremely subtle differentiation across elevation and between geographic replicates. We  
94 collected genomic, morphological, and biacoustic data to evaluate species limits and evidence for  
95 assortative mating, phylogenetic relationships, and demographic history. Under a hypothesis of  
96 parallel gradient speciation, we predicted that highland and lowland populations of *Syma* in  
97 isolated mountain ranges that have never been connected by montane forest (Benz, 2011) would  
98 be sister to one another and more distantly related to their allopatric congeners. Alternately,  
99 under the secondary contact hypothesis, we predicted all highland and lowland populations  
100 would be reciprocally monophyletic. We additionally evaluated the role of gene flow during  
101 divergence in *Syma*, and compile evidence relevant to these hypotheses from the published  
102 literature.

103  
104           **Materials and Methods: Study system.** As currently delimited, Yellow-billed and  
105 Mountain Kingfishers *Syma torotoro* and *S. megarhyncha* (Aves: Alcedinidae) are putative sister  
106 taxa that segregate by elevation (Pratt & Beehler, 2015; Diamond, 1972) across the island of  
107 New Guinea. Lowland forest species *S. torotoro* is reportedly smaller with a higher-pitched call,  
108 and primarily found below 700 m, while the slightly larger, deeper-voiced *S. megarhyncha* is  
109 primarily found above 1100 m to 2200 m or higher in mid-montane forest (Pratt & Beehler,  
110 2015). A third population, Yellow-billed Kingfisher subspecies *S. t. ochracea*, may also merit  
111 species status; intermediate in size and divergent in call, it is restricted to the oceanic islands of  
112 the D'Entrecasteaux Archipelago in southeastern Papua New Guinea. (For the remainder of the  
113 manuscript we refer to this taxon as *S. (t.) ochracea* to reflect this uncertainty but will ignore

114 subspecies level taxa in other cases.) All *Syma* taxa spend most of their time in the middle and  
115 upper strata of closed-canopy forests, where they feed on a mix of insects and small vertebrates  
116 (Pratt & Beehler, 2015).

117         While Mayr, Rand, and Diamond argued that elevational replacements in New Guinea  
118 form through secondary contact of differentiated lineages (Rand, 1936; Mayr, 1942; Diamond,  
119 1972), some evidence suggests *Syma megarhyncha* and *S. torotoro* may instead have undergone  
120 gradient speciation across elevation. First, both mainland *Syma* are territorial and sedentary,  
121 making it plausible that dispersal was sufficiently reduced to be overcome by divergent selection  
122 across the steep environmental gradient they inhabit (Endler, 1977). Second, *S. megarhyncha*'s  
123 larger size (Beehler & Pratt, 2016) is consistent with predictions of morphological adaptation to  
124 a cooler climate (Freeman, 2017). Additionally, geographically isolated populations of *S.*  
125 *megarhyncha* in the mountains of the Huon Peninsula and the Central Ranges have fixed  
126 differences in bill markings, hinting they may have formed independently through parallel  
127 divergence from local *S. torotoro* populations. However, species limits and range-wide variation  
128 have never been quantitatively assessed with either phenotypic or genetic data and observed  
129 differences might instead reflect phenotypic plasticity or clinal variation of a single widespread  
130 lineage (Caro et al., 2013).

131         *Morphological and bioacoustics data.* To evaluate phenotypic support for species limits,  
132 we measured bill length, bill depth, tarsus, wing chord, and tail length from 72 museum  
133 specimens of *Syma torotoro* ( $n=30$ ), *S. (t.) ochracea* ( $n=10$ ), and *S. megarhyncha* ( $n=32$ ) at the  
134 American Museum of Natural History, collected from 1894-1965 and including only individuals  
135 of known sex as originally identified by the preparator. Using these data, we performed principal  
136 component analyses in R (R Core Team 2018) with log-transformed and normalized variables

137 and used PC1 to build mixture models using the R package mclust v. 5.4.1, which we evaluated  
138 with a maximum likelihood classification approach (Scrucca, Fop, Murphy, & Rafferty, 2016).  
139 We downloaded all available vocalizations from *S. torotoro* ( $n=34$ ) and *S. megarhyncha* ( $n=14$ )  
140 from xeno-canto and Cornell's Macaulay Library. We filtered these data for quality and  
141 quantified 36 standard bioacoustic variables using the warbleR package v. 1.1.14 in R (Araya-  
142 Salas & Smith-Vidaurre, 2017), analyzing 278 distinct vocalizations from *S. torotoro* and 106  
143 from *S. megarhyncha* in total. We ran PCA with normalized variables on the output and used  
144 these data to alternate species delimitation models using the same approach as with our  
145 morphological data.

146 *Sampling, library preparation, and DNA sequencing.* To infer population genetic  
147 structure and phylogenetic history in *Syma*, we extracted DNA from fresh tissues ( $n=6$ ) and  
148 toepad samples from historical museum specimens ( $n=34$ ) from 28 individuals of *S. torotoro*, 2  
149 individuals of *S. (t.) ochracea*, and 10 individuals of *S. megarhyncha* ( $n=10$ ), including 3  
150 individuals collected in 1928 by Ernst Mayr himself. Though only partially overlapping with  
151 samples used in morphometric analyses, these individuals represented the full extent of both  
152 species' ranges in New Guinea and Australia (**Table S1**) and included all described subspecies.  
153 We extracted DNA from fresh tissues using a Qiagen DNAeasy kit and the manufacturer's  
154 recommended protocol. For historical toepad samples (collected 1877-1973), we extracted DNA  
155 using either a using a phenol-chloroform and centrifugal dialysis method (Dumbacher &  
156 Fleischer, 2001) (for reduced representation sequencing) or a standard salt extraction protocol  
157 (for whole genome sequencing). Due to constraints of cost and time, we employed two  
158 complementary sequencing approaches. On a subset of samples ( $n=20$ ), we performed reduced  
159 representation genome sequencing using a hybridization capture with RAD probes (hyRAD)

160 approach, described in detail elsewhere (Linck, Hanna, Sellas, & Dumbacher, 2017). We sent the  
161 remaining samples to the UC Berkeley's Vincent J. Coates Genomic Sequencing Laboratory,  
162 where laboratory staff prepared genomic libraries for low coverage whole genome sequencing  
163 (WGS) using Illumina TruSeq Nano kits and a modified protocol that enzymatically repaired  
164 fragments with RNase and skipped sonication. They then pooled ( $n=20$ ) and sequenced these  
165 samples with 150 base pair paired end reads on a single lane of an Illumina HiSeq 4000.

166 *Sequence assembly and variant calling.* We processed demultiplexed reads from both  
167 sequencing strategies together with a custom bioinformatic pipeline optimized for handling  
168 degraded DNA data and available at [https://github.com/elinck/syma\\_speciation](https://github.com/elinck/syma_speciation). Briefly, we  
169 trimmed raw reads for adapters and low-quality bases using bbduk from BBTools version 38.06  
170 suite of bioinformatics tools. We aligned these reads to an unpublished draft genome of  
171 Woodland Kingfisher *Halcyon senegalensis* from the Bird 10K Genome Project using bmap  
172 with a k-mer value of 12, a maximum indel length of 200 bp, and a minimum sequence identity  
173 of 0.65. These highly sensitive alignment parameters were necessary given the clade containing  
174 *Syma* diverged from the clade containing *Halcyon* approximately 15 mya (Andersen,  
175 McCullough, Mauck, Smith, & Moyle, 2018). We used PicardTools v. 2.17.8 and GATK v. 3.6.0  
176 (McKenna et al., 2010) to append read groups and perform local realignment on .bam files. We  
177 then used mapDamage 2.0.9 to account for postmortem damage to DNA from historical museum  
178 specimens by rescaling quality scores (Jónsson, Ginolhac, Schubert, Johnson, & Orlando, 2013).  
179 We performed multisample variant calling using the UnifiedGenotyper tool in GATK v. 3.6.0,  
180 and filtered our variant calls for missing data, coverage, and quality with VCFtools 0.1.16  
181 (Danecek et al., 2011). We generated multiple SNP datasets by implementing different filters to  
182 suit the requirements and goals of different analyses; these settings and their underlying rationale



183 are described in detail below. To complement our nuclear DNA sequences, we assembled near-  
184 complete and fully annotated mitochondrial genomes from a majority of individuals using  
185 mitofinder v. 1.0.2 (Allo et al., 2020) and a complete mtDNA genome from close relative  
186 *Todiramphus sanctus* as a reference (Andersen et al., 2015). We then extracted NADH  
187 dehydrogenase 2 (ND2) from these genomes and performed multiple sequence alignment using  
188 MAFFT v7.407 under its “-auto” parameter setting package (Katoh & Standley, 2013).

189 *Population structure inference.* We evaluated population genetic structure within and  
190 between species using a nonparametric clustering approach. We performed principal component  
191 analysis of genotypes (PCA) and identified putative genetic clusters for  $k=2$  through  $k=4$  using  
192 adegenet v. 2.1.1 (Jombart, 2008) and a 95% complete dataset with 66,917 SNPs from 35  
193 individuals of *Syma torotoro* ( $n=24$ ), *S. (t.) ochracea* ( $n=2$ ), and *S. megarhyncha* ( $n=9$ ) that  
194 passed quality filters. This included individuals from both sequencing approaches with a  
195 minimum depth of coverage of 3x per individual, a maximum depth of coverage of 120x per  
196 individual, a minimum quality score of 30, and a minimum minor allele frequency of 0.05. These  
197 relatively lenient filtering parameters were chosen to permit the inclusion of samples from both  
198 sequencing strategies in our initial assessment of probable species limits. We evaluated the best-  
199 fit clustering model using the Bayesian Information Criterion, again implemented in adegenet.  
200 To ensure our results were not an artifact of different library preparation methods or tissue type,  
201 we employed two complementary approaches. First, we performed pairwise Wilcoxon rank sum  
202 tests evaluating whether differences in coverage or the proportion of missing data were  
203 significantly associated with inferred clusters. Second, we reran PCA using the same filters as  
204 above but instead 1) included only whole genome sequence data or 2) excluded all modern

205 tissues. We then compared relationships among samples from these analyses with those observed  
206 in the full dataset.

207 *Phylogenetic inference.* As we lacked whole genome sequences from an appropriate  
208 outgroup species, we evaluated phylogenetic relationships among using our mitochondrial DNA  
209 (ND2) alignment, which was near-complete and did not feature correlations between missing  
210 data and library preparation method or tissue type. We inferred a time-calibrated phylogeny in  
211 BEAST 2.6.0 (Bouckaert et al., 2019) from the 37 samples of *Syma torotoro* ( $n=24$ ), *S. (t.)*  
212 *ochracea* ( $n=3$ ), and *S. megarhyncha* ( $n=10$ ) with sufficiently high quality ND2 sequence data,  
213 including Sacred Kingfisher *Todiramphus sanctus* as an outgroup (Andersen et al., 2015). We  
214 used a strict molecular clock with a rate of  $2.9 \times 10^{-8}$  following Lerner, Meyer, James, Hofreiter,  
215 & Fleischer (2011), a GTR+GAMMA model of nucleotide evolution, and a Yule species tree  
216 prior. We ran BEAST for 50 million generations, storing trees every 1000 generations, and  
217 assessed mixing, likelihood stationarity and adequate effective sample sizes (ESS) above 200 for  
218 all estimated parameters using Tracer v.1.7.1 (Rambaut, Drummond, Xie, Baele, & Suchard,  
219 2018). We then generated a maximum clade credibility tree using TreeAnnotator v.2.6.0,  
220 discarding the first 10% of trees as burnin (Bouckaert et al., 2019).

221 *Demographic inference.* We inferred the demographic history of the diverging lineages  
222 identified in our clustering and phylogenetic analyses using moments v.1.0.0, which uses  
223 ordinary differential equations to model the evolution of allele frequencies (Jouganous, Long,  
224 Ragsdale, & Gravel, 2017). We used the `joint_sfs_folded()` function in Python package scikit-  
225 allele v.1.2.1 (Miles, Ralph, Rae, & Pisupati, 2019) to calculate a folded joint site frequency  
226 spectrum (JSFS) using a SNP dataset that included only whole genome sequencing data, a  
227 minimum minor allele count of 1, no more than 10% missing data per site, a minimum depth of

228 6x per individual, and a minimum quality score of 30. We chose these filtering parameters to  
229 reduce the influence of sequencing error without strongly biasing the JSFS. This dataset did not  
230 include *S. t. ochracea*, and had been further thinned to include only variants in approximate  
231 linkage equilibrium (defined as sites where  $r^2 < 0.1$ ) using scikit-allel's `locate_unlinked()`  
232 function. To account for the remaining missing data, we projected the JSFS down to an effective  
233 sample size of 16 x 12 chromosomes for *S. megarhyncha* and *S. torotoro* respectively.

234 We then specified four basic demographic models chosen to represent plausible  
235 speciation scenarios in *Syma*, which differed by level and timing of gene flow. These were: an  
236 isolation-with-migration model (IM), which permitted gene flow throughout the history of  
237 diverging lineages; an isolation-with-ancestral-migration model (AM), which featured an initial  
238 period of gene flow followed by a period of isolation; a model of secondary contact (SC), which  
239 featured an initial period of isolation followed by a period of gene flow; and a model of strict  
240 isolation (SI), which allowed no gene flow following initial divergence. Next, we specified four  
241 additional models which differed from each of the above only by allowing exponential  
242 population growth in the most recent time period: an isolation-with-migration-and-growth model  
243 (IMg), an isolation-with-ancestral-migration-and-growth model (AMg), a secondary contact and  
244 growth model (SCg), and a strict isolation and growth model (SIg).

245 After initially optimizing parameters for fitting each model using the “optimize log”  
246 method and a maximum of 3 iterations, we ran 9 additional optimizations, randomly sampling  
247 starting values from a uniform distribution within the bounds of  $1 \times 10^{-4}$  and 10. We converted  
248 parameter values to real units using a genome-wide mutation rate of  $2.3 \times 10^{-9}$  (Smeds,  
249 Qvarnström, & Ellegren, 2016), an effective sequence length scaled to reflect our LD-thinned  
250 SNP dataset, and a generation time estimate of two years. We generated parameter uncertainty

251 estimates for the best-fit demographic model (selected based on AIC values) by fitting it to 200  
252 bootstrapped site frequency spectra generated using the `fs.sample()` function on our data, an  
253 approach which is appropriate when sites are unlinked (Jouganous et al., 2017).

254 *Literature review.* Last, we reviewed the published literature for studies explicitly or  
255 implicitly testing predictions of the gradient speciation hypothesis in elevational series of birds.  
256 We first performed a Web of Science search on 29 May 2020 using the terms “speciation AND  
257 elevation\* AND bird\*”. From the 122 results, we retained 15 studies that met three criteria: they  
258 (1) generated novel phylogenetic or population genetic data; (2) involved at least two congeneric  
259 species or otherwise classified reciprocally monophyletic lineages with different elevational  
260 ranges; and (3) focused on tropical or subtropical taxa. We augmented these results with 16  
261 relevant studies missed by the initial search that we were aware of from other contexts or  
262 discovered through the cited literature. In sum, our review included studies addressing a total of  
263 24 unique taxa (**Table 2**).

264  
265 **Results and Discussion:** Despite the aspects of the biology and distribution of *Syma*  
266 kingfishers that are consistent with parallel gradient speciation across elevation, phylogenetic  
267 and population genetic evidence lead us to unequivocally reject this hypothesis in *Syma*. A  
268 maximum likelihood phylogeny from the mitochondrial gene ND2 and a clustering analysis from  
269 genome-wide DNA sequence data indicate current species limits are largely correct: allopatric  
270 high-elevation populations of Mountain Kingfisher *Syma megahryncha* are indeed each other’s  
271 closest relative, as are all sampled populations of Yellow-billed Kingfisher *Syma torotoro*  
272 (**Figure 1b,d**). These results were robust to potential artifacts of sample type or sequencing  
273 strategy. PCA performed on only whole genome sequencing data or only historic samples

274 recovered qualitatively similar patterns (**Figure S1**), and pairwise Wilcoxon rank sum tests  
275 found no statistically significant association between coverage or the proportion of missing data  
276 in samples (all  $P > 0.05$ ). Inferred nuclear DNA clusters and mtDNA lineages were also reflected  
277 by phenotypic data, occupying divergent though overlapping areas of principal component space  
278 (**Figure 1e,f**). The first principal component of all bioacoustic and morphological data was  
279 bimodally distributed with respect to *S. torotoro* and *S. megarhyncha*, as were individual trait  
280 measurements (**Figure S2a**), a pattern predicted under assortative mating (Cadena, Zapata, &  
281 Jiménez, 2018). Strong negative loadings on PC1 for all measured morphological variables are  
282 more consistent with general body size differences than divergent selection on a specific  
283 ecologically relevant trait (i.e., bill width) (**Table S3**).

284         The clustering analysis and ND2 phylogeny alone do not exclude the possibility that  
285 gradient speciation occurred across a single slope followed by population expansion of the  
286 montane taxa to additional isolated montane regions. However, our best-fit demographic model  
287 of secondary contact (**Table 1**) is difficult to reconcile with speciation at the necessarily small  
288 spatial scale of a local mountainside. Furthermore, we note that our ND2 gene tree indicates an  
289 unexpected sister relationship between *S. megarhyncha* and the phenotypically distinctive  
290 Yellow-billed kingfisher subspecies *S. (t.) ochracea*, suggesting the latter may best be classified  
291 as a distinct biological species (**Figure 1b**). Though this relationship is consistent with multiple  
292 divergence histories—and reflects only the history of a single, nonrecombining locus—it  
293 presents a possible (if unparsimonious) scenario of allopatric speciation on an oceanic island  
294 followed by a subsequent reinvasion of the mainland and range displacement.

295         In spite of the lack of evidence for parallel speciation, we emphasize our data nonetheless  
296 indicate a prominent role for natural selection in driving the evolution of elevational

297 replacements. Parameter estimates from our model of secondary contact indicate *Syma torotoro*  
298 and *Syma megarhyncha* initially diverged in allopatry over 649,700 years ago (SD: 13,672  
299 years), a value remarkably similar to the estimate from the time-calibrated ND2 gene tree  
300 (**Figure 1b**, 790,020 years ago; 95% CI: 607,421-972,618 years). This period of isolation was  
301 followed by a period of secondary contact and gene flow that initiated 250,000 years ago (**Table**  
302 **1**). Our best estimate of the effective migration rate from *S. torotoro* to *S. megarhyncha*  
303 ( $Nm=1.14$ ; SD: 0.02) is low but above the widely cited rule of thumb that one-migrant-per-  
304 generation prevents population stratification (Wright, 1931; Slatkin, 1985; Wang, 2004). This  
305 level of migration indirectly implies that some force—which we suggest is divergent selection—  
306 prevents gene flow from leading to the collapse of distinct populations (Nosil, 2012). Effective  
307 migration from *S. megarhyncha* to *S. torotoro* was considerably lower ( $Nm=0.11$ ; SD=0.007), as  
308 expected given the lower inferred census population size of the smaller-ranged Mountain  
309 Kingfisher ( $N=368,390$ ; SD=8,528) compared to the Yellow-billed Kingfisher ( $N=1,909,985$ ;  
310 SD=26,028).

311         The apparent absence of recently admixed individuals in our dataset is unsurprising given  
312 the coarse grain of our sampling, but unfortunately precludes a rigorous assessment of the  
313 relative contribution of postzygotic (e.g., hybrid sterility) and prezygotic (habitat choice or  
314 assortative mating) isolation. However, the shallow divergence and long duration of gene flow  
315 between species leads us to suspect the former mechanism is at the very least only an incomplete  
316 reproductive barrier, suggesting extrinsic factors (particularly habitat requirements and selection  
317 against maladapted immigrants from different elevations) may be important. Evidence from  
318 recent field surveys that the species' elevational range limits may not be truly parapatric in some

319 cases but instead separated by hundreds of meters could further limit effective migration in either  
320 direction (Freeman & Freeman, 2014; Sam, Koane, & Novotny, 2014).

321         Ultimately, our assessment of speciation in *Syma* is consistent with trends revealed by a  
322 review of the literature on the origin of elevational series of tropical birds (**Table 2**). Of the 24  
323 taxa included in our review, 17 concluded secondary contact was the exclusive mechanism  
324 behind the formation of elevational replacements. While seven taxa that showed patterns  
325 consistent with gradient speciation between at least two species, only a single study suggested  
326 gradient speciation was more common than allopatric divergence within elevational zones in a  
327 particular clade. All putative cases of gradient speciation lacked corroborating population  
328 genomic evidence. Of the eight taxa that had been explicitly tested for gene flow between  
329 elevational replacements, six detected it at appreciable levels. Though this figure is likely  
330 inflated by ascertainment bias, it nonetheless suggests hybridization between elevational  
331 replacements may be more than previously appreciated (e.g., Cadena & Céspedes, 2019). We  
332 believe that while these findings confirm that divergence in allopatry likely generates the  
333 overwhelming majority of elevational series and support a shift away from *in situ* gradient  
334 speciation in labile organisms such as birds, they should reinforce a focus on divergent selection  
335 in the origin of tropical beta diversity. As the discovery of gene flow between diverging lineages  
336 at some point in the speciation continuum becomes the norm rather than the exception (Nosil,  
337 2008; Kumar et al. 2017; Schumer et al., 2018; Edelman et al., 2019), we suggest that ecological  
338 and phenotypic differences that are evident during early divergence are likely to be adaptive and  
339 to function as drivers of the speciation process.

340



341           **Data availability:** All code used in this study can be found at  
342 [https://github.com/elinck/syma\\_speciation](https://github.com/elinck/syma_speciation). Processed data are available from Dryad [doi:  
343 10.5061/dryad.4f4qrfj9b], and sequence data are available from the NCBI SRA [accession:  
344 pending].

345

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577 **Table 1.** Demographic model test results.

| Model   | Log-likelihood | AIC      |
|---|----------------|----------|
| Secondary contact (SC)                              | -564.062       | 1140.124 |
| Isolation-with-ancestral-migration (AM)             | -590.0233      | 1192.047 |
| Strict isolation and growth (SIg)                   | -611.0493      | 1228.099 |
| Isolation-with-migration and growth (IMg)           | -617.116       | 1244.232 |
| Isolation-with-migration (IM)                       | -631.8897      | 1273.779 |
| Strict isolation (SI)                               | -637.185       | 1280.37  |
| Isolation-with-ancestral-migration and growth (AMg) | -653.5472      | 1319.094 |
| Secondary contact and growth (SCg)                  | -716.0587      | 1444.117 |

578

579 **Table 2.** Previous studies addressing the origin of elevational series of tropical birds using  
580 molecular data. The column “Speciation mode” describes the inferred model of divergence for  
581 each clade; when multiple species pairs in a study demonstrated conflicting histories, we  
582 describe the more common process but acknowledge heterogeneity using the word “mostly”. In  
583 some cases, we have assigned speciation mode based on our interpretation of the data rather than  
584 an explicitly stated conclusion by the original authors. The column “Gene flow” indicates  
585 whether gene flow between elevational replacements was tested for, and if so, whether or not it  
586 was detected.

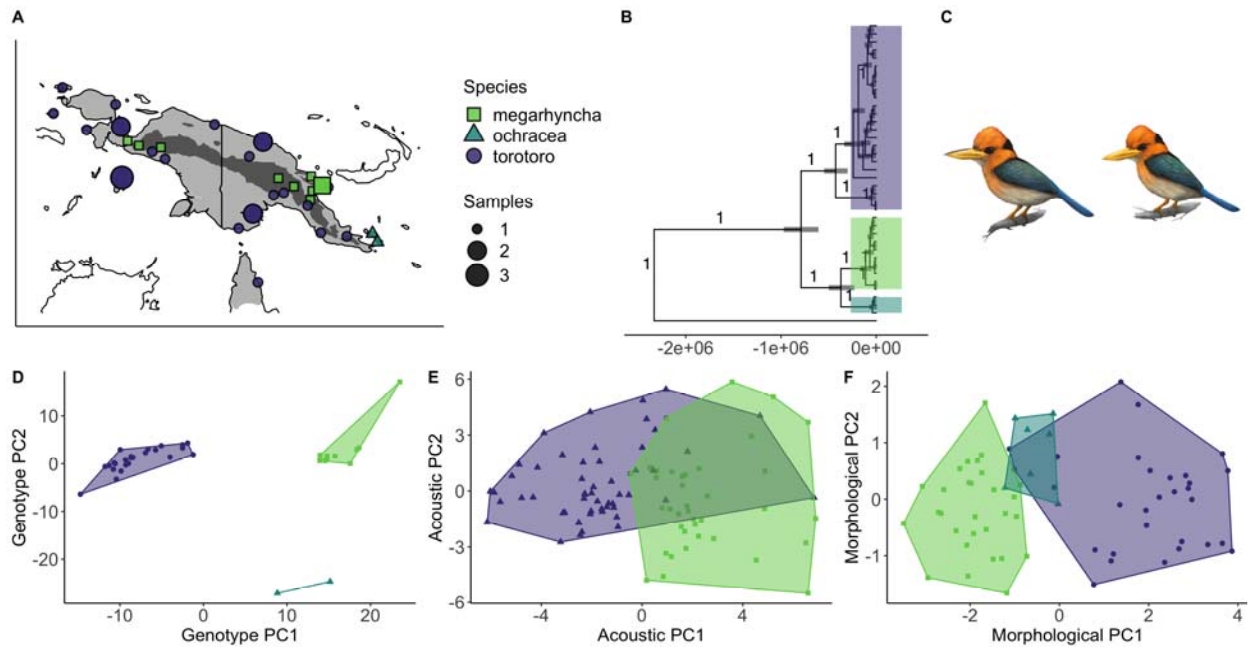
587

| Citations  | Taxon                            | Region | Speciation mode             | Gene flow |
|--|----------------------------------|--------|-----------------------------|-----------|
| Bates & Zink, 1994   | <i>Leptogon</i><br>flycatchers   | Andes  | Gradient<br>speciation      | N/A       |
| Arctander & Fjeldsa,<br>1994; Cadena et al.,<br>2020   | <i>Scytalopus</i><br>tapaculos   | Andes  | Mostly secondary<br>contact | N/A       |
| Garcia-Moreno et al.,<br>1998  | <i>Ochthoeca</i><br>chat-tyrants | Andes  | Secondary contact           | N/A       |
| Garcia-Moreno et al.,<br>1999a   | <i>Cranioleuca</i><br>spinetails | Andes  | Secondary contact           | N/A       |
| Garcia-Moreno et al.,<br>1999b; Benham et<br>al., 2014   | <i>Metallura</i><br>hummingbirds | Andes  | Secondary contact           | N/A       |
| Garcia-Moreno et al.,<br>2001  | <i>Hemispingus</i><br>tanagers   | Andes  | Secondary contact           | N/A       |
| Burns & Naoki, 2004  | <i>Tangara</i><br>tanagers       | Andes  | Secondary contact           | N/A       |
| Dingle et al., 2006;<br>Caro et al., 2013;<br>Halfwerk et al.,<br>2016; Cadena et al.,<br>2019 | <i>Henicorhina</i><br>wood wrens | Andes  | Secondary contact           | Yes       |
| Ribas et al., 2007   | <i>Pionus</i> parrots            | Andes  | Secondary contact           | N/A       |
| Cadena, 2007   | <i>Buarremon</i>                 | Andes  | Secondary contact           | N/A       |

|  |   |                |                              |     |
|--|---|----------------|------------------------------|-----|
|  | brush-finches                                     |                |                              |     |
| Chaves et al., 2007                            | <i>Adelomyia melanogenys</i> hummingbird lineages | Andes          | Possibly gradient speciation | Yes |
| Norman et al., 2007                            | <i>Meliphaga</i> honeyeaters                      | Australasia    | Mostly secondary contact     | N/A |
| Parra et al., 2009                             | <i>Coeligena</i> hummingbirds                     | Andes          | Secondary contact            | N/A |
| Sedano & Burns, 2010                           | 93 tanager species                                | Andes          | Mostly secondary contact     | N/A |
| Fuchs et al., 2011                             | <i>Phyllastrephus debilis</i> lineages            | Afrotropics    | Secondary contact            | Yes |
| Päckert et al., 2011                           | Seven passerine groups                            | Himalaya       | Secondary contact            | N/A |
| Dubay & Witt, 2012; Dubay & Witt, 2014         | <i>Anareites</i> tit-tyrants                      | Andes          | Secondary contact            | Yes |
| Wu et al., 2014                                | Leiotherichinae babblers                          | Himalaya       | Secondary contact            | N/A |
| Winger et al., 2014                            | <i>Grallaria</i> antpittas                        | Andes          | Secondary contact            | N/A |
| Voelker et al., 2015                           | <i>Pheonicurus</i> redstarts                      | Himalaya       | Mostly secondary contact     | N/A |
| Moyle et al., 2017                             | Five pairs of passerine taxa                      | Borneo         | Secondary contact            | No  |
| Morales-Rozo et al., 2017                      | <i>Ramphocelus</i> tanagers                       | Andes          | Secondary contact            | Yes |
| Cowles & Uy, 2019                              | <i>Zosterops</i> white-eyes                       | Australasia    | Secondary contact            | No  |
| Gabrielli et al., 2020; Bourgeois et al., 2020 | <i>Zosterops bourbonicus</i> white-eyes           | Reunion Island | Possibly gradient speciation | Yes |

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591 **Figure 1.** A) Sampling localities for *Symbia* kingfishers across New Guinea and Australia, color-  
592 coded by genotype PC1 and scaled by number of individuals. B) A time-calibrated ND2  
593 phylogeny supports reciprocal monophyly of *S. torotoro* and a clade with *S. megarhyncha* and *S.*  
594 *(t.) ochracea*, both themselves monophyletic. C) Illustration of *S. megarhyncha* (top) and *S.*  
595 *torotoro* (bottom), by Kevin Epperly. D). Principal component analysis of genotypes, clustered  
596 by the best fit *k*-means result ( $K=3$ ) and color-coded by the mean PC1 value for all individuals in  
597 a given cluster. E) Principal component analysis of bioacoustic parameters, color-coded by  
598 species. F) Principal component analysis of morphological data, color-coded by species.



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**Supplemental material.**

**Table S1.** Sampling, sequencing, and analysis information. WP=Indonesian New Guinea; PNG=Papua New Guinea.

| Specimen          | Species  | Subspecies | Locality                    | Tissue source | Date collected | Sequencing strategy | PCA | mtDNA phylogeny | Demographic inference |
|-------------------|----------|------------|-----------------------------|---------------|----------------|---------------------|-----|-----------------|-----------------------|
| AMNH:Birds:293714 | torotoro | torotoro   | Ifaar, WP                   | toepad        | 1928           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:293715 | torotoro | torotoro   | Kepaur, WP                  | toepad        | 1897           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:300723 | torotoro | torotoro   | Kepaur, WP                  | toepad        | 1897           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:300723 | torotoro | torotoro   | Misol Island, WP            | toepad        | 1900           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:329542 | torotoro | torotoro   | Wasior, WP                  | toepad        | 1928           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:437798 | torotoro | torotoro   | Amberbaki, WP               | toepad        | 1877           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637429 | torotoro | torotoro   | Humbolt Bay, WP             | toepad        | 1928           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637441 | torotoro | torotoro   | Mt. Mori, WP                | toepad        | 1899           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637445 | torotoro | torotoro   | East Sepik Province, PNG    | toepad        | 2003           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637446 | torotoro | torotoro   | Waigeu Island, WP           | toepad        | 1900           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637450 | torotoro | tentelare  | Aru Islands, WP             | toepad        | 1896           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637464 | torotoro | tentelare  | Aru Islands, WP             | toepad        | 1900           | hyRAD               | yes | yes             | no                    |
| AMNH:Birds:637464 | torotoro | ochracea   | Normanby Island, PNG        | toepad        | 1934           | hyRAD               | yes | yes             | no                    |
| CAS:Birds:7131    | torotoro | pseuestes  | Gulf Province, PNG          | muscle        | 2002           | hyRAD               | yes | yes             | no                    |
| KU:Birds:5215     | torotoro | pseuestes  | Gulf Province, PNG          | muscle        | 2003           | hyRAD               | yes | yes             | no                    |
| KU:Birds:5464     | torotoro | pseuestes  | Gulf Province, PNG          | muscle        | 2003           | hyRAD               | yes | yes             | no                    |
| KU:Birds:626      | torotoro | meeki      | Varirata National Park, PNG | muscle        | 2011           | hyRAD               | yes | yes             | no                    |
| KU:Birds:6927     | torotoro | meeki      | Mt. Suckling, PNG           | muscle        | 2011           | hyRAD               | yes | yes             | no                    |

|                            |             |              |                          |        |      |       |     |     |     |
|----------------------------|-------------|--------------|--------------------------|--------|------|-------|-----|-----|-----|
| NHMUK:Birds:1911.12.20.822 | torotoro    | pseustes     | Satakwa River, WP        | toepad | 1911 | hyRAD | yes | yes | no  |
| NHMUK:Birds:1911.12.20.823 | torotoro    | pseustes     | Mimika River, WP         | toepad | 1913 | hyRAD | yes | yes | no  |
| AMNH:Birds:301861          | megarhyncha | wellsi       | Weylendgep Kunupi, WP    | toepad | 1931 | WGS   | yes | yes | yes |
| AMNH:Birds:302859          | megarhyncha | wellsi       | Mt. Derimapa, WP         | toepad | 1930 | WGS   | yes | yes | yes |
| AMNH:Birds:339957          | megarhyncha | wellsi       | Bernhard Camp, WP        | toepad | 1939 | WGS   | yes | yes | yes |
| AMNH:Birds:808986          | megarhyncha | megarhyncha  | Okapa, PNG               | toepad | 1965 | WGS   | yes | yes | yes |
| ANWC:Birds:B02192          | megarhyncha | megrhyncha   | Western Highlands, PNG   | toepad | 1963 | WGS   | yes | yes | yes |
| ANWC:Birds:B04293          | megarhyncha | megarhyncha  | Morobe, PNG              | toepad | 1966 | WGS   | yes | yes | yes |
| ANWC:Birds:B25307          | megarhyncha | megarhyncha  | Wagau, PNG               | toepad | 1973 | WGS   | yes | yes | yes |
| ANWC:Birds:B25652          | megarhyncha | sellamontis  | Huon Peninsula, PNG      | toepad | 1973 | WGS   | yes | yes | yes |
| ANWC:Birds:B26140          | megarhyncha | sellamontis  | Huon Peninsula, PNG      | toepad | 1973 | WGS   | yes | yes | yes |
| YPM:Birds:91444            | megarhyncha | sellamontis  | Huon Peninsula, PNG      | toepad | 1969 | WGS   | yes | yes | yes |
| AMNH:Birds:329539          | torotoro    | ochracea     | Fergusson Island, PNG    | toepad | 1928 | WGS   | yes | yes | yes |
| AMNH:Birds:329540          | torotoro    | ochracea     | Fergusson Island, PNG    | toepad | 1928 | WGS   | yes | yes | yes |
| AMNH:Birds:426095          | torotoro    | flavirostris | Fly River, PNG           | toepad | 1936 | WGS   | yes | yes | yes |
| AMNH:Birds:426096          | torotoro    | flavirostris | Fly River, PNG           | toepad | 1936 | WGS   | yes | yes | yes |
| AMNH:Birds:426121          | torotoro    | meeki        | Wassi Kussi River, PNG   | toepad | 1937 | WGS   | yes | yes | yes |
| AMNH:Birds:637460          | torotoro    | tentelare    | Aru Islands, WP          | toepad | 1900 | WGS   | yes | yes | yes |
| AMNH:Birds:637471          | torotoro    | meeki        | Simbang, PNG             | toepad | 1899 | WGS   | yes | yes | yes |
| AMNH:Birds:637511          | torotoro    | flavirostris | Cape York Peninsula, AU  | toepad | 1913 | WGS   | yes | yes | yes |
| ANWC:Birds:B07293          | torotoro    | torotoro     | East Sepik Province, PNG | toepad | 1966 | WGS   | yes | yes | yes |
| ANWC:Birds:B07499          | torotoro    | torotoro     | East Sepik Province, PNG | toepad | 1966 | WGS   | yes | yes | yes |

603 **Table S2.** The proportion of missing data (genotypes) and mean fold sequencing coverage by  
604 library preparation method and tissue type. Missing data rates calculated from the 95% complete  
605 SNP matrix used in *k*-means clustering analyses.  
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| <b>Sample subset</b> | <b>Missing data (<math>\pm</math>SD)</b> | <b>Sequencing coverage (<math>\pm</math>SD)</b> |
|----------------------|--|---|
| WGS libraries        | 0.007 ( $\pm$ 0.010)                     | 6.022x ( $\pm$ 3.567x)                          |
| hyRAD libraries      | 0.015 ( $\pm$ 0.013)                     | 4.427x ( $\pm$ 2.795x)                          |
| Modern tissues       | 0.007 ( $\pm$ 0.009)                     | 5.452x ( $\pm$ 3.321x)                          |
| Toepad tissues       | 0.011 ( $\pm$ 0.013)                     | 4.427x ( $\pm$ 3.235x)                          |

607 **Table S3.** PCA loadings of log-transformed morphological variables for all *Syma* taxa.  
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| <b>Trait</b>        | <b>PC1</b> | <b>PC2</b>  | <b>PC3</b>  |
|---------------------|------------|-------------|-------------|
| Bill (from nostril) | -0.4249207 | 0.33505714  | 0.22963905  |
| Bill width          | -0.4367190 | 0.27088119  | 0.08777821  |
| Bill depth          | -0.4389784 | 0.14310077  | 0.30742375  |
| Tarsus              | -0.3046142 | -0.85803722 | 0.37960606  |
| Wing chord          | -0.4467254 | 0.05852709  | -0.16954328 |
| Tail length         | -0.3790295 | -0.23287113 | -0.81988160 |

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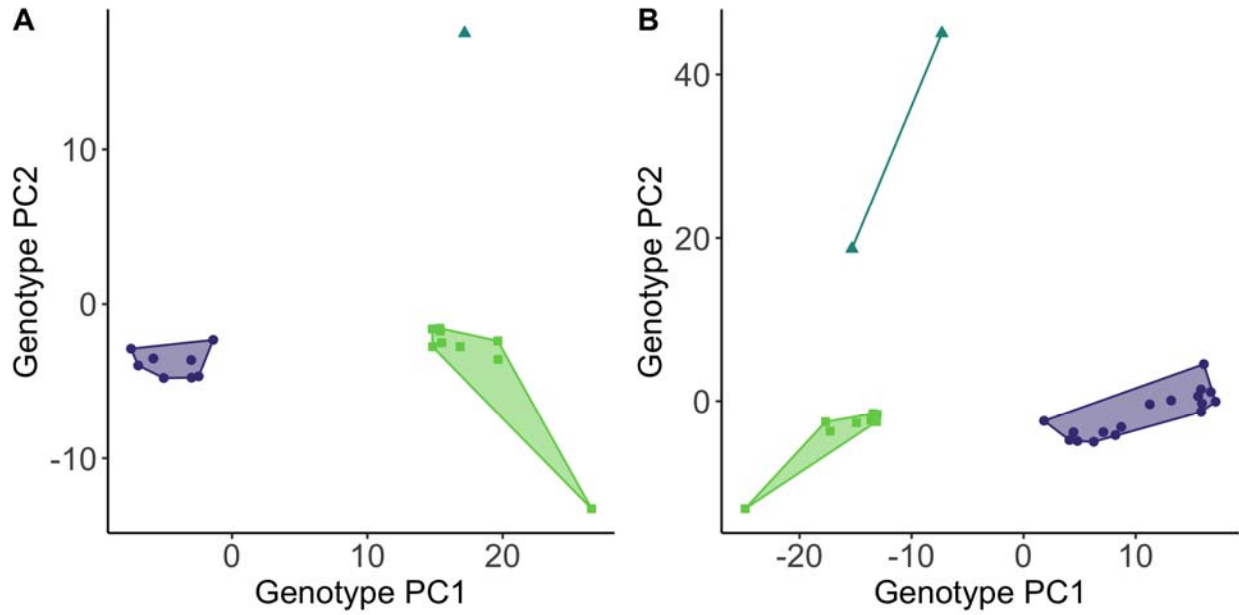
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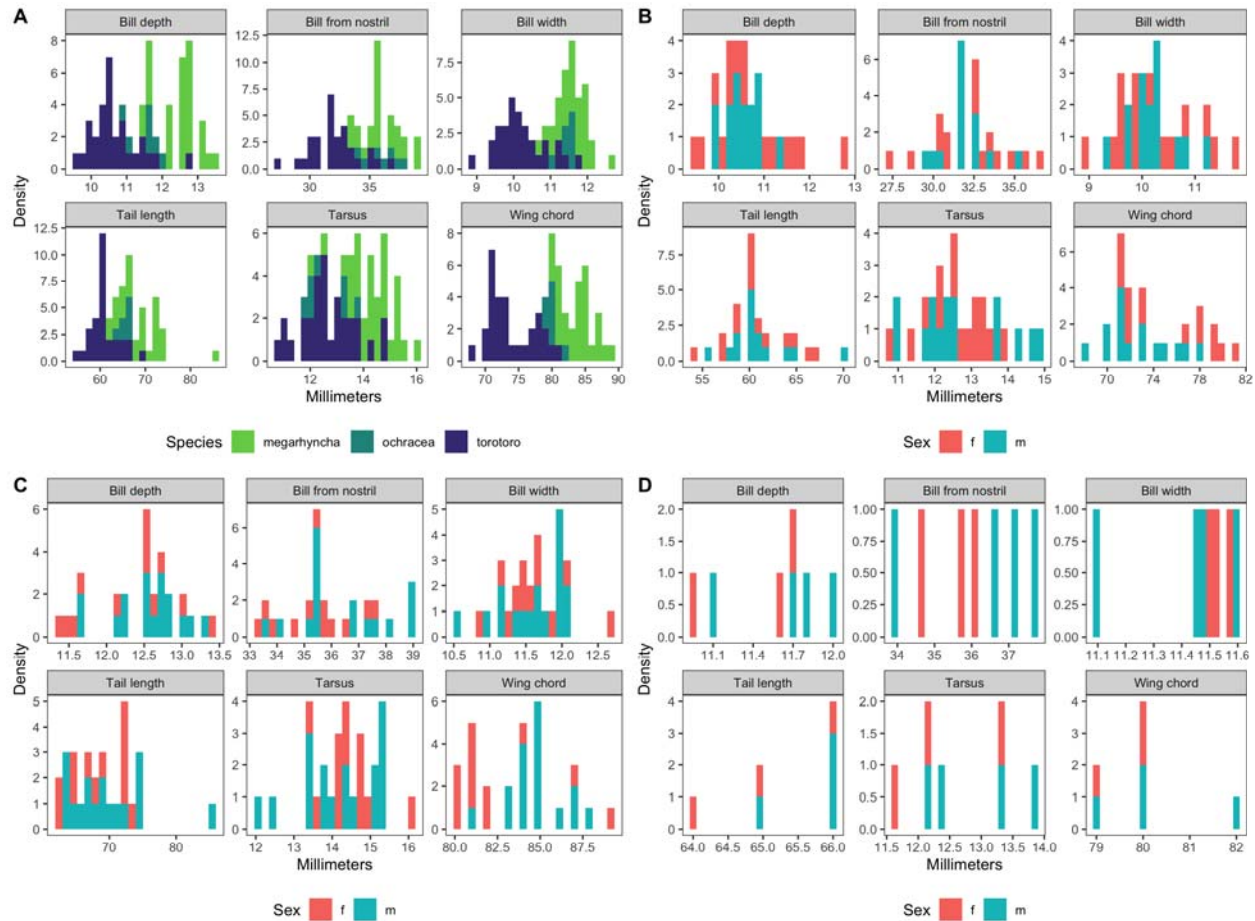
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625 **Figure S1.** Principal component analysis of genotypes from A) whole-genome sequence data  
626 alone or B) only historic samples regardless of sequencing method, color-coded to match *k*-  
627 means results in Figure 1D. Note the single *S. (t) ochracea* individual high on PC2 in panel A.  
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630 **Figure S2.** A) The distribution of log-transformed morphological trait measurements by taxon.  
 631 B-D) The distribution of log-transformed morphological trait measurements by sex for *S.*  
 632 *toroto*, *S. megarhyncha*, and *S. (t.) ochracea*, respectively. Note the limited sample size of  
 633 *S. (t.) ochracea*.  
 634